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„RNAi screen to identify SUN-1 interaction
partners in meiosis of *Caenorhabditis elegans* “

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„Zwei Dinge sollen Kinder von ihren Eltern bekommen: Wurzeln und Flügel.“

– J. W. von Goethe

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Introduction

Meiosis – a specialized cell division

Prior to fertilization, sexually reproducing organisms have to halve their genome in order to maintain a stable number of chromosomes over generations. To fulfill this task, organisms rely on meiosis, a specialized cell division, in which one round of replication is followed by two rounds of consecutive nuclear divisions. After a round of pre-meiotic DNA replication cell nuclei enter meiosis. Homologous chromosomes have to pair and be subsequently held in close proximity to establish a physical linkage between them. The required juxtaposition is stabilized by formation of the synaptonemal complex (SC), a proteinaceous structure, composed of lateral and axial elements between homologous chromosomes. Crossovers (COs) arise from introduction of double strand breaks (DSBs) and their repair by homologous recombination, using the homologous parental chromosome as a template. The establishment of crossovers initiates the physical linkage between parental homologs and it is maintained by cohesion. CO formation also leads to an exchange of genetic material between homologs. Alongside random segregation of chromatids during meiosis II, this process leads to genetic variation among sexually produced offspring.

In metaphase I, chromosomes align at the equatorial plate and homologs are segregated from each other towards opposite poles of the meiotic spindle in anaphase I. Finally, telophase I marks the end of the first meiotic division, where two new nuclear envelopes are formed. The second meiotic division is similar to a mitotic division; by separating the two sister chromatids, it gives rise to four haploid meiotic products that can develop into gametes or get dismissed as polar bodies (for review see: (Petronczki, Siomos et al. 2003; Gerton and Hawley 2005) and Fig.1).

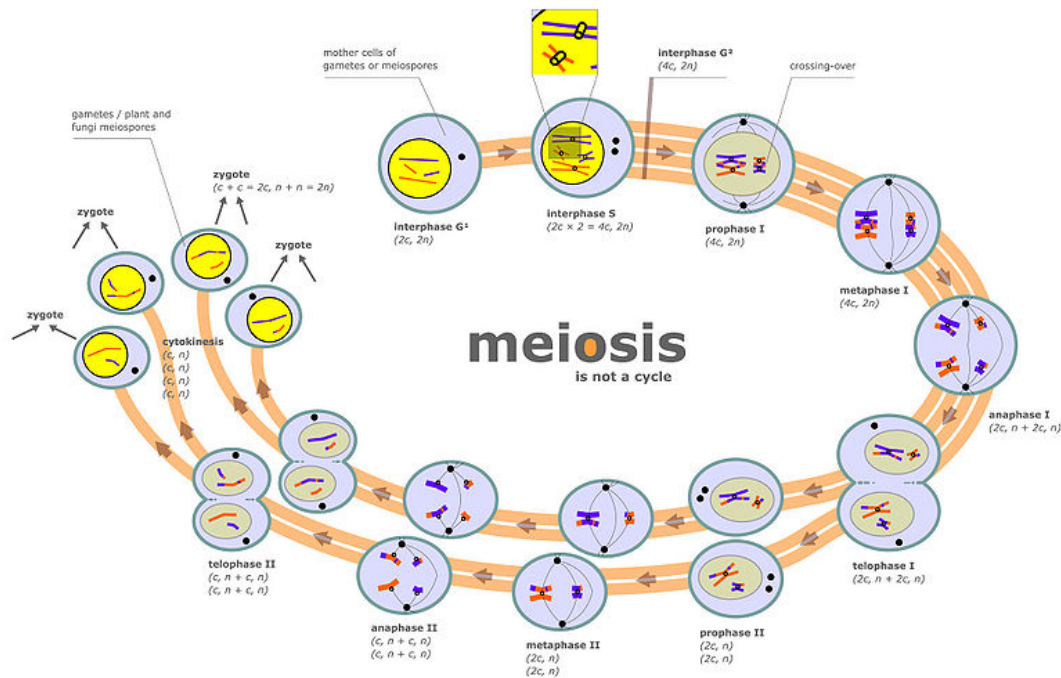


Figure 1: Schematic drawing of meiosis. One round of duplication, and two rounds of division lead to four haploid gametes. In meiosis I, the homologous chromosomes (blue and red) pair, exchange genetic material via crossovers and get separated. After completed separation and formation of a new nuclear envelope, the chromosomes undergo the second meiotic division, which is similar to a mitotic division. This results in formation of four haploid gametes. (E. P. Solomon)

***Caenorhabditis elegans* – a model organism for studying meiosis – Prophase I**

Caenorhabditis elegans (*C. elegans*) is a 1mm long free-living soil nematode (roundworm) with a genome consisting of about 23.000 genes or 10^9 base pairs, organized on 5 autosome pairs and one sex chromosome (XX for the hermaphrodite and Xo for the male) (Wormatlas). The advantages of this model organism are the short reproductive cycle of about 3.5 days at 20°C, its small amount of cells and simple anatomy, a fully sequenced genome, easy culture conditions and its

transparency, which makes it highly competent for analysis by live-imaging. About half of the nuclei in an adult worm undergo meiosis, constituting this organism as very suitable for investigating this specialized cell division. Meiosis takes place in the two symmetrical gonads, which can be dissected for closer examination. At the distal end of the gonad, the distal tip cell (DTC) induces a stem cell niche of cells undergoing mitotic divisions prior to meiotic entry, leading to a continuous supply of newly dividing cells during the fertile period of the worm. Rows of meiotic cells, move down to the proximal end of the gonad tube, at a speed of about one row per hour, while they undergo the differed stages of prophase I (Kimble and White 1981; Watt and Hogan 2000; Crittenden, Leonhard et al. 2006). Consequently, nuclei within the same row are at the same stage of the meiotic cycle (Hirsh, Oppenheim et al. 1976) (reviewed in (Hansen and Schedl 2013)). Hence, *C. elegans* can be used to observe the meiotic time course of prophase I in a spatially organized manner.

C. elegans, males occur by spontaneous miss-segregation (0.02%) of the X-chromosomes during meiosis of the self-fertilizing hermaphrodites (Hodgkin, Horvitz et al. 1979). A higher incidence in males (HIM phenotype), can therefore be used to screen for mutants with problems in chromosome segregation.

Life cycle of *C.elegans*

An adult hermaphrodite lays about 300 eggs or up to 1000 after fertilization by males. At the time of hatching, the embryo consists of 558 cells, which later give rise to 959 somatic and a variable number of germ cells in the adult hermaphrodite. Development of *C. elegans*, from the fertilized egg to the adult, follows a strict pattern, a predetermined lineage of cell divisions and programmed cell deaths. The nematode develops through 4 different larval stages, which are separated by a lethargic molting phase before reaching adulthood. The velocity of development can

be influenced by different growing temperatures from 16-25°C, which leads to a life-cycle length from 200 – 90 hours (Wormatlas).

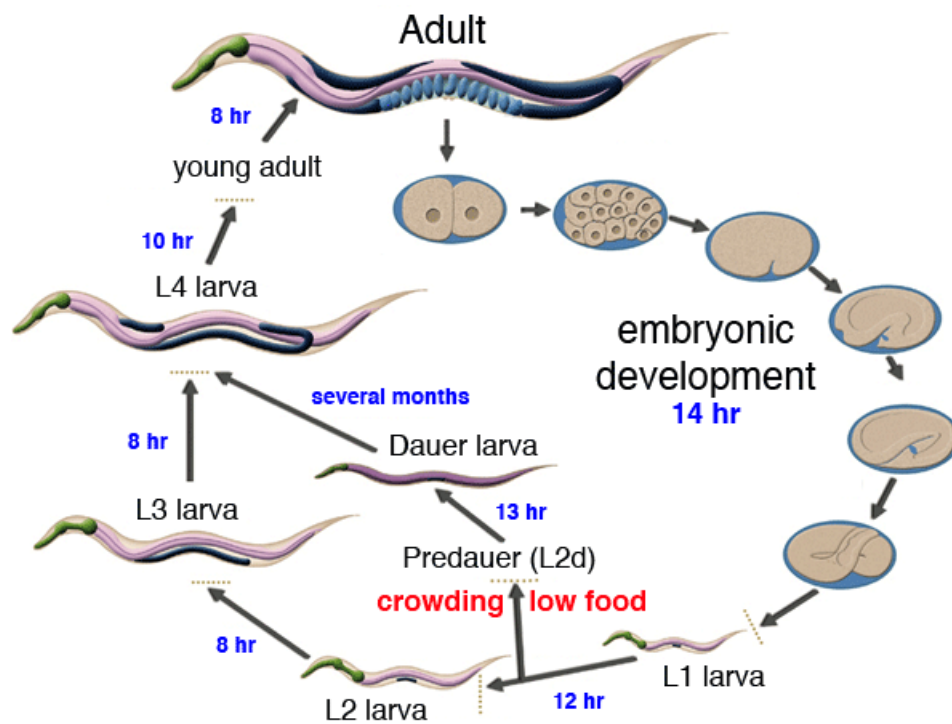


Figure 2: *C. elegans* life cycle at 22°C (artwork by Altun and Hall, © Wormatlas). After egg laying and hatching, the worm develops through 4 different larval stages until entering adulthood. In times of low food supply or crowding L1 larvae can enter predauer and dauer stages, which they can exit as L4 larvae (Cassada and Russell 1975).

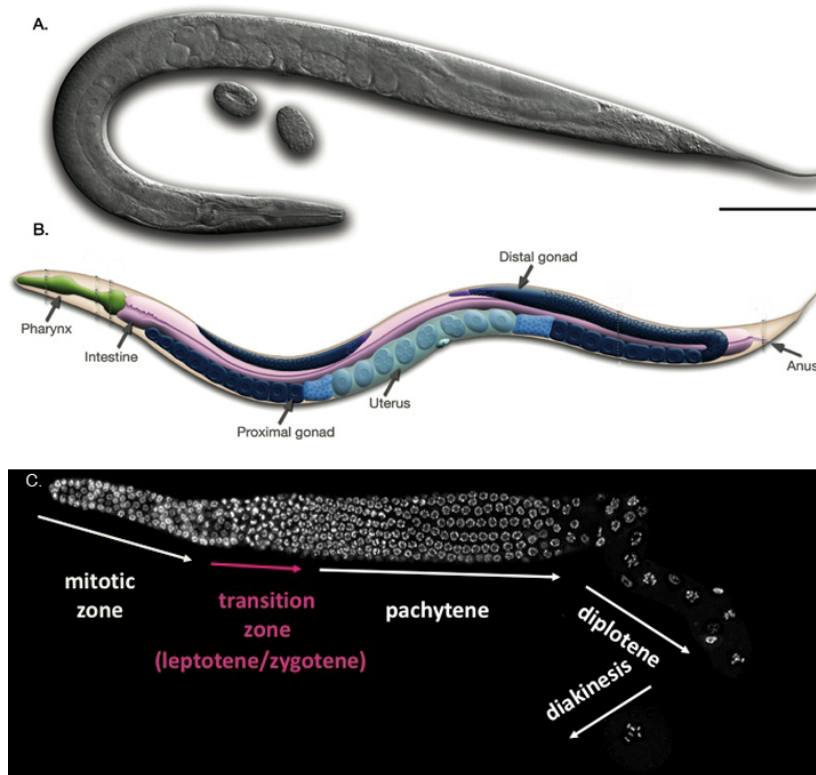


Figure 3: A+B Anatomy of an adult hermaphrodite. (Wormatlas) **A.** DIC image of an adult hermaphrodite, left lateral side. Scale bar 0.1mm. **B.** Schematic drawing of anatomical structures, left lateral side. **C.** DAPI stained gonad showing the mitotic zone and zones of prophase I of the first mitotic division (from A. Penkner, adapted).

Prophase I in *C. elegans*

After pre-meiotic DNA-replication, nuclei enter meiosis in the leptotene/zygotene stage. In *C. elegans*, in contrast to other organisms, these first two stages of meiotic prophase I cannot be cytologically distinguished. The transition zone (TZ) in the gonad likely represents leptotene/zygotene. At this point, chromosomes are pushed to one site of the nucleus, forming a typical half moon shaped chromatin. The intense chromosome movement, at this stage, has been found required for homologous pairing and inhibition of synapsis between non-homologs (Penkner, Tang et al. 2007; Penkner, Fridkin et al. 2009; Sato, Isaac et al. 2009).

Kinetic forces, originated in the cytoplasm, drive TZ chromosome movement.
Reviewed in (Doris Y. Lui 2013)

To transmit these forces through the nuclear envelope, chromosomes are tethered to the nuclear envelope via one end of each chromosome (Reichenow 1927; Goldstein and Slaton 1982; Scherthan 2007). Pairing center (PC) or homolog recognition regions (HRR) are found at the sub-telomeric region of chromosomes (Rosenbluth and Baillie 1981; McKim, Howell et al. 1988; Herman and Kari 1989; Villeneuve 1994). These sites, which stabilize homologous pairing and promote SC formation contain highly repetitive sequences (McKim, Peters et al. 1993; Villeneuve 1994; MacQueen, Phillips et al. 2005; Phillips, Meng et al. 2009) Meiotically expressed C2H2 zinc-finger proteins bind to PCs and are required for homolog pairing. These are: ZIM 1-3, which are enriched on autosomes and HIM-8, which is exclusively found at the X-chromosome (throughout this thesis I will refer to the PC binding proteins as 'ZIMs') (Phillips, Wong et al. 2005; Phillips and Dernburg 2006; Phillips, Meng et al. 2009). After binding to the PCs, the ZIMs recruit the polo-kinases PLK-2 (and PLK-1) to the PC. This leads to relocation of SUN-1 aggregates at chromosome ends and furthermore mediates chromosome end-led movement. A particularly interesting allele of *plk-2* (*vv44*) revealed that PLK-2 mediates timely synapsis and chromosome pairing (Labella, Woglar et al. 2011) The ZIMs might also be responsible for recognition of the homologs, but as two ZIMs bind to two different chromosomes another factor for homology identification might be needed. On the other hand, it has been speculated that the specific spacing of the ZIM binding heterochromatic repeats confers chromosome identity (Phillips, Meng et al. 2009).

Subsequent to this early pairing events, homologous chromosomes have to be held in close juxtaposition to ensure CO formation; this is established by the formation of the zygotene assembled synaptonemal complex (SC), a proteinous structure between homologous chromosomes consisting of central element proteins

(SYP-1, SYP-2, SYP-3 and SYP-4) (MacQueen, Colaiacovo et al. 2002; Colaiacovo, MacQueen et al. 2003; Smolikov, Eizinger et al. 2007; Smolikov, Schild-Prufert et al. 2009; Schild-Prufert, Saito et al. 2011) and lateral elements (Pasierbek, Fodermayr et al. 2003). The cohesin complex is at the basis of the lateral elements and is required to load axial element components. This complex is primarily needed for sister chromatid cohesion and consists of rings structures made of SMC-1, SMC3 and SCC-3 cohesion molecules and the kleisin subunit SCC-1, or REC-8 respectively (REC-8 is meiosis specific) (Pasierbek, Jantsch et al. 2001; Wood, Severson et al. 2010). However the dependencies of axes protein deposition are more complex than previously thought. It has been found that axes components such as HTP-3 can also contribute to sister chromatid cohesion by being required for cohesion loading. (Severson, Ling et al. 2009) The axial element protein HIM-3 together with its paralogs HTP-1-3 are essential for chromosome axis formation, homolog pairing, DSB formation and imposing a barrier to use the sister chromatid as a repair template during homologous recombination (Zetka, Kawasaki et al. 1999; Couteau and Zetka 2005; MacQueen, Phillips et al. 2005; Martinez-Perez and Villeneuve 2005; Goodyer, Kaitna et al. 2008; Hillers and Villeneuve 2009). Axis formation is initiated in the TZ and is followed by the immediate establishment of the synaptonemal complex (MacQueen, Colaiacovo et al. 2002). Depletion of HIM-3 leads to impaired synapsis and lack of chiasmata formation. (Zetka, Kawasaki et al. 1999). The closely related HTP-1 prevents formation of the SC between non-homologous chromosomes and aims synapsis at mature chromosomal axis. In the *htp-1/htp-2* double mutant, synapsis fails entirely (Couteau and Zetka 2005; Martinez-Perez and Villeneuve 2005). HTP-3 is found in a complex with the DSB forming proteins MRE-11/RAD-50 and the axis component HIM-3; therefore the HORMA domain proteins play a central role in linking DSB formation with homolog pairing, synapsis and crossing over (Goodyer, Kaitna et al. 2008).

