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Deciphering Meiotic Entry: The Interplay of the Phosphatase PPM-1