In contrast to other organisms, pairing and SC-formation in *C. elegans* are independent of double strand breaks (DSBs) (Dernburg, McDonald et al. 1998). DSBs are induced by SPO-11, a topoisomerase (Dernburg, McDonald et al. 1998). RAD-51, a protein that binds ssDNA and is able to recognize and invade homologous DNA, marks processed DSBs (Alpi, Pasierbek et al. 2003; Colaiacovo, MacQueen et al. 2003). Disappearance of Rad-51 foci in end pachytene indicates complete repair of DSBs. In *C. elegans*, on average 4 DSBs are induced in early pachytene and up to 8 per chromosome in late pachytene. A single DSB on each chromosome gives then rise to a functional CO (Mets and Meyer 2009; Rosu, Libuda et al. 2011). The others are either resolved as non-crossover products, a specific pathway that results in homologous repair without exchange of genetic material, or with the aid of the sister chromatid as a repair template (Zalevsky, MacQueen et al. 1999; Smolikov, Eizinger et al. 2007; Rosu, Libuda et al. 2011). Following pairing nuclei enter pachytene, where the SC is fully elongated along chromosomal axes. The chromatin is redistributed and all ZIMs, except HIM-8, (it stays until the end of pachytene), dissociate from the PCs. Consequently leptotene/zygotene characteristic chromosome movement stops in pachytene.

CO formation relies on SC formation and likely takes place in pachytene as a consequence of processing and maturation of DSB repair intermediates and final resolution of double holiday junctions. Alongside with synapsis, CO specific DSB repair is dependent on MSH-5, MSH-4, COSA-1 and ZHP-3 (Kelly, Dernburg et al. 2000; Colaiacovo, MacQueen et al. 2003; Jantsch, Pasierbek et al. 2004; Rosu, Libuda et al. 2011). How CO precursors are resolved to give rise to functional CO remains enigmatic in the worm. However, a resolvase must be implicated, since the *him-18* mutant, shows features of unresolved bivalents connected by DNA threads. HIM-18 is a MUS321/Slx4 ortholog that is needed for converting homologous recombination intermediates into COs (Saito, Youds et al. 2009). In contrast to mid pachytene, where axial elements are evenly distributed along the SC, they

redistribute in late pachytene/diplotene in response to CO formation. In diplotene the chromosomes condense further and the SC disassembles asymmetrically (MacQueen, Phillips et al. 2005; Martinez-Perez and Villeneuve 2005; Nabeshima, Villeneuve et al. 2005) and chiasmata indicate COs. This leads to maturation of asymmetric bivalents, which are ready for cohesion cleavage along the short arm. SYP-1 is concentrated at the short arm at the site of homologous connection, whereas HTP-1 and LAB-1 get concentrated at the long arm sister chromatids (Nabeshima, Villeneuve et al. 2005; Tzur, Egydio de Carvalho et al. 2012). In diakinesis, chromosomes are fully condensed and are visible as 6 DAPI positive structures, which represent the 6 pairs of homologs in *C. elegans*. As the oocytes migrate down the gonad, they pass by the spermatheca where they get fertilized by a sperm. Oocytes are arrested at metaphase I up to fertilization, which in turn triggers the two meiotic divisions (Ward and Carrel 1979; Singson 2001).

SUN-1-dependent movement of chromosomes in prophase I in *C. elegans*

After meiotic entry, chromatin adopts a characteristic halve moon shape, usually detectable at one side of the nucleus. This region is referred to as transition zone (TZ) and corresponds to leptotene/zygotene. This clustering is a consequence of chromosome movement driven by the cytoskeleton. Forces from the cytoplasm are transmitted through the nuclear membranes by the SUN/KASH bridge. SUN-1 is found at the inner nuclear membrane and connects to PCs in an unknown manner; ZYG-12 is a KASH domain bearing protein and is located at the outer nuclear membrane. At the stage of chromosome movement, SUN-1 accumulates in prominent aggregates at chromosome ends (Jantsch, Pasierbek et al. 2004; Penkner, Tang et al. 2007; Penkner, Fridkin et al. 2009; Phillips, Meng et al. 2009; Sato, Isaac et al. 2009), reminiscent of chromosome attachment plaques, a prominent structure also seen in vertebrate EM sections (Schmitt et al., 2007). In this region, the chromosome

ends become attached to the nuclear envelope. It has been shown that introduction of extrachromosomal arrays bearing the PC heterochromatic repeats can induce SUN-1 aggregates (Phillips, Meng et al. 2009). Dynein, as well as other NE components are required for synapsis and restriction of SC formation to appropriate homolog pairs (Sato, Isaac et al. 2009). The inner nuclear envelope trans-membrane protein SUN-1 is expressed in germ cells and early embryos, where it is maternally provided. The C-terminal SUN domain in the perinuclear lumen binds and locates the KASH-domain protein (Klarsicht, Anc-1, Syne-homologue) ZYG-12, which crosses the outer nuclear membrane (Malone, Misner et al. 2003; Penkner, Tang et al. 2007). The SUN/KASH pair, therefore, spans the nuclear envelope and mediates the cytoskeletal force to the nuclear interior. In *C.elegans* the cytoskeletal force is provided by microtubules; as shown by Sato et. al, colchicine treatment (a microtubule inhibiting drug) leads to loss of homolog pairing. ZYG-12 aggregates mirror SUN-1 aggregates and strong foci of the microtubule motor, the dynein complex and foci of LIS-1 accompany ZYG-12 aggregates (Sato, Isaac et al. 2009). However, if forces are generated either by sliding on existing microtubules or active microtubule nucleation remains to be studied. Additional functions of the SUN/KASH bridge include, nucleus and centrosome positioning, germ cell development and centrosome attachment in embryos. (Malone, Misner et al. 2003; Fridkin, Mills et al. 2004; Minn, Rolls et al. 2009).

In the TZ, SUN-1/ZYG-12 form movement-competent aggregates, which strictly co-localize with the ZIMs (Penkner, Fridkin et al. 2009; Sato, Isaac et al. 2009). This movement supports the finding of homologous chromosomes and inhibits non-homologous pairing (Penkner, Tang et al. 2007). SUN-1 aggregates are formed at the beginning of TZ and dissolve in mid-pachytene, in the case of autosome-associated aggregates. In contrast to this, aggregates that colocalize with the HIM-8 associated X chromosome, persist until late pachytene (Penkner, Fridkin et al. 2009). Time lapse imaging of SUN-1::GFP aggregates allowed visualization of the high dynamic behavior of the chromosome ends. Aggregates showed a high

tendency to come together into clusters, however aggregates were also seen to leave existing aggregate clusters and move to join another one. An aggregate speed of more than 160 nm/s with up to 15 fusion and splitting events within the first 15 minutes of filming was observed. Speed distribution of aggregates revealed that around 10% moved with fast trajectories. Compared to this, aggregates of a SUN-1 (G311V)::GFP fusion in a *sun-1 (ok1282)* deletion background, move at a speed of only 10 nm/s - 100 nm/s and displayed no fusion and splitting events at all. Interestingly movement remained the same throughout the TZ despite progressing pairing. One can therefore hypothesize that full synapsis releases a nucleus wide signal to shut down chromosome movement. It was also shown, that formation of SUN-1 aggregates depends on chromosome axes. (Penkner, Fridkin et al. 2009; Baudrimont, Penkner et al. 2010; Labella, Woglar et al. 2011)

SUN-1 gets phosphorylated at multiple sites at its amino-terminus (S8, S12, S24, T36, S43, S58 and S62) during ongoing meiosis ((Penkner, Fridkin et al. 2009) and unpublished data)). This, CHK-2 and PLK-2 depended (Penkner, Fridkin et al. 2009; Labella, Woglar et al. 2011), phosphorylation appears sharply at the beginning of TZ, whereby the phosphorylation on S12 is only detectable on SUN-1 molecules organized into aggregates and also disappears as aggregates dissolve. In contrast to S12, modifications of S8, 24, and 43 can be seen on the entire population of SUN-1 molecules. Phosphorylation of S12 exclusively depends on PLK-2 (Harper, Rillo et al. 2011; Labella, Woglar et al. 2011). Along with phosphorylation, PLK-2 is also responsible for SUN-1 aggregate formation. (Harper, Rillo et al. 2011; Labella, Woglar et al. 2011). SUN-1 gets de-phosphorylated with progressing meiosis and is found free of phospho-signals in mid/late pachytene, when the last aggregate dissolved.

The *sun-1* null allele displays a wide range of phenotypes: gonads are completely disorganized, meiotic stages cannot be made out, gonads contain fewer

germ cells, indicative of a proliferation defect and the progeny die as embryos or larvae. Also very few eggs are laid; often *sun-1* mutants are completely sterile (Fridkin, Mills et al. 2004). A point mutation in the SUN-domain of SUN-1, as in the *jf18* allele, interrupts the SUN/KASH bridge by displacing ZYG-12 from the outer nuclear envelope, leading to abolishment of directed chromosome movements and therefore, impairs the typical chromosomal clustering at the leptotene/zygotene stage of prophase I. As a consequence, synapsis between non-homologous chromosomes takes place (Penkner, Tang et al. 2007; Penkner, Fridkin et al. 2009; Baudrimont, Penkner et al. 2010). Additionally, *sun-1 (jf18)* fails to locate ZYG-12 to the outer nuclear membrane, leading to accumulation of ZYG-12 in structures resembling the endoplasmatic reticulum (Penkner, Tang et al. 2007).

Meiotic movement of chromosomes in other organisms

Tethering of chromosomal telomeres to the nuclear envelope and rapid meiotic prophase chromosomal movement (RPMs) are highly conserved features of early prophase I. During meiotic prophase I of many organisms, chromosomes end-led movements are leading to a visual chromosomal bouquet with prominent telomere concentration at the periphery of the nuclei. A SUN/KASH protein complex provides the essential linkage between the nuclear interior and cytoskeletal forces. In animals and fungi this site is defined by the location of the microtubule organization center (MTOC), whereas in plants, which lack a defined MTOC, bouquet formation accompany the polarization of the whole cell (Hiraoka 1993). Depletion of components responsible for force transmission leads to impaired bouquet formation.

Restriction of telomeres to a distinct volume of the nucleus reduces the area of homolog search to two dimensions and could therefore aid pairing and synapsis of homologs. This explanation has not only recently become a matter of debate. Other models suggest that movement has also a role in teasing chromosome entanglements

apart and in supporting the recombination process (reviewed in (Koszul, Kim et al. 2008)).

Saccharomyces cerevisiae

In *S. cerevisiae*, the meiosis specific telomere protein Ndj1p mediates bouquet formation and interacts with Mps3p, a SUN-domain protein. Mps3p is important for spindle pole duplication, telomere clustering, homologous pairing and synapsis (Trelles-Sticken, Dresser et al. 2000; Rao, Shinohara et al. 2011). The Ndj1p/Mps3p interaction aids bouquet formation (Trelles-Sticken, Dresser et al. 2000). Full depletion of Ndj1p delays synapsis and homolog pairing and reduces spore formation and viability (Chua and Roeder 1997; Conrad, Dominguez et al. 1997; Rockmill and Roeder 1998). Interaction of Ndj1p and Mps3p is essential for bouquet formation and timely progression of meiosis. Deletion of the Ndj1p-interaction domain of Mps3 (residues 2-64 at its n-terminus), leads to impaired bouquet formation (Conrad, Lee et al. (2007). Mps3^{Δ2-64} as well as depletion of Ndj1p leads to unspecific localization of telomeres within the nucleus instead of localization at the periphery (Conrad, Lee et al. 2007; Conrad, Lee et al. 2008). Cms4 is found in one complex with Mps3/Ndj1 at the site of telomere attachment and facilitates bouquet formation. In absence of Cms4, telomeres are still bound to the nuclear envelope by Ndj1, but chromatin clustering is impaired. Additionally, Cms4 seems to play a role in distinct steps during cross-over formation and meiotic recombination, such as resolution of double Holiday junctions (Kosaka, Shinohara et al. 2008).

Visualization of chromosome movement in *S. cerevisiae* was achieved by live-imaging of Rap1::GFP, a telomere binding protein. Additionally, GFP tagged Mps3 and Rec-8 independently confirmed the theory of telomere-led chromosome movement (Trelles-Sticken, Dresser et al. 2000; Conrad, Lee et al. 2008; Koszul, Kim et al. 2008). As expected the chromosome movement is strongly reduced in Ndj1 depletion and Mps3^{Δ2-64}, respectively (Conrad, Lee et al. 2007; Scherthan, Wang et

al. 2007; Conrad, Lee et al. 2008). In contrast to other organisms, as fission yeast, plants or mammals, movement is sensitive to actin destabilizing drugs, but insensitive to microtubule de-polymerization drugs (Trelles-Sticken, Dresser et al. 2000).

Sacharomyces pombe

The spindle pole body (SPB) in fission yeast functions as microtubule organization center, similar to the centrosome (in contrast to mammals, centriole structures have not been observed). Kms1, a KASH protein in the outer membrane of the nucleus binds to the SPB and to Sad1, which is a SUN protein. Sad1/Kms1 form a bridge through the nuclear envelope analogous to SUN-1/ZYG-12 in *C. elegans*. Bouquet formation in fission yeast depends on the meiotic proteins Bqt1 and Bqt2 (Chikashige, Tsutsumi et al. 2006), which are both required to link telomeres to the spindle pole body (SPB). Taz1 serves as telomere binding factor and is responsible for the connection between chromosomal ends and Rad1, which in turn interacts with Bqt1 and Bqt2 (Cooper, Watanabe et al. 1998; Nimmo, Pidoux et al. 1998; Kanoh and Ishikawa 2001). In fission yeast, the bouquet has an important function in SPB organization and maintenance as discussed by Tomita and Cooper (Tomita and Cooper 2007).

Chromosomes of the Prophase I in fission yeast exhibit a unique horsetail movement. The nucleus is oscillating back and forth with the spindle pole at the leading edge and chromosomes clustered opposite to the SBP (Chikashige, Ding et al. 1994; Chikashige, Ding et al. 1997). The clustering of chromosomes and horsetail movement is genetically separable but both contribute to chromosomal pairing. Horsetail movement is depended on astral microtubules and the MT motor Dhc1 (Ding, Chikashige et al. 1998; Davis and Smith 2006). Currently it is thought, that clustering of chromosomes in fission yeast is required for chromosome pairing and

early interaction and the horsetail movement contributes to recombination, which need close juxtapositioning of chromosomes.

Mammals

Higher eukaryotes, like mammals, possess orthologous proteins important for bridging the cytoskeletal force into the nucleus, similar to the worm SUN/KASH pair. However, knockout of these do not always display the same severe consequences, as the increased complexity of the genome of mammals may have led to new or overlapping functions (La Volpe and Barchi 2012). The mammalian Sun1 protein is, unlike its homolog Sun2, exclusively expressed in meiocytes and interacts with the KASH proteins Nesprin 1 and 2 (Haque, Lloyd et al. 2006). Knockdown of Sun1 in mice prevents telomere attachment to the nuclear envelope and impairs homolog pairing and synapsis formation. Moreover, this leads to an increased induction of apoptosis and deficiency oogenesis and spermatogenesis (Ding, Xu et al. 2007). Sun2 is exclusively found at chromosome attachment sites in prophase I of meiosis (Schmitt, Benavente et al. 2007). The SUN/KASH bridge is not only required in chromosomal movement, but also for nuclear positioning, depletion leads to a set of diseases termed as laminopathies; including Hutchinson-Gilford progeria, Meckel-Gruber syndrome and Emery Dreifuss muscular dystrophy in humans (reviewed in (Starr 2011). The proteins that provide the necessary linkage of Sun1/Sun2 to the chromosomes remain unknown in mammals.

There is still little data about how chromosomes move in vertebrates. In hamster, meiotic movements were followed by filming of chromatin highlighted with Hoechst and it appears as if the whole nucleus would show a rotational movement (Yao and Ellingson 1969; Parvinen and Soderstrom 1976). It was shown only recently that KASH5 a KASH-domain protein, that interacts with Sun1 localizes specifically to telomeres. KASH5 is thought to connect the mammalian Sun1 to the cytoskeletal force and therefore promotes chromosome movement. As in *C.elegans* and budding

yeast, chromosome movement depends on microtubules (Morimoto, Shibuya et al. 2012).

Aim of this thesis

In meiosis, homolog chromosomes have, to recognize and pair each other in order to exchange genetic material and be separated at the end of meiosis I. The trans-membrane protein SUN-1 transfers the force, which is necessary for chromosome movement and homology search in leptotene/zygotene of prophase I. SUN-1 connects the cytoskeleton to the interior of the nucleus via ZYG-12 (Malone, Misner et al. 2003; Minn, Rolls et al. 2009), but by now, we do not know how the cytoskeletal forces are transferred onto the chromosomes. Immuno-precipitation and yeast-two-hybrid studies showed no direct interaction between SUN-1 and the ZIMs. Also the fact that SUN-1 lacks a DNA-binding domain, rules out a direct interaction between SUN-1 and the PCs. Therefore, the question was raised whether there is an interacting protein that connects SUN-1 with the chromosomes; either directly or over an intermediate proteins such as the ZIMs. In addition, this hypothesis is supported by the fact that such 'linker' proteins exist in other organisms such as yeast. In *S. pombe* the connection between the SUN protein, Sad-1 and the telomeres is established by Rad-1, Bqt-1, Bqt-2 and Taz-1 (Cooper, Watanabe et al. 1998; Nimmo, Pidoux et al. 1998; Kanoh and Ishikawa 2001). In *S. cerevisiae* the telomere specific protein Ndj1p is responsible for bouquet formation and connection to the SUN protein Mps3p (Trelles-Sticken, Dresser et al. 2000). Until present no homolog of such a linker proteins has been found in *C. elegans*.

For further investigation of this hypothesis, the aim of this project was to find an interaction partner of SUN-1 that transfers the moving forces of the cytoskeleton to the chromosomes. This factor X would connect the moving force of the cytoskeleton (transferred by SUN-1) to the chromosome ends of each chromosome. Knockout of this factor X would lead to impaired chromosome movement and

therefore to absence of chromatin clustering at the TZ of the gonad. Moreover, homolog chromosomes would not pair and meiosis would fail.

Experimental design

The candidates for the screen were selected from proteins that immuno precipitated with SUN-1 (See: “Results” Table 2) and were identified by mass spectrometry. To get an insight into the function of a particular gene, we performed phenotypic analysis of either the RNAi knockdown or the deletion strain obtained from the Caenorhabditis Genetics Center (CGC). In case of an RNAi knockdown, we fed the worms for 48 hours post larval stage L4 on RNAi plates and dissected the gonads for closer examination by immuno staining. In addition, the F1 generations were analyzed, if existing. After the initial screen, we selected the most promising candidates with regard to chromatin clustering in the TZ. In cases where the RNAi led to embryonic lethality we subjected the PO to RNAi earlier (L1 larvae) and dissected the PO gonads. This was done to optimize the feeding conditions and to obtain a more penetrant phenotype (see Fig.1). For a more detailed explanation of the RNAi feeding procedure see materials and methods.

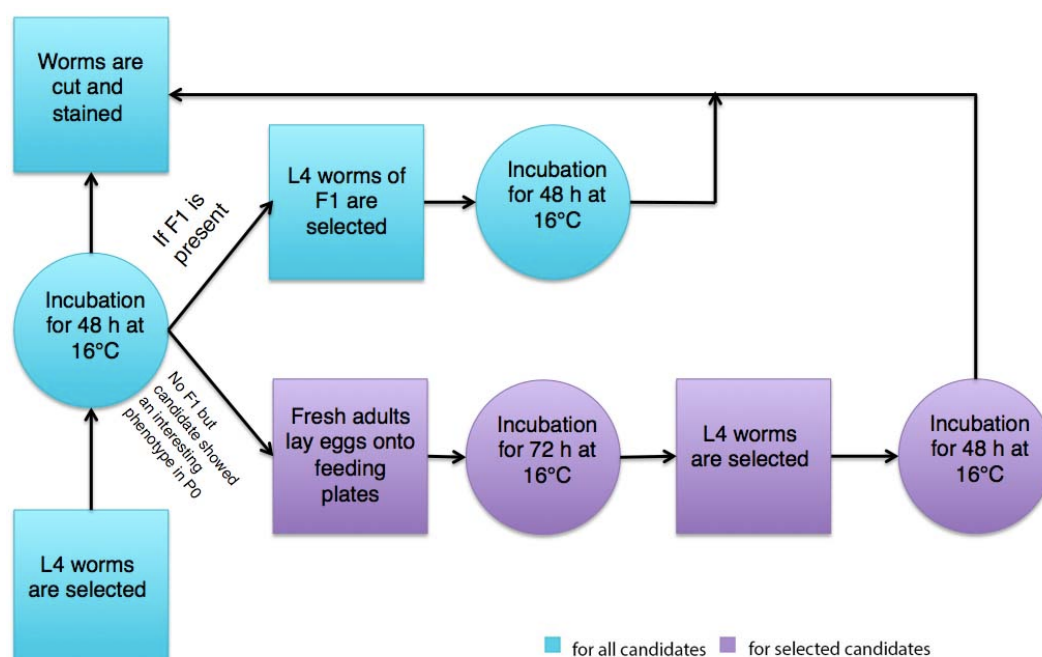


Figure 4: Workflow for the RNAi screen. Light blue squares and circles indicate procedures done for every candidate of the initial screen. Violet squares and circles indicate experiments only done with selected candidates.

Phenotypic analysis

In this screen, we used four different readouts for the phenotypic analysis of RNAi knockdown and deletion strains, which are briefly summarized below:

Viability and appearance of the worms

After feeding we checked the overall state of the worms, including their progeny, for observable differences like: embryonic viability, reduced growth/developmental speed, larval arrest, an increase of males in the viable progeny and the overall appearance of the worm (altered body shape or length, any visible phenotype). Most importantly, the viability of F1 was calculated as percentage of hatched larvae. This was done because alterations in body shape would rather indicate an important function of the particular gene in somatic tissue, whereas dead eggs and a high incidence of males hint to a meiotic failure.

After dissection of the gonads, we used the following assays and markers for immunohistochemistry:

Chromatin

DNA was stained by DAPI. With this staining, we examined the characteristic shape and structure of the chromatin/chromosomes in the different stages of meiosis. Our main read out of chromosome movement was the typical half-moon shaped chromatin at the entry of TZ. Moreover, we counted the number of DAPI-positive structures at diakinesis, to monitor the establishment of a crossover. DAPI staining is in particular important to observe the overall state of the chromatin. It is known that in meiotic mutants the clustering in TZ can be prolonged until the end of the gonad (like for instance in *syp-1(me17)* (MacQueen, Colaiacovo et al. 2002) or not observable at all (as in *sun-1(jf18)* (Penkner, Tang et al. 2007)). In addition, a clear

sign of meiotic failure would be more than 6 DAPI positive structures in diakinesis (12 univalent are observed in *syp-1(me17)* (MacQueen, Colaiacovo et al. 2002))

Phosphorylation of Serin8 of SUN-1 at serine 8

For this staining we used an antibody, which recognizes phosphorylated SUN-1 S8. This phosphorylation comes up at the entry of TZ and is present in the whole population of SUN-1 at the nuclear envelope (Penkner, Fridkin et al. 2009). We checked for any alteration of the kinetics of phosphorylation/de-phosphorylation and SUN-1 aggregate formation. An alteration of this kinetics, as we know so far, reflects problems in meiosis with regard to chromosome movement and/or the chromosome homology search, synapsis and progression of recombination. De-novo phosphorylation is only possible in TZ and mid pachytene. Hence, phosphorylated SUN-1 S8 in later stages can be an indication of a meiotic defect.

RAD-51

An antibody against RAD-51 was used to follow the formation and repair of DSBs, as RAD-51 binds ssDNA and promotes strand invasion in mid pachytene (Rinaldo, Bazzicalupo et al. 2002; Alpi, Pasierbek et al. 2003; Colaiacovo, MacQueen et al. 2003). Hence, a change of the dynamics of RAD-51 monitors an alteration of meiotic processes at the level of DSBs and their processing (West 2003). Moreover, in mid pachytene nuclei with increased RAD-51 foci accompanied by phosphorylated SUN-1 indicate an arrest in meiosis. With this read out we could observe any defect in meiotic DSBs repair. As the DSBs are only repaired, if homologs pair correctly this monitors also meiotic defects at the level of homolog recognition and synapsis. The example of SUN-1 knockdown by RNAi (see results Tab. 3, done as a positive control) showed the expected increase of RAD-51 foci, as shown in Penkner et al., 2007, indicating that meiotic repair was impaired due to the lack of the homolog (*sun-1 RNAi* led to non-homologous synapsis as has been observed for the *sun-1 (jf18)*

allele). We expect that abrogation of a factor that connects chromosomes to the movement apparatus might partially phenocopy the *sun-1 (jfi8)* phenotype.

The results of the phenotypic analysis of P0, F1 and the deletion alleles (if available) were combined.

Expectations for knockdown of factor X

We here define the factor X, as the protein that connects SUN-1 to the chromosomes, either by binding to the chromosomes themselves or by binding to a connective factor. Either way, this factor would be responsible for linking the cytoskeletal forces to the chromosomes. Upon knockdown of factor X we would expect severe impairment of chromosome movement in TZ, due to the missing connection of the chromosomes to the nuclear envelope. Our main read-out for movement was the typical half moon shaped chromatin in the TZ, which results from clustering of chromosomes to one pole of the nucleus. Knockdown of factor X would result in a TZ that either shows reduced or no visible clustering, as the connection, which transmits the cytoskeletal force is disrupted. Moreover, we would also expect more than 6 DAPI positive structures in diakinesis if we extrapolate that non-homologous synapsis takes place in the absence of chromosome movement.

Furthermore, knockdown of factor X could lead to a prolonged zone of phosphorylated SUN-1 S8, as SUN-1 S8 stays phosphorylated until the search for the homolog chromosome would be finished and crossover recombination would have proceeded to a certain intermediate. In addition, one could also speculate that the factor X could be needed for initial recruitment of the ZIMs, thus no PLK-2 would be recruited upon knockdown of the factor X and SUN-1 S12 phosphorylation could be hampered.

In a knockdown of our desired target gene, we would expect that RAD-51 stays until late pachytene, as the homolog chromosome cannot serve as a repair

template for the breaks and in this time window a block to use the sister chromatid as a repair template is still in place. In addition, there might be an increased number of nuclei in late pachytene that show signs of meiotic arrest (increased RAD-51 accompanied with phosphorylated SUN-1 S8).

Moreover, knockdown of factor X would lead to strongly decreased viability of F1 and/or an increase of male progeny, as both are signs of aneuploidy, meiotic failure and X chromosome nondisjunction.

Meiotic entry and homologue pairing

In our secondary screen we analyzed meiotic entry and chromosome pairing of outstanding candidates. For this we used candidates that showed no or a very reduced clustering of chromatin in TZ. To make sure that the knockdown of a respective protein did influence the movement of chromosomes in meiosis rather than entry of meiosis itself, we stained for the meiotic marker ZIM-3. ZIM-3 is expressed at the beginning of meiosis and localizes to the PCs of chromosome IV and I (Phillips and Dernburg 2006).

Homologue pairing was analyzed, using a FISH-probe against the rDNA locus on chromosome V. Again, we subjected knockdowns of the candidate ORFs, which showed less or no chromatin clustering in the transition zone, to FISH analysis.

Again, for the knockdown of factor X we would expect failure of homologue pairing but normal entry into meiosis.

Results

Preparations for the screen

Immuno-precipitation and mass spectrometry

Immuno-precipitation of SUN-1 was done under two different conditions to obtain a suitable list of screening candidates. Proteins were either pulled down under native conditions or after cross-linking by formaldehyde prior to the pull down. The proteins contained in the precipitate were then identified by mass-spectrometry and those found under both IP/pull down conditions were compiled as candidates for the RNAi screen.

Optimization of the screening strain

In order to find the optimal strain for our purpose, we tested three different strains with regard to RNAi sensitivity: N₂ (wild type), *rrf-1 (pk1417) I.* and *rrf-3 (pk1426) II.* We fed RNAi against *syp-1*, counted the viability of F1 and dissected the gonads of P0 and F1 animals to follow the influence of RNAi over two generations. *syp-1* encoded for an important central element protein of the SC. Chromatin clustering of the TZ in *syp-1(me17)* knockout mutants is prolonged until the end of pachytene. The offspring viability is reduced to 5% of the wild-type level and hatching worms show an increased incidence in males (36%) due to non-disjunction of the X-chromosome (MacQueen, Colaiacovo et al. 2002).

We found that the viability of F1 animals upon feeding with RNAi against *syp-1* was higher (72%) in the wild-type background than those of *rrf-1 (pk1417) I.* (42%) or *rrf-3(pk1426) II* (45%). The dissected DAPI stained gonads of P0 of all three strains showed prolonged clustering of the chromatin. An almost equal amount of cell rows in the meiotic zone (rows from the TZ to the end of pachytene) of all tree

genotypes showed TZ clustering (N2: 51%, *rrf-1 (pk1417) I.*: 45%, *rrf-3(pk1426) II.* : 42%.; $n=8-10$). This phenotype was even stronger in F1 animals. The TZ of F1 in all three strains was prolonged until the end of pachytene. Concerning diakinesis, we observed 6 DAPI positive structures in Po and slightly more in F₁ (8.1 signals in N2, 7.5 signals in *rrf-1 (pk1417) I.* and 6.5 signals in *rrf-3(pk1426) II.*). As the penetrance of RNAi can vary strongly between different target genes and RNAs used for the target genes, such a test can only be a hint for choosing the most sensitive strain. Different prior screens discovered *rrf-3(pk426)* as suitable for genome wide RNAi screenings (Simmer, Tijsterman et al. 2002). Thus, we used *rrf-3 (pk426)* for our screen.

RNAi against *syp-1* was always used as a quality control for the feeding plates to ensure that the induction of dsRNA transcription worked. Worms fed with regular OP₅₀ bacteria of the *rrf-3 (pk426)* strain were used as a wild-type control.

Primary screen

Features of the controls, *rrf-3* not subjected to RNAi

The *rrf-3 (pk426)* strain fed with OP₅₀ bacteria was used as a wildtype control. The gonads of the control were dissected in parallel to the candidates of the screen. We observed following control phenotype (see figure 1): The mitotic zone was about 20 rows long. Entry of TZ is characterized by tight clustering of the chromatin to one side of the nucleus. The TZ is about 10 rows long and shows loosening of the chromatin towards the end. At the entry of pachytene homologous chromosomes become visible as long cord-like parallel aligned structures. Theses structures get shortened and denser in diplotene and give finally rise to 6 DAPI positive structures in diakinesis.

The phosphorylation of SUN-1 at serine 8 (SUN-1 S8Pi) was first seen at the entry of TZ, where the chromatin clusters to one side of the nucleus. All the SUN-1 S8Pi is located at the nuclear envelope at this stage. However, SUN-1 S8Pi was also sometimes observed in the mitotic zone (1-2 nuclei per gonad-arm), where it highlights the centrosome in mitotic anaphases. SUN-1 S8Pi in the TZ is not only distributed over the entire nuclear envelope, but also seen in aggregates of SUN-1 S8Pi concentrated at chromosome end attachments. These aggregates range from small dots to bigger ones in the beginning of TZ and became less in number but bigger in size in mid/end of TZ. In early pachytene all aggregates, apart from one (in former studies those single aggregates were found to co-localize with HIM-8), disappear in the mid of pachytene. SUN-1 S8Pi gets de-phosphorylated (visible by a fainter antibody signal) within mid pachytene within about 2-3 rows. I also observed strong signals of SUN-1 S8Pi at the end of pachytene, ranging from 1-3 nuclei per gonad. Additionally, these nuclei showed an accumulation of RAD-51 foci. As de-novo phosphorylation of SUN-1 S8 was found to only take place in TZ and early/mid pachytene (unpublished data), we assumed that this pattern represents nuclei with unfinished meiotic tasks and seem to be somehow arrested in meiosis. These 'arrested nuclei' can vary in their chromatin state from tightly clustered chromatin (as seen in TZ) to loosely (mid pachytene) clustered or non-clustered chromatin.

RAD-51 was first seen in the beginning of pachytene as 3-5 foci per nucleus. This number increases to about 10 foci per nucleus in mid pachytene and RAD-51 disappears quickly within about two rows in late/mid pachytene. As stated before there were about 1-3 nuclei in late pachytene, which showed a high density of RAD-51 foci and SUN-1 S8Pi; those nuclei are most likely arrested in leptotene/zygotene while continuously being pushed downwards the gonadal tube.

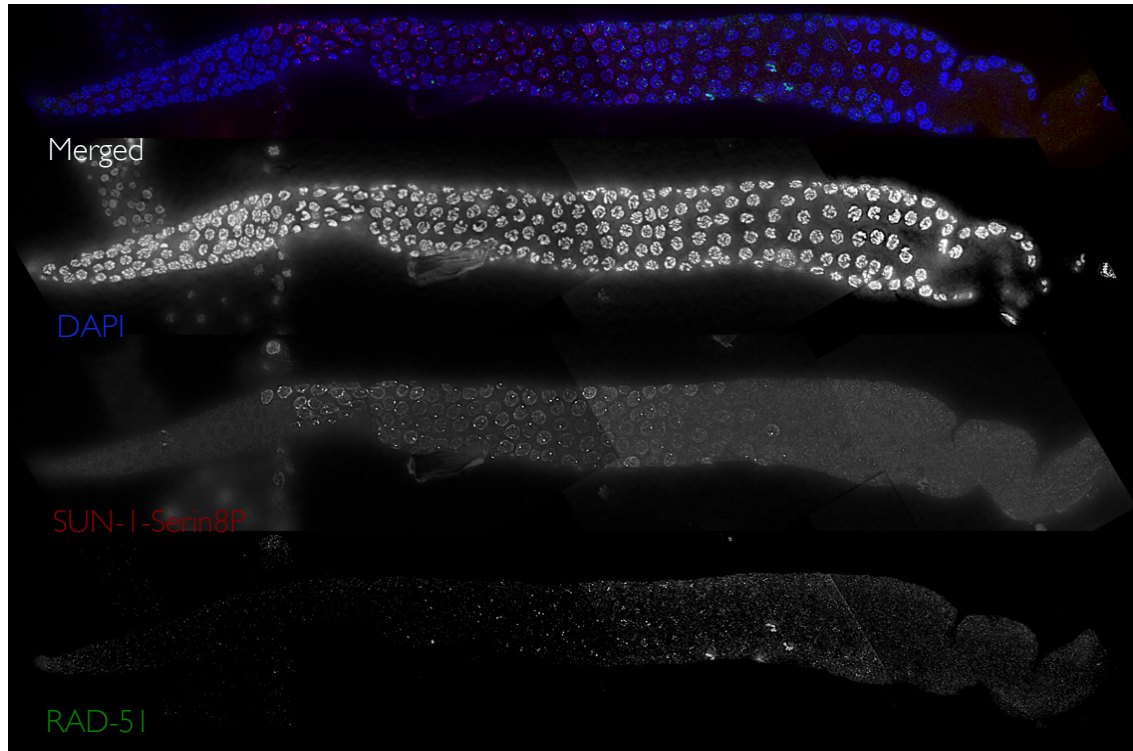


Figure 5: Triple staining of *rrf-3*, fed on OP50: DAPI (blue): After about 17 rows of mitotic zone, the nuclei enter TZ, which is marked by tight clustering of chromatin to one side of the nucleus. At the transition from the mitotic region to the TZ SUN-1 S8 gets phosphorylated and SUN-1 S8Pi (red) aggregates concentrated to chromosome ends are visible. RAD-51 (green) staining starts in the mid of TZ. 4 nuclei in mid/late pachytene show high incidence of RAD-51 along with phosphorylation of SUN-1 S8.

Results summary

All dissected gonads were stained with DAPI and an antibody against SUN-1 S8Pi as well as RAD-51. This staining was chosen because a lot of different meiotic features can be analyzed and compared. Furthermore, almost all meiotic mutants that I analyzed showed an aberration in at least one of the markers I used. In the initial screen, a total of 39 candidates (see Table 2) were analyzed. For this purpose, the gene products were either depleted by RNAi feeding or genomic deletion strains for the respective gene generated by random mutagenesis were obtained from the Caenorhabditis Genetics Center (CGC).

Table 1 shows an overview of observed phenotypes and Table 3 a more detailed list of the investigated candidates. In summary, an aberrant phenotype was

observed in 25 out of 39 candidates. We observed developmental defects, resulting in larval arrest, in 7 of the candidates. Larval arrest was most of the time (6 out of 7) accompanied with very low (less than 50%) viability of the progeny. We found 9 candidates with a shortening of chromatin clustering in TZ. This was correlated with prolonged SUN-1 S8Pi in three instances. The TZ clustering was prolonged in five examined candidates. Alteration of the phosphorylation pattern of SUN-1 S8Pi occurred 8 times. It was prolonged in 7 cases and it correlated with larval arrest in 4 cases. Shortening of the phosphorylation was seen only once in the screen. RAD-51 foci kinetics was altered in 13 candidates. In 8 cases the numbers of nuclei in late pachytene with high number of RAD-51 were increased. In these nuclei also SUN-1 S8Pi persisted. One candidate displayed a shortening of the zone displaying RAD-51. One candidate exhibited a prolonged RAD-51 staining. The results of the examined candidates are summarized table 3 (short version in table 1) and highlight the very wide range of different phenotypes observed.

Phenotype	Number of candidates
Larval arrest	7
Decreased viability	10
Aberrant RAD-51 staining	13
Aberrant SUN-1 S8Pi staining	8
Shortening/absence of TZ	9
Prolongation of TZ	6
wild type	14

Table 1: Overview of observed phenotypes

Nr.	Gen name	Systematic name	Description of wormbase
1	<i>cbd-1</i>	<i>H02I12.1</i>	Chitin-Binding Domain protein family member (cbd-1)
2	<i>Y6B3B.9</i>	<i>Y6B3B.9</i>	hypothetical protein Y6B3B.9
3	<i>symk-1</i>	<i>F25G6.2</i>	hypothetical protein F25G6.2
4	<i>rpn-6.1</i>	<i>F57B9.10</i>	Proteasome regulatory particle, non-atpase-like protein 6, isoform a
5	<i>msh-6</i>	<i>Y47G6A.11</i>	Msh (muts homolog) family protein 6
6	<i>mmaa-1</i>	<i>T02G5.13</i>	MethylMalonic Aciduria type A protein family member (mmaa-1)
7	<i>sun-1</i>	<i>F57B1.2</i>	SUN (S. pombe sad1/Ce-UNC-84) domain protein family member (sun-1)
8	<i>dpm-1</i>	<i>Y66H1A.2</i>	Dolichol Phosphate Mannosyltransferase family member (dpm-1)
9	<i>rpn-12</i>	<i>ZK20.5</i>	proteasome Regulatory Particle, Non-ATPase-like family member (rpn-12)
10	<i>F38A1.8</i>	<i>F38A1.8</i>	hypothetical protein F38A1.8
11	<i>tag-76</i>	<i>ZK757.3</i>	Temporarily Assigned Gene name family member
12	<i>syp-1</i>	<i>F26D2.2</i>	SYP-1 is a synaptonemal complex component
13	<i>glna-1</i>	<i>C09F9.3</i>	hypothetical protein C09F9.3
14	<i>rab-11.1</i>	<i>F53G12.1</i>	rab11-like
15	<i>F13D12.5</i>	<i>F13D12.5</i>	hypothetical protein F13D12.5
16	<i>npp-10</i>	<i>ZK328.5</i>	Nuclear pore complex protein protein 10, isoform b, ZYGote defective: embryonic lethal family member
17	<i>zyg-9</i>	<i>F22B5.7</i>	(zyg-9)
18	<i>rpt-5</i>	<i>F56H1.4</i>	proteasome Regulatory Particle, ATPase-like family member (rpt-5)
19	<i>mog-4</i>	<i>C04H5.6</i>	Masculinisation of Germline family member (mog-4)
20	<i>xpo-1</i>	<i>ZK742.1</i>	eXPOrtin (nuclear export receptor) family member (xpo-1)
21	<i>prp-21</i>	<i>W07E6.4</i>	yeast PRP (splicing factor) related family member (prp-21) similar to yeast pre-mRNA splicing factor PRP21,
22	<i>clcc-222</i>	<i>C03E10.6</i>	C-type LECTin family member
23	<i>spe-15</i>	<i>F47G6.4</i>	defective SPERmatogenesis family member (spe-15)
24	<i>ZK632.7</i>	<i>ZK632.7</i>	hypothetical protein ZK632.7
25	<i>C25D7.12</i>	<i>C25D7.12</i>	hypothetical protein C25D7.12
26	<i>ugt-46</i>	<i>B0310.5</i>	UDP-GlucuronosylTransferase family member
27	<i>pkc-2</i>	<i>E01H11.1</i>	protein kinase C2 A isoform
28	<i>xpo-2</i>	<i>Y48G1A.5</i>	eXPOrtin (nuclear export receptor) family member (xpo-2) Importin beta family protein 5
29	<i>F13H10.4</i>	<i>F13H10.4</i>	hypothetical protein F13H10.4
30	<i>capg-1</i>	<i>F29D11.2</i>	hypothetical protein F29D11.2
31	<i>Y54G2A.2</i>	<i>Y54G2A.2</i>	hypothetical protein Y54G2A.2
32	<i>faah-1</i>	<i>Y56A3A.12</i>	hypothetical protein Y56A3A.12
33	<i>W02B12.15</i>	<i>W02B12.15</i>	hypothetical protein W02B12.15
34	<i>W03F11.1</i>	<i>W03F11.1</i>	hypothetical protein W03F11.1
35	<i>Y92H12BR.3</i>	<i>Y92H12BR.3</i>	hypothetical protein Y92H12BR.3

36	<i>mms-19</i>	<i>C24G6.3</i>	hypothetical protein C24G6.3
37	<i>Y54E10BR.5</i>	<i>Y54E10BR.5</i>	hypothetical protein Y54E10BR.5
38	<i>D2045.2</i>	<i>D2045.2</i>	hypothetical protein D2045.2
39	<i>W08F4.3</i>	<i>W08F4.3</i>	hypothetical protein W08F4.3

Table 2: Screened candidates subjected to the primary screen. ‘Description found in wormbase’ refers to the entries in wormbase (<http://www.wormbase.org/>) in the section ‘overview’ for a particular gene.

No.	Gen name	Viability F1	TZ length	SUN-1 S8Pi	RAD-51	F1	further findings	CGC
1	<i>cbd-1</i>	46%	---				less progeny	+
2	<i>Y6B3B.9</i>	99%	---	+++	-			
3	<i>symk-1</i>	35%	--	++	A	-	larval arrest	
4	<i>rpn-6.1</i>	1%	--			-	arrested MN	
5	<i>msh-6</i>	89%	--	+++	A		shortened gonad	+
6	<i>mmaa-1</i>	99%	--					+
7	<i>sun-1</i>	75%	--	depleted	++		LN	
8	<i>dpm-1</i>	93%	--					
9	<i>rpn-12</i>	91%	--					+
10	<i>F38A1.8</i>	1%	++	++	----	-	larval arrest	
11	<i>tag-76</i>	50%	++					+
12	<i>syp-1</i>	96%	+++	+++				
13	<i>glna-1</i>	98%	++		A		SM	
14	<i>rab-11.1</i>	88%	++	+				
15	<i>F13D12.5</i>	99%	++	+				
16	<i>npp-10</i>	1%	DO	++	A	-	larval arrest	+
17	<i>zyg-9</i>	46%	-	++		-	larval arrest	
18	<i>rpt-5</i>	3%	DG			-		
19	<i>mog-4</i>	8%	DG	--	A	-	larval arrest	+
20	<i>xpo-1</i>	14%		-	A	-	larval arrest	
21	<i>prp-21</i>	37%	+		A		slow growth, PM	
22	<i>clcc-222</i>	97%			A			
23	<i>spe-15</i>	99%			A			

24	<i>ZK632.7</i>	100%	--	+	A			
25	<i>C25D7.12</i>	97%	-		A			
26	<i>ugt-46</i>	82%				-	larval arrest	
27	<i>pkc-2</i>	88%						+
28	<i>xpo-2</i>	82%	+	+			shortened gonad sick, shortened gonad	+
29	<i>F13H10.4</i>	97%						
30	<i>capg-1</i>	100%						
31	<i>Y54G2A.2</i>	98%	-					
32	<i>faah-1</i>	100%						+
33	<i>W02B12.15</i>	98%						
34	<i>W03F11.1</i>	93%						
35	<i>Y92H12BR.3</i>	100%	-					
36	<i>mms-19</i>	100%						+
37	<i>Y54E10BR.5</i>	100%	-					
38	<i>D2045.2</i>	95%					slow growth	
39	<i>W08F4.3</i>	95%	-					

Table 3: Observed phenotypes of the screened candidates. Viability of the F1 generation is displayed as percentage of hatched larvae compared to the amount of laid eggs. **TZ:** +++ refers to a TZ that is prolonged until the end of the gonad, ++ an intermediate prolongation of about 50-75% of the wild-type length, + a slight prolongation of less than 50% of the wildtype. --- Reflects an absence of the typical clustering in TZ, -- a medium shortening of the clustering of up to 4 rows of clustering – a moderate shortening of TZ to about 5-7 rows. The same code was used for describing the shortening and prolongation of the zone positive for SUN-1 S8Pi; AC... aberrant chromatin shows small pieces of clustered and non clustered chromatin in the whole gonad, DO... disorganized gonad show clustered and non-clustered nuclei not confined to TZ. **RAD-51:** A...arrested nuclei indicates an increase of nuclei in late pachytene that still have a lot of RAD-51 along with SUN-1 S8Pi. **Gonad:** - indicates a shorter gonad (about 2/3 of the wildtype). SM...shortened mitotic zone, PM... prolonged mitotic zone, LN... less nuclei, arrested MN...arrested nuclei in metaphase in the mitotic zone; Absence of F1 is indicated by -. A + in the “CGC” column indicates that a deletion strain from CGC was analyzed. Aberrant phenotypes are highlighted in yellow.

Outstanding candidates, remarkable phenotypes, existing information on the candidates and conclusion

After the initial screen, we selected certain candidates for closer examination. We chose the candidates that showed no or very few nuclei with clustered chromatin in the TZ to examine whether we could observe any defects in the meiotic entry or homolog pairing.

Y6B3B.9 – a Las1 homolog

The chromatin in the TZ of *Y6B3B.9i* was distributed throughout the nuclei and SUN-1 S8 remained phosphorylated until the end of the gonad (see Fig. 2). DSB turnover as monitored by RAD-51 staining, exhibited wild-type kinetics. Both the viability and the total number of laid eggs were similar to the control reference strain. We did not observe any larval arrest or developmental defect.

Y6B3B.9 has two different transcripts: *Y6B3B.9a* (485aa) and *Y6B3B.9b* (37aa). Bioinformatics revealed *Y6B3B.9* as a homolog of the evolutionary conserved yeast *Las1*, which is required for pre-rRNA processing at both ends of ITS2 (internal transcribed spacer 2) (Schillewaert, Wacheul et al. 2012). ITS2 separates the 5.8S and 28S rRNA and its cleavage is essential for correct formation of rRNA.

We chose this candidate because, of the fact that no clustering (see Fig. 3) was observed in TZ and phosphorylation of SUN-1 S8 stayed until the end of the gonad. This suggests a role of *Las1* in meiosis. On the other hand, viability of the offspring was not influenced by the knockdown. This could mean that the phosphorylation of SUN-1 S8 has only a minor role in establishment of homologous pairing. Also the clustering in the TZ appeared not to be essential for this task. Besides, *Las1* codes for a protein that is widely used in rRNA processing, which could implicate that its

knockdown has an affect on multiple other proteins and leads to a phenotype that reflects the knockdown of multiple genes.

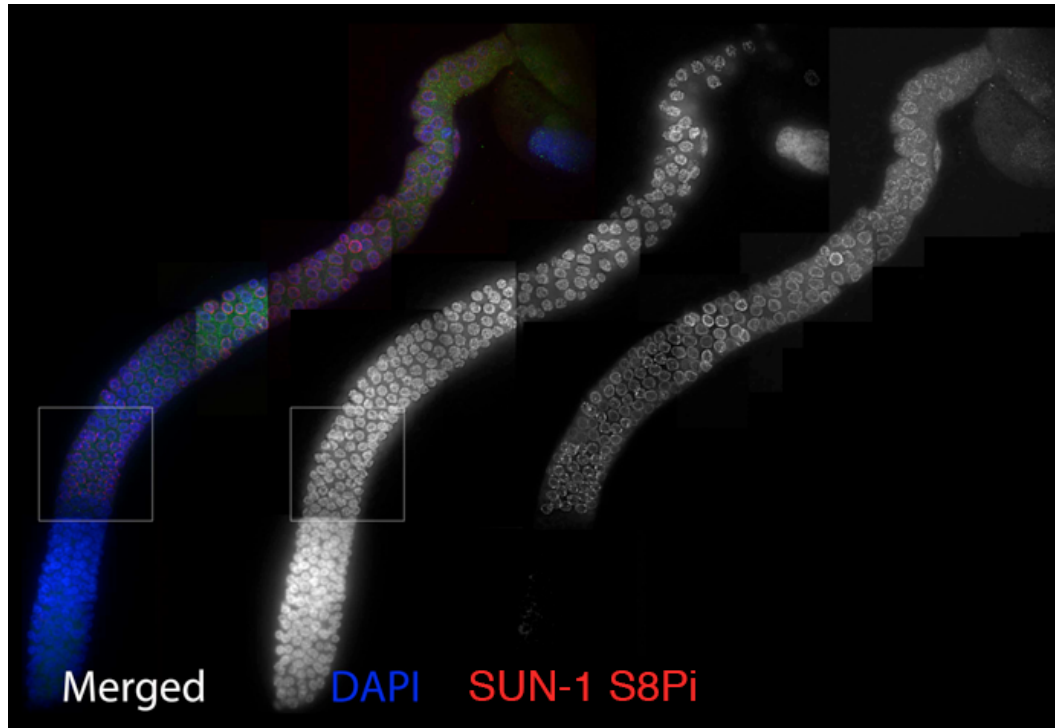


Figure 6: Gonad of *Y6B3B.9i*: The chromosomes (blue) show no visible clustering in the TZ (marked by grey squares). SUN-1 S8 (red) is phosphorylated and aggregates form as seen in the reference control strain. Despite disappearance of SUN-1 S8Pi concentrated into aggregates at chromosome ends, SUN-1 S8Pi persisted until the end of the gonad.

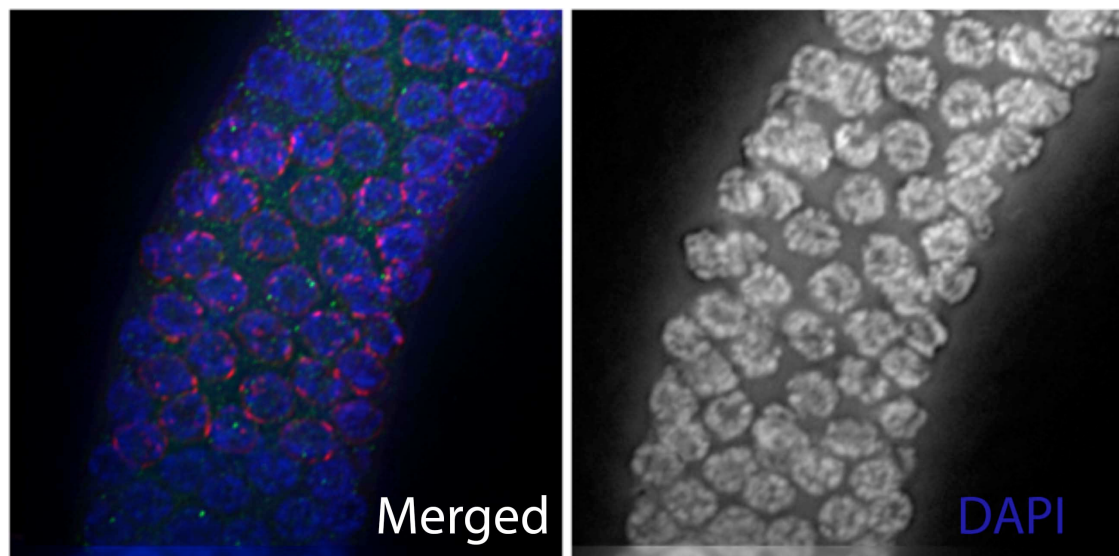


Figure 7: Close-up of the TZ of *Y6B3B.9i*: **Merged:** The entry of TZ is clearly highlighted by SUN-1 S8Pi (red). **DAPI (blue):** The chromatin of TZ nuclei never adopted

the half moon shape. Nuclei of the mid and late TZ show stretched DAPI structures that resemble pachytene chromosomes.

cbd-1

Knockdown of *cbd-1* had a dramatic impact on maternal egg-laying and offspring viability with an egg-laying rate as low as about 20% of the wild type and a hatch rate of 45%. The TZ of this knockdown exhibited un-clustered chromatin (see Fig.5), similar to the mitotic zone. SUN-1 S8Pi started in a wild-type manner with SUN-1 S8Pi aggregates formation right at the entry of the meiotic zone. However the zone with nuclei with SUN-1 S8Pi aggregates seemed shortened. DSB formation and repair showed wild-type kinetics (see Fig. 4).

cbd-1 encodes a 1319 aa large protein with 12 chitin binding residues. CBD-1 is needed to ensure the osmotic stability of the eggshell and the embryo (Sonnichsen, Koski et al. 2005). It is probably also needed for eggshell synthesis and mechanical cross linking of chitin (Tjoelker, Gosting et al. 2000) in the eggshell and prevention of polyspermy (Johnston, Krizus et al. 2010).

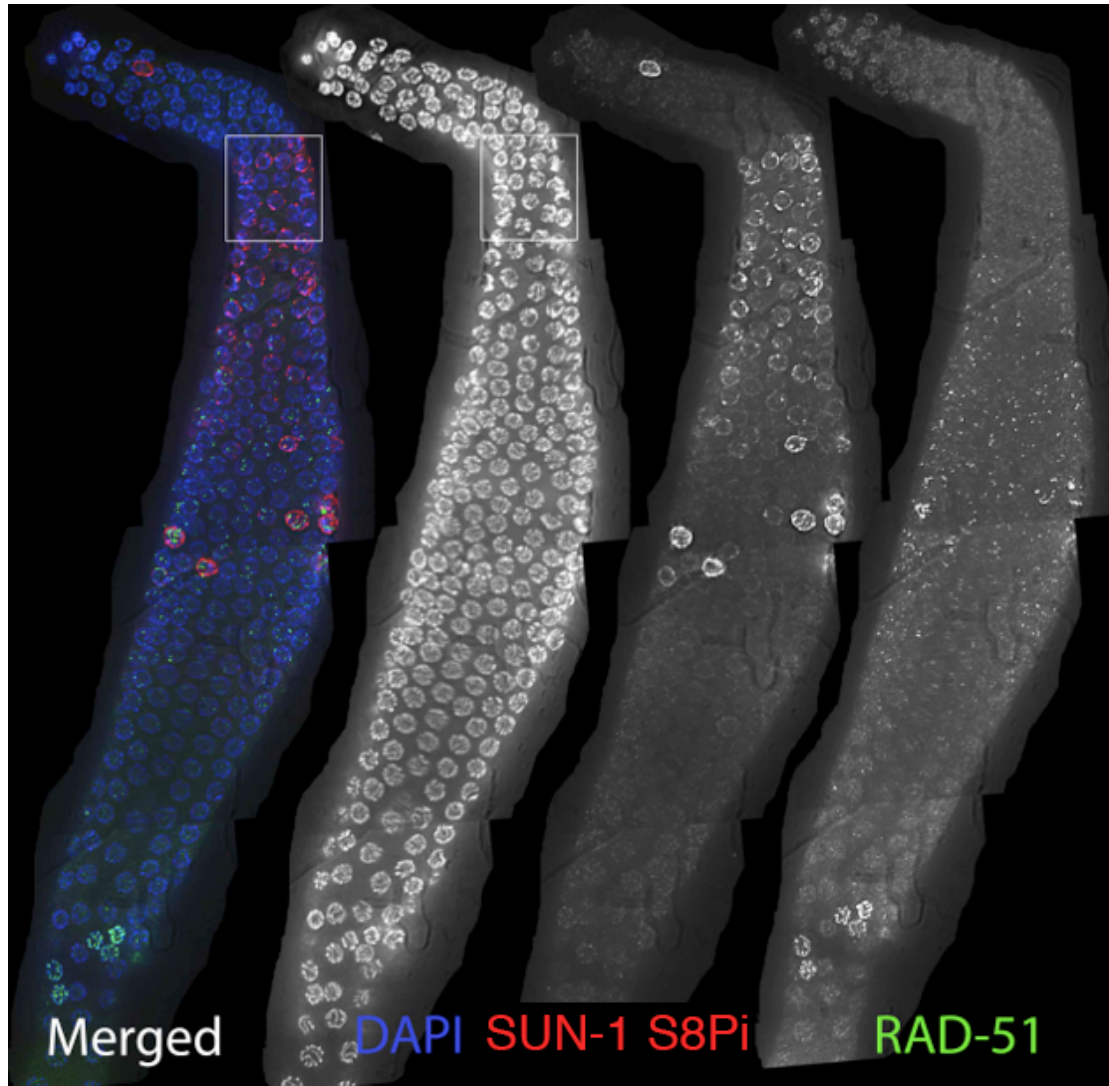


Figure 8: Gonad of *cbd-1i*: Despite SUN-1 S8Pi (red), the chromatin (blue) at the TZ entry (marked by grey squares) stays distributed over the entire nucleus. The zone of SUN-1 S8Pi seemed shortened in this gonad. RAD-51 (green) foci appear at the end of TZ and peaked in mid pachytene. Some nuclei of the mid pachytene showed accumulated RAD-51 along with SUN-1 S8Pi.

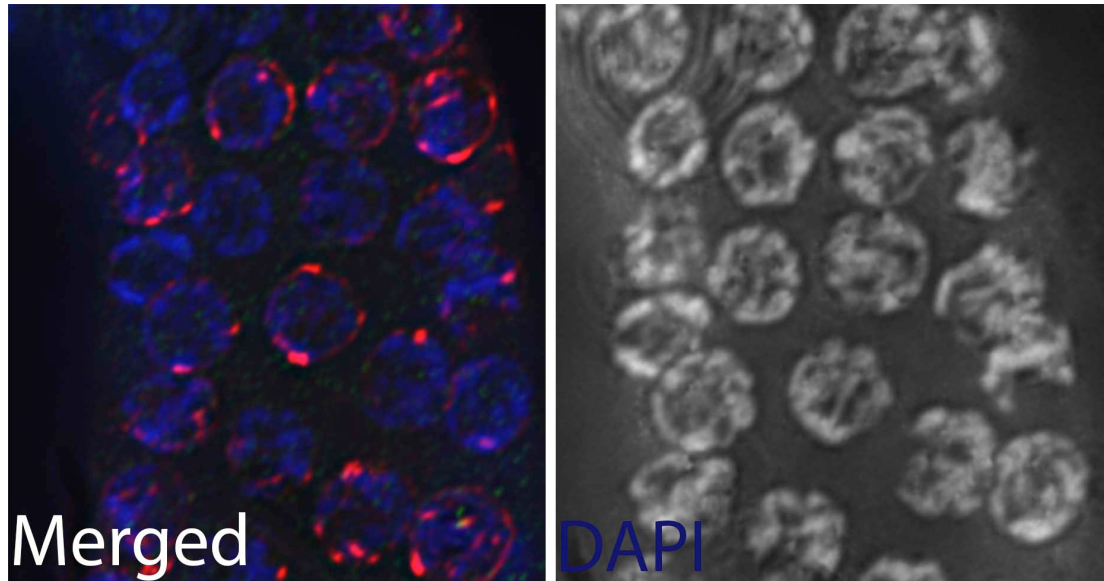


Figure 9: Close-up of *cbd-1i* TZ: Merged: SUN-1 S8Pi (red) marks the entry of meiosis; SUN-1 S8 Pi aggregates were formed. **DAPI (blue):** Mitotic and meiotic nuclei show the same visual appearance.

The analyzed CBD-1::GFP strain, a kind gift from James Dennis, showed expression of CBD-1 only in the outer membrane of the oocytes with some diffuse cytoplasmic signal (see Fig.6). This strain was not for the analysis of CBD-1 in the gonad due to germ line silencing.

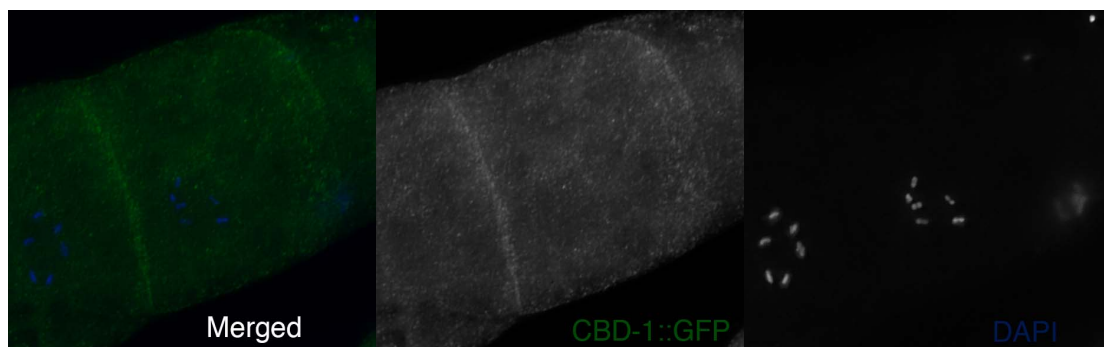


Figure 10: Diakinesis of a wild type gonad expressing CBD-1::GFP. DAPI (blue): Diakinesis nuclei show 6 DAPI positive structure, reflecting the 6 paired homologous chromosomes. CBD-1::GFP (green) is expressed only in diakinesis and localizes at the membrane of the oocyte.

The strongly reduced egg-laying frequency (about 20% compared to the control) of the knockdown could not only indicate a problem in meiosis, but is

probably due to the essential function of CBD-1 in eggshell synthesis, which prevents polyspermy (Tjoelker, Gosting et al. 2000; Johnston, Krizus et al. 2010). Additionally, the very low hatch rate (45% of the control) could be explained by the contribution of CBD-1 to osmotic stability of the embryo. Successfully hatched embryos developed normally, suggesting that the CBD-1 acts exclusively in eggs and embryos.

Unfortunately, the TZ phenotype (absence of clustering at the entry of TZ) could not be reproduced. The egg laying defect as well as the viability defect was reproduced three times.

symk-1

symk-1 depletion led to very low viability (36%) and larval arrest of the hatched progeny. SUN-1 S8 was phosphorylated at the beginning of TZ accompanied with a less pronounced chromatin clustering than in the respective zone of the reference control gonads. Also nuclei that exhibited absence of chromatin clustering were observed. SUN-1 S8Pi aggregates were very small which could either mean that less phosphorylated SUN-1 S8Pi was concentrated into aggregates at chromosome ends or the expression of SUN-1 per se was lower. In addition, the mitotic zone was shortened to about 2/3 of the wild-type length. In the wild type, the mitotic zone extended over about 20 cell rows, whereas this knockdown exhibited a mitotic zone shortened to about 12-13 cell rows (n=5 for each genotype).

symk-1 encodes an evolutionary conserved 1143 aa long cleavage and adenylation factor. Knockdown of *symk-1* by RNAi revealed a function in distal tip cell (DTC) migration (Cram, Shang et al. 2006). *symk-1* is also involved in life span regulation (Curran and Ruvkun 2007) with an increased life span of 25% compared to control, if depleted.

The DTC is needed and sufficient for induction of the mitotic stem cell niche. Therefore, a variation of the migration of the DTC could be an explanation for the shortened mitotic zone in this knockdown. The larval arrest and low viability (34%) of this mutant indicate important functions in development and growth; this would be consistent with its essential function of mRNA stability and regulation. The phenotypes of the *symk-1* knockdown were reproducible and showed only little variation concerning the extent of clustering in TZ.

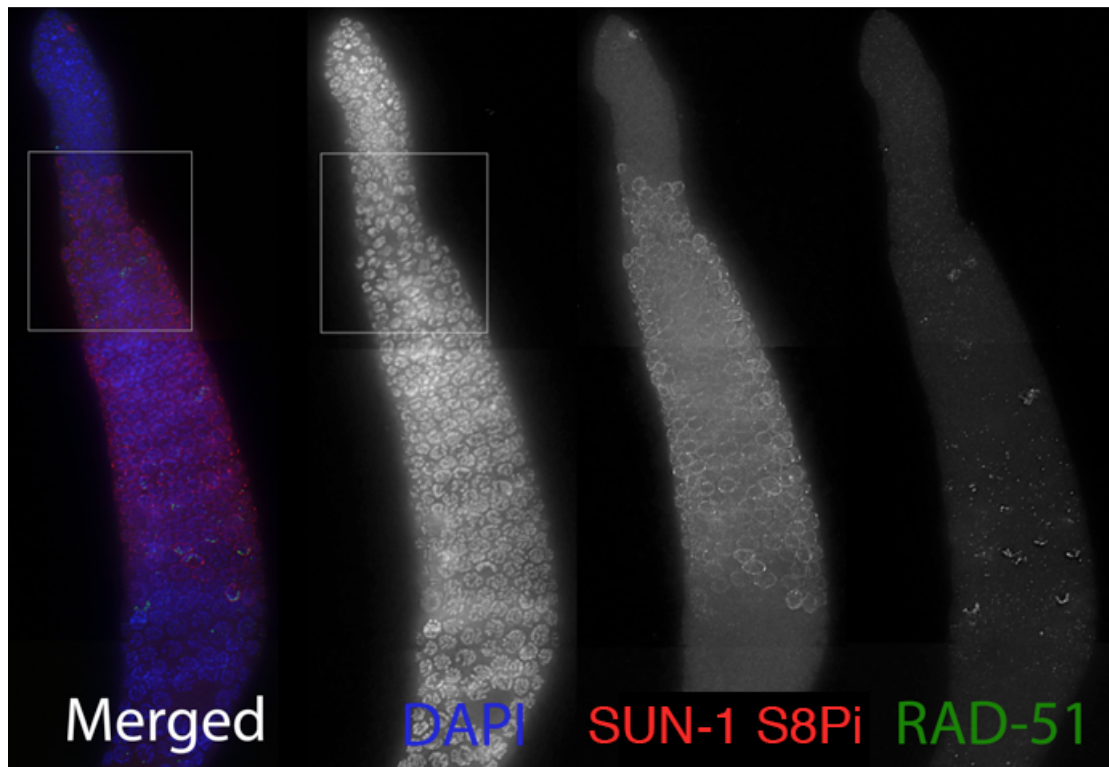


Figure 11: Gonad of *symk-1i*: In this candidate the phenotype was variable concerning the extent of chromatin (blue) clustering in the TZ. Some gonads showed almost wild-type clustering while others showed no clustering at all. Less phosphorylated SUN-1 S8 (red) was organized in aggregates, therefore the staining of the nuclear membrane by SUN-1 S8Pi was by far stronger than in the control. RAD-51 (green) turn over was normal, but there were some isolated nuclei with increased RAD-51 foci which was accompanied by SUN-1 S8Pi.

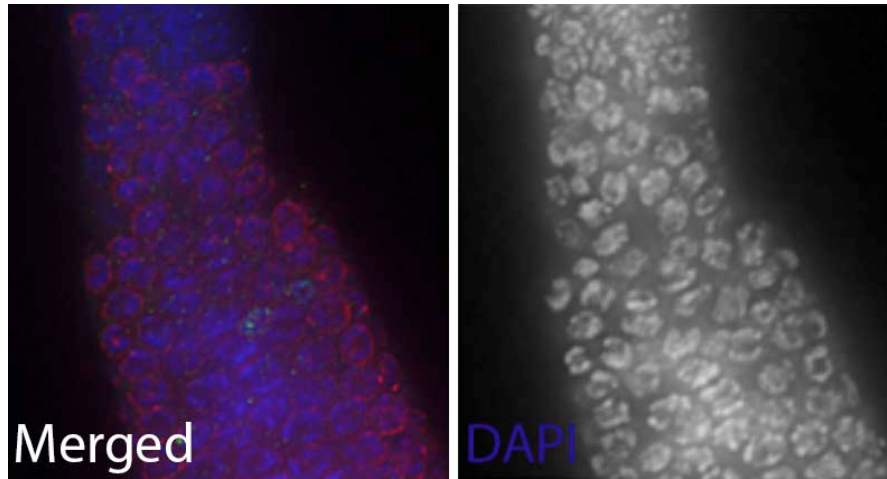


Figure 12: TZ close-up of *Symk-1i*: Chromatin (blue) clustering in the TZ is not as distinct as in wild type. Less SUN-1 S8Pi (red) is organized into aggregates.

Knockdown of proteasome components *rpn-6.1* and *rpt-5*, respectively

Knockdown of components of the proteasome or the proteasome-regulating particle in this screen (like in *rpn6.1i* and *rpn5i*) led to a drastic prolongation of the mitotic zone. The mitotic zone of both knockdowns had about the double length of the wildtype mitotic zone. In addition, a high number of mitotic nuclei showed strongly condensed DAPI signals, accompanied by dots of phosphorylated SUN-1 S8Pi. This phosphorylated SUN-1 S8Pi most likely highlight the centrosomes and therefore indicate a mitotic arrest in the anaphase. The viability was extremely low and hatching offspring showed growth defects and larval arrests. The TZ was marked by phosphorylated SUN-1 S8Pi and consistent with a prolonged mitotic zone, started later in the middle of the gonad (the region corresponding to pachytene in wild type gonads). The chromatin clustering in the TZ seemed reduced in these knockdowns. Only some nuclei were clustered in the TZ (see Fig.8).

rpn 6.1 codes for a non-ATPase subunit of the 19S regulatory complex of the proteasome. Former RNAi studies revealed various numbers of phenotypes associated with a gene knockdown including: variation of chromatin condensation,

impaired expansion and anucleation of the germline compartment (Green, Kao et al. 2011). The same phenotypes were observed for *rpt 5i*, which encodes a subunit of the 19S proteasome regulating particle.

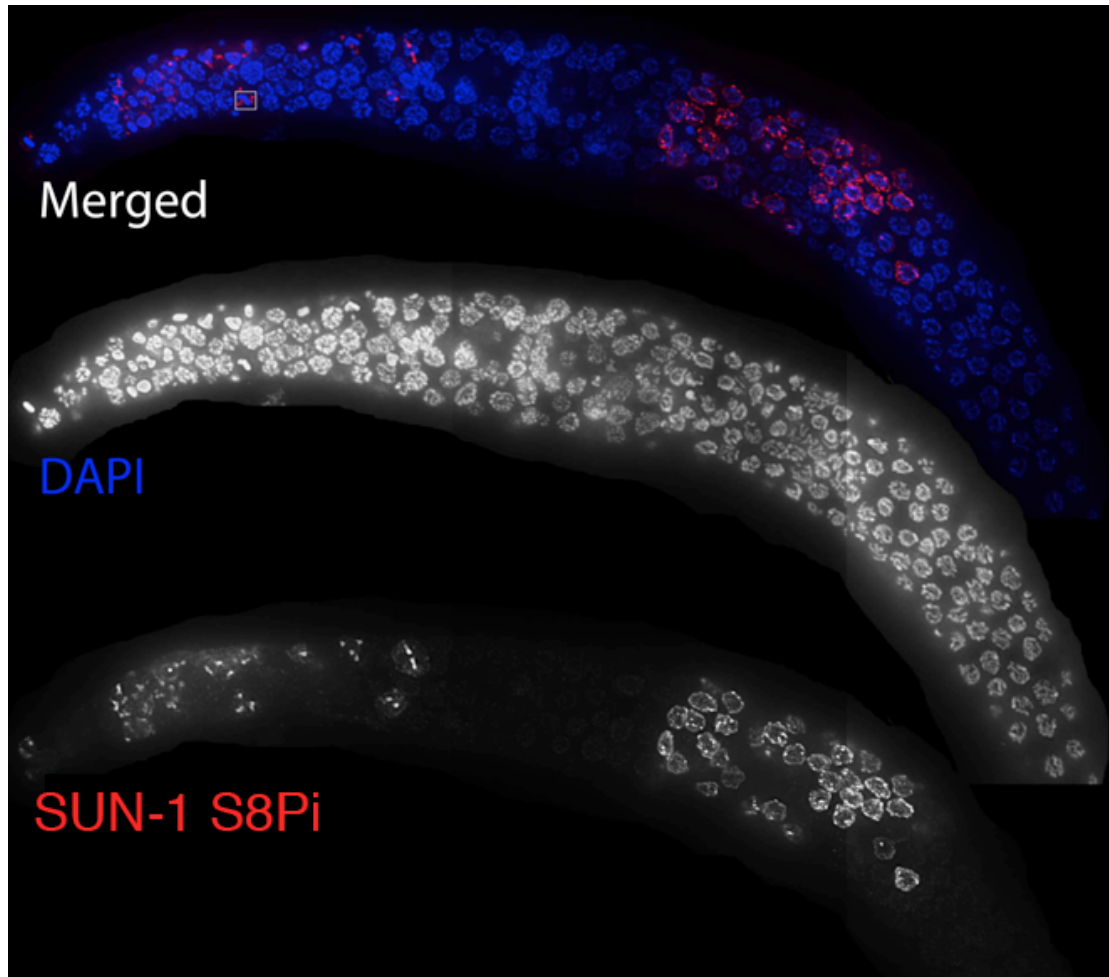


Figure 13: Gonad of *rpn6.1i*: The mitotic zone was extremely prolonged. Condensed chromatin (blue) in the mitotic zone was accompanied by dots SUN-1 S8Pi (red). The clustering in the TZ was disorganized. Phosphorylation of SUN-1 S8 was not always parallel by clustering of the chromatin.

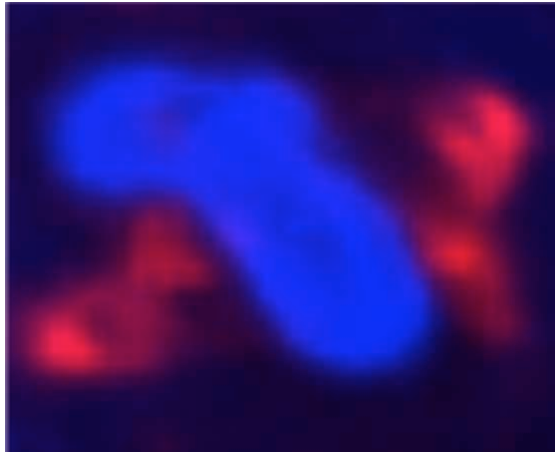


Figure 14: Close-up of a nucleus of the prolonged mitotic zone of *rpn 6.1i*: The chromatin (blue) of this nuclei was condensed as seen in anaphase nuclei and the centrosomes were highlighted by SUN-1 S8Pi (red).

Analysis of meiotic entry and chromosome pairing

ZIM-3 antibody staining

To determine whether the loss of clustering in the transition zone (as seen in *Y6B3B.9i* and *symk-1i*) was due to a failure to enter meiosis, we stained for ZIM-3, a marker for the meiotic entry (Phillips and Dernburg 2006). ZIM-3 is expressed at the beginning of meiosis and localizes with the PCs of chromosome IV and I. We found that ZIM-3 was expressed in both of the tested knockdowns. ZIM-3 was also stained in the knockdowns of proteasome components *rpn6.1* and *rpn5*, respectively. ZIM-3 was expressed in both knockdowns, however with a delay. The ZIM-3 staining localized with clustered chromatin in the TZ in those gonads.

FISH analysis

We used a probe, which highlighted the rDNA locus on chromosome V to determine the kinetics of homologous pairing in the candidates that showed absence of clustered chromatin in the TZ. Both *symk-1i* and *Y6B3B.9i* showed wild-type pairing. An example of this staining is shown in figure 11. The nuclei at the tip of the gonad showed distribution of the chromatin as typically seen in mitotic nuclei. The

FISH staining showed two dots per nuclei, indicating unpaired homolog chromosomes. After a very short zone in which nuclei showed two FISH signals per nuclei, the FISH signal merged into one after only 10 rows of nuclei. This sharp edge (see figure 12) would suggest that this is also the site of meiotic entry. Because of the shortened mitotic entry zone this FISH staining cannot be directly compared to a wild type control. For this purpose an additional marker for the meiotic entry is needed. Thus, the next step would be to establish immuno-FISH staining which would allow detection of meiotic entry together with a FISH probe to assess the two parameters simultaneously. Concerning the Immuno-FISH, we could establish the antibody staining, but the FISH staining shows only a faint signal and high background. Thus, this technique needs further improvement.

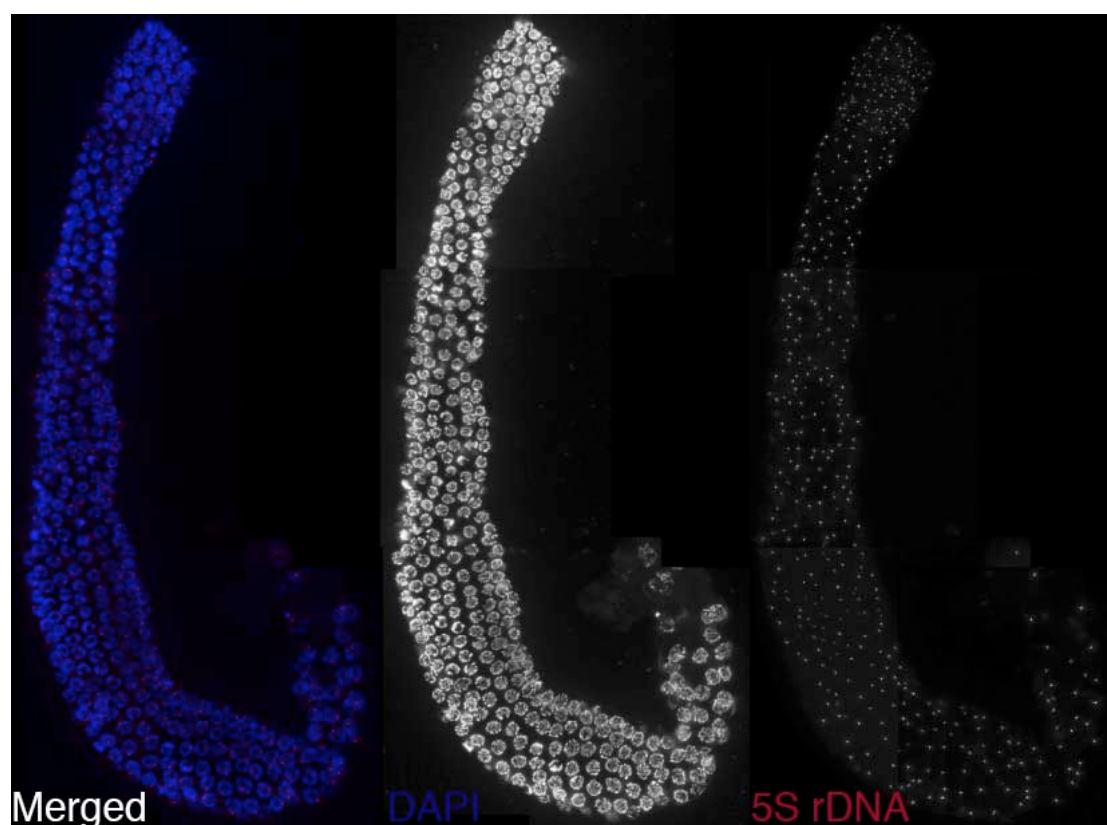


Figure 15: FISH staining for *symk-1i*. 5S rDNA probe (red) was used to analyze pairing in this knockdown. Chromatin (blue) shows no clustering at the site of paired FISH signals

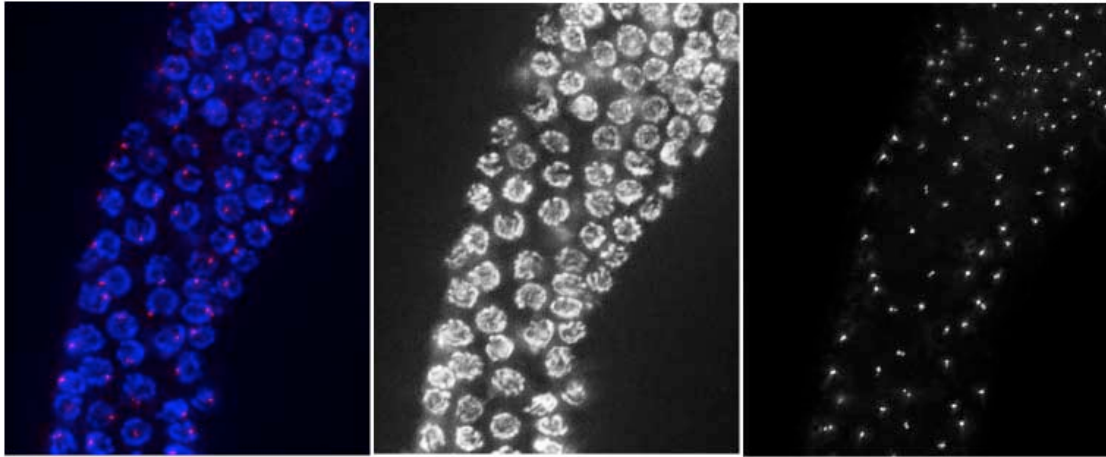


Figure 16: Close-up of the FISH staining in *symk-1i*: Close-up of the region corresponding to the TZ as judge by the pairing of FISH signals (red).

Discussion

Conclusions and findings in the screen

About the aim of this thesis

The initial aim of this thesis was to identify a linker protein (factor X) between the SUN/KASH bridge of the nuclear envelope and the chromosomes inside the nucleus. This hypothetical protein would have the task of transferring the cytoskeletal force onto the chromosomes by mediating a linkage between SUN-1 and the chromosomes and would therefore be essential for the movement of the chromosomes in early meiosis. In the well studied example of horsetail movement in *S. pombe* the Bqt-1 and 2 protein complex has been identified as such a linker protein complex between telomeres and the SUN domain bearing protein Sad1 (for review see, Tomita and Cooper, 2006). We hypothesized that the depletion of an analogous factor in *C. elegans* would lead to cessation of chromosome movement. Since chromatin clustering in leptotene/zygotene is a consequence of chromosome movement we expected absence of “TZ like nuclei” in such a mutant. Although we found promising candidates (*symk-1i* and *Y6B3B.9i*) with respect to the impairing of chromatin clustering in TZ, none of them fulfilled all the expected phenotypes of this potential linker protein. Those expectations, apart from the reduced clustering in TZ, would also include: failure of homologous pairing, a prolonged zone of SUN-1 S8Pi and a failure of DSB repair leading to persistent RAD-51 foci.

There are various explanations that we might have missed the linker protein with this screen. A likely explanation would be that the factor X was not included in the list of candidates that we subjected to our screen. Taking in account the way the list was obtained (two immune-precipitations and mass-spectrometry), reveals the possibility that the searched protein was not stable in one of the precipitation

conditions and was therefore ruled out at the beginning. This would also be true, if the interaction between SUN-1 and the linker protein was not stable or strong enough to be pulled down with SUN-1 under the IP conditions that were used. Considering the forces exerted on chromosomes during chromosome movement, we expected an interaction between SUN-1 and a linker protein to be quite robust and stable.

In a future approach we could chose to use a different approach and use a system that proved to be very successful in detecting protein interactions of the nuclear lamina (Gerace and Huber 2012; Roux, Kim et al. 2012). With this technique a certain target protein (e.g. SUN-1) would be fused to BirA*, a biotin ligase modified to act in a promiscuous way and would therefore enable the protein to labeling its nearby partners with biotin (a small protein also known as vitamin H or co-enzyme R). The labeled proteins would then be recovered by affinity purification by streptavidin-coated beads and very harsh and denaturing conditions can be used with the advantage of also isolating proteins that would be insoluble under regular IP conditions. (Roux, Kim et al. 2012). This labeling technique has already proven very specific therefore might be more suitable to isolate candidates to be subjected to our primary screen to find the factor X. This method shares one disadvantage with other pull-down approaches (e.g. immuno-precipitation), namely the problem of scale, meaning that low abundance proteins are harder to detect, as their concentration in the pull-down might too low, implicating that a lot of starting material would be needed to enrich the protein of interest. In general, immune precipitations for *C. elegans* germline factors are difficult, since meiotic stages cannot be synchronized and the germline tissue cannot easily be separated from somatic tissue. Another disadvantage comes with the nature of this method, meaning the addition of BirA* to the target protein (e.g. SUN-1) may affect its behavior in terms of activity, localization and interaction with other proteins. However, it was observed that a deletion of the first 60 amino acids of SUN-1 reaching into the nucleus did not result in the *sun-1* null phenotype (Woglar pers. communication). This is the expected portion of the

SUN-1 protein to interact with chromosomes. We therefore believe that the addition of the BirA* would not be too detrimental for SUN-1. The big advantages of this screen would be, in contrast to a Y2H approach (which I will discuss separately below), that the protein interactions would be detected in their native environment and cellular context, meaning that we might detect less false positive interactions, since only interacting proteins would be subjected to biotinylation. In contrast to this, in a Y2H screen interactions are tested with overexpressing two specific proteins leading to random interactions. Along with this, another disadvantage of the Y2H system is avoided, namely the problem of targeting the prey and bait proteins to the nucleus (only here the specific tasks of the Y2H screen can be fulfilled). As the biotinylation happens in the natural environment there is no concern about the natural concentration and localization of the interacting proteins. Screening methods such as Y2H or Y3H library screening would be a less efficient alternative to obtain a more complete screening list for the linker protein.

In the Y2H assay a protein of interested (e.g.SUN-1) would be fused to a bait molecule and used to screen a prey library of *C. elegans* open reading frames. (Young 1998). The disadvantage of the Y2H system lies in the high number of false negative and false positive results and the possibility of rendering proteins non-functional by the fusion with a prey or bait molecule. The exact rate of false positive matches in the Y2H system is not known, but is believed to be up to 70% (Deane, Salwinski et al. 2002). These false positives can occur, because the overexpression of the potential interacting partners can lead to an unnatural concentration of the proteins and the interaction may only specifically take place in yeast whereas the interacting proteins are not even expressed in the same cell type of the investigated host organism. There is also a high potential of false negative interactions resulting from the fact that the bait and prey molecule may alter the protein interacting surface or the proteins are not expressed or modified in the same way as in the host organisms (in yeast proteins might fold differently or the post-translational modifications might not be properly

added). Specific protein modifications, required for successful protein/protein interactions might also not be taking place upon expression in a heterologous system.

Another explanation for the outcome of this screen would be that the hypothetical linker protein does not exist or the task might simply be performed by another protein that already has specific other functions in SUN-1 biology and its knockdown might not result in the particular phenotype we postulated. An example would be PLK-2, which is responsible for the phosphorylation of SUN-1 at the entry of meiosis (Harper, Rillo et al. 2011; Labella, Woglar et al. 2011). PLK-2 is therefore present, when movement of chromosomes is initiated. Thus, it appears possible that PLK-2 is not only responsible for the phosphorylation of SUN-1, but also functions as a physical linker between SUN-1 and the chromosome bound ZIMs. On the other hand, we did not find any evidence for direct binding of PLK-2 with SUN-1 (unpublished data did not show any interaction in a yeast-two-hybrid interaction experiment). However, it was shown that PLK-2 and SUN-1 can be found in a protein complex. (Harper, Rillo et al. 2011).

A further option would be that the linker protein exists and was in the screening list, but the RNAi simply did not work or only small amounts of this protein is sufficient to fulfill its action. In both cases a knockdown would not reveal a specific phenotype, which hints to the linker function of this certain protein. In this case one could consider a more penetrant way of dsRNA delivery, such as dsRNA injections into the germline. Alternatively one could redo the screen with deletion mutant alleles of the candidates that did not show a phenotype with RNAi, however the availability of the deletion alleles might be limited. It could also be that the linker protein has multiple functions during development and its knockout phenotype was pleiotropic and did not allow us to detect its contribution during meiotic chromosome movement.

The last, explanation for the failure of this screen could be that redundant proteins exist, which perform the function of the factor X. In that case, the knockdown of one of these components would not elicit a mutant phenotype because another factor compensates for it.

In summary, the search for this hypothetical linker protein needs further investigation. This would include both, the optimization of the screening candidates and the screening criteria. As stated below, chromatin clustering might not be the best read-out for chromosome movement during leptotene/zygotene.

Chromatin clustering might not be needed for homologous pairing

Although, we found candidates that lacked chromatin clustering at the entry of TZ (marked by SUN-1 S8Pi) we found that they had no obvious problems in homologous pairing at the same time. As for example *symk-1i* showed clear pairing signals of the FISH probe within non-clustered nuclei. This might suggest that the clustering of chromatin to one pole of the nucleus in the TZ is not strictly necessary for homologous pairing. We would therefore, also conclude that the absence of clustering does not automatically lead to an impairing of chromosome movement, as the homolog chromosomes still pair with each other.

More general, this finding shows that the clustering to one side of the nucleus in the TZ might not be a suitable read-out for movement. This hypothesis could be tested by an experiment to follow chromosome end led movement in non-clustered nuclei in the TZ (e.g. *symk-1i*). This could be tested by time-laps microscopy using a SUN-1::GFP reporter to mark chromosome ends. Recently it was found in yeast that the kinetics of chromosome pairing only depended on rapid chromosome movements (RPMs) but not on bouquet formation (Lee, Conrad et al. 2012). This result suggest that chromosomal movement and bouquet formation are two independent actions in the early prophase I. In analogy in worms the chromosome end led movement in the

TZ could be separated into two different features: the movement itself and the restriction of the movement to one pole of the nucleus. Two different sorts of proteins might fulfill these distinguishable features. Therefore, the movement of the chromosomes is essential for homologue pairing but not the restriction of this movement to a limited area in the nucleus. Although these two independent features (individual movement of chromosome ends and limiting the movement to a restricted area) show similarities in the two model systems, the fact that *C.elegans* does not show a typical chromosome bouquet as seen in yeast, might suggest that different regulatory mechanisms in these two organisms are in place.

Components of the proteasome are needed for correct entry of meiosis

Knockdown of proteasome components leads to a largely prolonged mitotic zone due to an arrest of the meiotic pre-cursor cells in the anaphase of mitosis. It was known before that the proteasome plays a major role in the cell cycle progression. In wild type cells the anaphase promoting complex or cyclosome (APC/C), a large, multisubunit E3 ubiquitin ligase, supports the transition from meta- to anaphase as well as the M-phase exit. The APC/C marks its targets with a poly-ubiquitin chain for proteasome-mediated degradation (reviewed in (Peters 2006; Simpson-Lavy, Oren et al. 2010)). One of these mitotic and meiotic targets is securin, whose degradation leads to the release of separase, which for its part cleaves the cohesin complex between sister chromatids in the meta-/anaphase transition or the cyclin B at the exit of M-phase. Absence of a working proteasome therefore impairs the polyubiquitin-mediated degradation of securin and leads to arrest in the meta-/anaphase transition or M-phase exit, respectively. By now most of components of APC/C or the proteasome were identified by an arrest of the one cell embryo in meiosis I. This is also true for components of the proteasome.

Materials and methods

Worm strains used in this thesis

No.	Description
1	Wild Type
845	<i>JfSi1[Psun-1::GFP cb-unc-119(+)] II; sun-1(ok1282) V.</i>
992	<i>rrf-1(pk1417) I.</i>
993	<i>rrf-3(pk1426) II.</i>
1014	<i>msh-6(pk2504) I</i>
1015	<i>tag-76(ok1041) V.</i>
1016	<i>C24G6.3(ok3345) V.</i>
1017	<i>pkc-2(ok328) X.</i>
1018	<i>F13H10.4(ok2135) IV. /nT1[qIs51](IV;V)</i>
1019	<i>mmaa-1(ok2514)/mIn1[mIs14 dpy-10(e128)] II.</i>
1020	<i>mog-4(ok2708)/mT1 II; +/mT1[dpy-10(e128)] III.</i>
1021	<i>cbd-1(ok2913) IV.</i>
1034	<i>+/mT1 II; npp-10(ok467)/mT1[dpy-10(e128)] III.</i>
1063	<i>xpo-2(tm1437) I.</i>
1091	<i>faah-1(tm5011) I.</i>

If not stated otherwise, worms were cultured under standard conditions described by Brenner et al. (Brenner 1974)

Lysis of worms

Worms were lysed to obtain DNA templates for PCR amplification. Single adult worms were picked and placed into a PCR tube, containing 10µl of lysis buffer plus protease K. The samples were then slowly frozen at -80°C for 10 minutes. In the PCR machine the samples were heated up to 65°C for 40 minutes and to 95°C for 15 minutes. After cooling to room temperature (RT) they were ready for PCR amplification.

RNAi feeding

Concept of RNAi in *C. elegans*

The concept of a gene knockdown by RNAi is based on the degradation of its mRNA (Nellen and Lichtenstein 1993). siRNA (small interfering RNA) is produced by a specialized bacteria strain such as HT115 (for more information see *Preparation of feeding plates*), which is used as feeding strain. Upon intake of siRNA by the worms' cells, the siRNA-protein-complex RISC (RNA-induced silencing complex) is formed. This leads to siRNA mediated target recognition and degradation of the mRNA (see Fig.1) (Montgomery and Fire 1998) (Montgomery, Xu et al. 1998) (Fire, Xu et al. 1998). In *C. elegans* there are three different ways to apply siRNA to the worm: feeding, injection and soaking (Tabara, Grishok et al. 1998). To obtain optimal results concerning the knockdown we used a worm strain, which is more sensitive to RNAi treatment (*rrf-3 (pk1426) II.*). *rrf-3* encodes an RNA-directed RNA Polymerase and its knockout leads to enhanced sensitivity to RNAi in divers tissues (Simmer, Tijsterman et al. 2002).

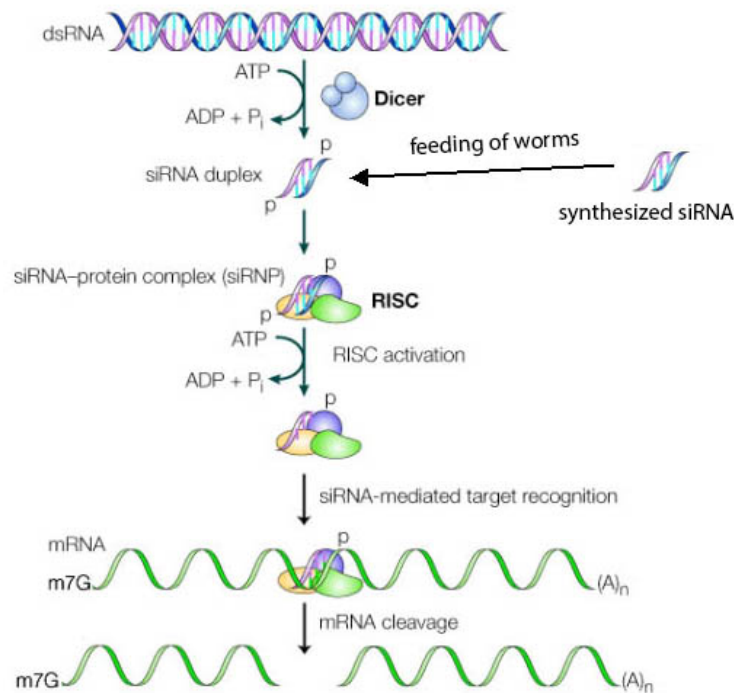


Figure 17: Concept of gene knockdown by RNAi (Dykxhoorn, Novina et al. 2003).

Small interfering RNA (siRNA), are either produced by degradation of a longer dsRNA precursor or are artificially synthesized. The siRNA is bound by a RISC, is single stranded and paired to the single stranded mRNA of the target gene. This leads to mRNA cleavage and therefore, knocks down the target gene.

RNAi feeding

About 20 L4 larvae were cleaned from residual OP50 bacteria by putting them on unseeded plates. Afterwards, the worms were transferred to RNAi-feeding plates and incubated at 16°C for 48 hours. Gonads of those worms were then dissected and prepared as described in the immuno-staining protocol. In case the Po produced viable offspring, these progeny were transferred to new feeding plates and the next generation was subjected to RNAi by feeding.

Cloning of RNAi clones

RNAi stains that were not available in the Ahringer library, were constructed according to the following protocol:

An exon of the candidate gene was amplified via PCR, using a fresh lysate of N2 worms as template (primers see table 4). The expression vector L4440 (Cardwell)

was cut with Sma I (according to the protocol available on Fermentas© homepage; see Fig. 18) to create blunt ends. The vector was then T-tailed and ligated with the PCR-product:

Gene name	3' Primer	5' Primer	Product size	Internal primer number
Dpm-1	gaaaatttgccgatttgc	cacgtgcaaacgataatcca	214bp	VJ926/927
Wo2B12.15	cgtgcaactacaagatcca	acattgtcgccagtcctt	164bp	VJ928/929
Y92H12BR.3	aaacctctccgacctgaagc	ctttcagcgacacgaacaa	164bp	VJ930/931
Y54E10BR.5	aactttgcgatggctgtctc	acgcgtactggatctcaag	167bp	VJ932/933
C25C7.12	aaccaagtcgtctctgtctt	tgtccttggtcacgtctcagc	203bp	VJ934/935
Y54G2A.2	cggagccaaaatccatgtta	accgtccaagaattgcac	237bp	VJ936/937

Table 4: Primer for the ligation product of L4440.

Cutting of L4440

5µl (0.95 µg/µl) L4440, 1µl Sma I, 5µl 10x TANGO buffer and 39µl H₂O were mixed and incubated at 30°C for 2 hours.

T-tailing of L4440

0.4µl TAQ, 5µl 10x Buffer, 4µl dTTPs (10mM) and 40µl of the cut vector were joined and incubated at 72°C for 2 hours

Ligation of PCR product and vector

Set up the ligation with: 1µl Ligase (5u/µl), 2µl 10xBuffer, x µl of Insert, 3.5µl L4440 (15µg/µl), and H₂O to a final volume of 20µl.

The Insert to Vector ratio was 3:1 and 50ng of the vector were used. The amount of insert was calculated according to following equation:

$$Insert(ng) = \frac{(Vector(ng) \times Insert(kb))}{Vector(kb)} \times 3$$

Ligation was done on 14°C over night. All steps were confirmed by gel electrophoresis.

Afterwards, the size of the insertion was checked by PCR and gel electrophoresis, using the following primer:

pL4440-dest-RNAi-FOR (5'-GTTTCCCAGTCACGACGTT-3')

pL4440-dest-RNAi-REV (5'-TGGATAACCGTATTACCGCC-3')

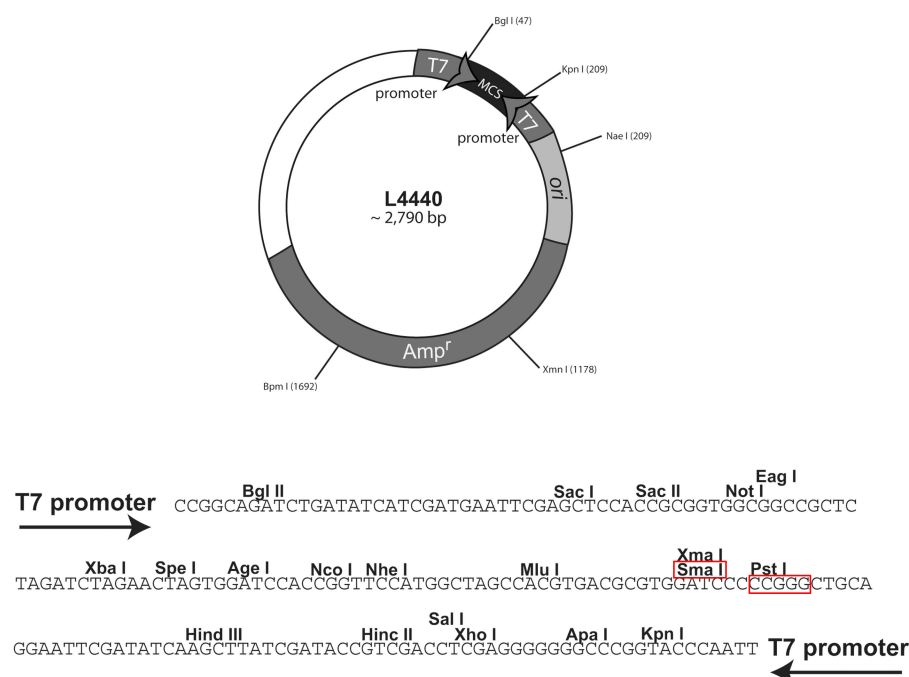


Figure 18: L4440: Map of L4440 and its sequence between the T7 promoter sequence. Cutting site and used restriction enzyme are highlighted in red squares.

Transformation of XL-1 Blue/ HT115

We used XL-1 Blue E. coli strain to grow sufficient amounts of plasmid DNA. The plasmids were then re-transformed to the HT115, which is more suitable for RNAi.

16µl of the ligation mix was combined with 100µl chemically competent cells and put on ice for 15 minutes. A heat-shock on 42°C for 1.5 minutes was followed by an incubation on 37°C for 1 hour. Finally, the cells were incubated on selection plates, containing 60µl X-Gal, 60µl IPTG (1M) and amp (50µl/ml), over night at 37°C.

On the next day, a single white colony was inoculated and prepared for miniprep. Minipreps were done according to the QUIAGEN © protocol (QUIAGEN Midi kit Cat. No. 12143). The plasmid was then re-transformed into the feeding strain HT115, using 2xTY+amp (1µl/ml of a 100mM stock)+tet (1µl/ml of a 15mM stock) as selective media.

Fluorescence in-situ hybridization (FISH)

Preparation of FISH probe (5S) by PCR and nick-translation

<u>PCR mix</u>	<u>PCR program</u>	<u>Nick-translation:</u>
2.5µl 10xTAQ Buffer	94°C 2'	34µl DNA from the previous PCR
1 µl fresh lysate of N ₂	94°C 30''	5µl 2mM dNTP's (GCA)
1µl dNTPs (2,5mM)	55°C 30''	dUTP Cy3
1µl primer mix	72°C 50''	5µl 10x TAQ buffer
(5µl 3'primer+ 5µl 5' primer+40µl H ₂ O	72°C 5'	1.5µl β-mercaptoethanol (0.28M)
19.2µl H ₂ O	4°C ∞	1µl TAQ

A highly repetitive sequence, coding for rDNA on chromosome V served as FISH probe.

Nick-translation was performed for 2 hours at 16°C and checked by gel electrophoresis, where it should show a smear between 100 and 500bp.

Sample preparation

Unless stated otherwise, all steps were done at RT. The gonad preparation was done in 10µl 1xPBS (optional: 1:10 -1:20 dilution of 100mM levamisole in PBS) with about 6-8 worms per slide. The samples were fixed with the same volume of 7,4% formaldehyde and covered with a cover slip. After incubation for 5 minutes, the specimens were frozen in liquid nitrogen. Furthermore, the slides were incubated in methanol, acetone/methanol (1:1) and acetone (pre cooled to -20°C) each for 5 minutes to fix the gonads. After three washes in 1xPBST (each 5 minutes), 50µl of 1M NaSCN were pipetted onto a plastic foil and carefully put onto the specimen. 10 minutes of incubation on 78°C was followed by three washing steps in 1xPBST. This was followed by stepwise dehydration by 5 minutes incubations in: 30%, 50%, 70%, 96%, 96% ethanol. Afterwards slides were dried over night or during probe preparation.

Probe preparation

To ensure that the salmon sperm DNA was single stranded, it is heated up to 95°C (for 5 minutes) and cooled on ice. 1µl of salmon sperm was mixed with 1µl of the 5S probe and dried in the vacuum centrifuge for 15 minutes at 45°C. Afterwards, 7µl formamid (100%) mixed with 7µl hybridization mix was added to the dried probes and incubated at RT for 2 hours while shaking. After a denaturation step at 95°C for 5 minutes, the probe is cooled on ice for another 5 minutes and applied to the gonads. Specimen were covered with a cover slide and fixed with Fixogum ©. Subsequently, they were denatured at 70°C for 7 minutes, and incubated at 37°C in a humid chamber over night.

On the next day, the Fixogum © was peeled off and cover slides were washed of in 2xSSC. Re-hydration was done on 42°C (water bath) in 1xSSC, 0.2xSSC and 0.1xSSC for 5 minutes each.

Finally, 12µl of Vectashield anti-fade containing 2 µg/ml DAPI was put onto the samples. The gonads were covered with a cover slide, which was fixed with nail polish.

Immunostaining

The samples for immunostaining were prepared according to the FISH protocol except for fixation with 2% formaldehyde. The slides were incubated in pre-cooled methanol (-20°C) for 1 minute. After three washing steps in 1xPBST for 5 minutes each, the slides were blocked with 20µl blocking buffer in a humid chamber for 15-30 minutes. 10µl of the first antibody dilution was applied to the slide and incubated over night at 4°C. On the next day, the slides were washed three times in 1x PBS and 10µl of the secondary antibody dilution was added. An incubation of 3 hours at RT was followed by three washes with 1x PBS for 5 minutes. Finally, 10µl of Vectashield anti-fade (Vector Laboratories Inc., Burlingame, CA) containing 2 µg/ml 4',6-diamidino-2-phenylindole (DAPI) were put onto the gonads, covered with a 24x24mm cover slip and sealed with nail polish.

Antibodies used

Primary antibodies			
Antigen	SUN-1 S8Pi	RAD-51	ZIM-3
Animal source	Guinea pig	Rabbit	Guinea pig
Source	Eurogentec	Adriana Lavolpe	Eurogentec
Dilution	1:500	1:125	1:100

Secondary antibodies		
Antigen	Guinea pig	Rabbit
Animal source	Goat	Goat
Dilution	1:500	1:500
Source	Invitrogen	Invitrogen
Fluorescence	Alexa Fluor 568	Alexa Fluor 488

Immuno-FISH

To detect antibody and FISH signals on the same sample, we used a combined immune-FISH protocol.

Gonad preparation was done as stated in the Immunostaining protocol. After freezing in liquid nitrogen the slides were put in -20°C pre-cooled methanol for 1 minute and washed three times in 1xPBST for 5 minutes each. Blocking was done in a slide jar in 0.7% BSA in PBST for one hour. 50µl of an antibody dilution was put onto the slides and covered with plastic foil and incubated for 3 hours at RT and over night at 4°C. The slides were then washed in PBST for 15 minutes, changing the buffer two times. The secondary antibody was added and incubation at RT for 2 hours followed. After additional washing steps (3x 5 minutes in 1xPBST), the samples were fixed in 3.7% paraformaldehyde in PBST for 15 minutes. The slides were then washed once in 1xPBST and twice in 2xSSCT for 5 minutes each. Subsequently, the slides were placed in 50% formamid/2xSSC at 37°C for 1 hour.

Meanwhile, the FISH probe was prepared as stated in the FISH protocol.

The denatured FISH probe was applied to the slides, covered with a glass cover slide and put at 78°C for 10 minutes. Later, the slides were placed in a humid chamber and hybridisation was done over night at 37°C.

The slides were then washed two times in 50% formamide/2xSSC at 37°C for 15 minutes and three times in 2xSSCT for 5 minutes on RT. About 12µl Vectashield anti-fade containing 2 µg/ml DAPI was added to the slides for DNA staining, covered with a cover slide and sealed with nail polish.

Enrique Martinez-Perez established Immunostaining, as well as the immune-FISH protocol.

Bleaching of worms

To eliminate contamination, worm strains were bleached from time to time: Worms from about 4 medium plates were washed off with a few ml of M9 buffer and collected into a 50ml falcon tube. The worms were allowed to settle down, liquid was removed carefully (leave 10ml in the tube) and filled up to 45 ml with bleaching buffer. The falcon was shaken for 7 minutes and washed 2-3 times with M9 buffer. Worms were collected by centrifugation at 1200 rpm for 4 minutes. After the last centrifugation the liquid was removed leaving worms in 1 ml to spread them onto new NGM-plates.

Buffers and solutions

10x PBS

1,37 M (1.37g/L) NaCl, 27mM (2g/L) KCl, 43mM (6.1g/L) Na₂HPO₄, 14 mM (1.9g/L) KH₂PO₄

PBST

PBS + 0,1% Tween 20

20xSSC

3M (175g/L) NaCl, 0.3M (88g/L) tri-Sodium citrate dehydrate, pH=7.0 by adding 1M HCl

Hybridization buffer

4xSSC, 20% dextransulfate

Bleaching solution

1 part Danchlor (blue), 2 parts 5M NaOH, 7 parts dH₂O

Mg buffer

Na₂HPO₄ 6g/L, KH₂PO₄ 3g/L, NaCl 5g/L

2xTY

16g/L Trypton, 10g/L Yeast extract, 5g/L NaCl, pH=7.3

Lysis buffer

50mM KCl, 10mM Tris (pH=8.2), 2.5mM MgCl₂, 0.45% Tween 20, 0.45% NP-40

NGM-plates

2.5g/L Trypsic Peptone, 3g/L NaCl, 17g/L agar; After autoclaving add : 1M MgSO₄, 0.8M CaCl₂, 1M KH₂PO₄, 1mL Cholesterol (5mg/mL in ethanol), 2mL Streptomycin (15mg/mL), 5mL Nystatin (10mg/mL in a 1:1 mixture of ethanol and 7.5M ammoniumacetate. Plates are seeded with a fresh over night culture of OP50.

NGM-Feeding plates

The same recipe as for the NGM plates was used. Modifications: 2mL/L Ampicillin (50mg/ml) instead of 2mL/L Streptomycin (15mg/ml). Additional 3 mL of 1M IPTG is added.

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Anhang

Zusammenfassung

Meiose (vom grich. meionon = vermindern) bezeichnet eine spezielle Form der Zellteilung, durch die es zur Halbierung des ursprünglichen Chromosomensatzes kommt. Diese Reduktionsteilung dient, sich geschlechtlich vermehrenden Organismen zur Erhaltung der Genomgröße über Generationen und der Durchmischung des Erbgutes. Diese Variation, fungiert als Motor der Evolution, da die Anpassungsfähigkeit an veränderte Habitate und Umweltbedingungen gefördert und die Wahrscheinlichkeit für ein Überleben der gesamt Population stark erhöht wird.

Die Meiose lässt sich grob in eine prä-meiotische Duplikation und zwei meiotische Teilungen gliedern, wobei die zweite meiotische Teilung grundsätzlich einer mitotischen Teilung entspricht. Anschließend an die Duplikation des Genoms, tritt die Zelle in die sogenannte Prophase der ersten meiotischen Teilung ein. Die wesentlichen Ziele dieser Phase sind die Paarung der homologen Chromosomen und der Austausch genetischen Materials mittels „crossingover“. Die Paarung von Homologen verlangt die gerichtete Bewegung, Erkennung und Synapse der Chromosomen. Dies wird durch das nukleäre Transmembranprotein SUN-1 gefördert. SUN-1 bildet gemeinsam mit ZYG-12 eine Brücke vom Zytoskelett zu den Chromosomen und übermittelt so die zytosolischen Bewegungen in das innere des Zellkerns.

Diese Diplomarbeit war der Suche nach potentiellen Interaktionspartnern von SUN-1 gewidmet. Hierfür wurden 39 Kandidaten, welche zuvor in einem Präzipitat mit SUN-1 gefunden wurden, auf eine mögliche Rolle in der Meiose

untersucht. Die Genprodukte wurden mittels RNA Interferenz depletiert und die Folgen dieser Depletierung, auf die Meiose, untersucht.

Als Modellorganismus wurde der Fadenwurm *Caenorhabditis elegans* gewählt, da dieser, neben grundlegenden Vorteilen als Modellorganismus, durch den großen Anteil an meiotischen Kernen und ihre außergewöhnliche Anordnung im adulten Tier, einen optimalen Einblick in den zeitlichen Ablauf der Meiose erlaubt.

Abstract

Meiosis (from the greek meionon = decrease) refers to a specialized form of cell division by which the initial set of chromosomes is halved. This reductive division is used by sexually reproducing organisms, in order to maintain genome size over generations and exchange genetic material. The variation of the genome functions as a motor of evolution. Since the ability to adapt to changing habitats and environmental conditions increases and therefore, survival of the total population is greatly increased.

Meiosis can be divided into a pre-meiotic duplication and two meiotic divisions, whereas the second meiotic division resembles a mitotic division. Subsequent to the duplication of the genome, the cell enters prophase of the first meiotic division. The main objectives of this phase are the pairing of homologous chromosomes and the exchange of genetic material by "crossing over". The pairing of homologs requires directed movement, recognition and synapsis of chromosomes. The nuclear trans membrane protein SUN-1 supports this essential movement. SUN-1, together with ZYG-12, forms a bridge between the cytoskeleton and the chromosomes and therefore transmits cytoplasmic forces into the interior of the nucleus.

This thesis was devoted to the search for potential interaction partners of SUN-1. For this purpose 39 candidates that were previously found in a precipitate with SUN-1, were examined for a possible role in meiosis. The gene products were depleted by RNA interference and the consequences of this depletion, examined.

We chose *Caenorhabditis elegans* as a model organism. Because, in addition to its fundamental advantages as a model organism, it allows the observation of meiotic stages due to the temporal and special order of germ cells in the gonad.

Curriculum vitae

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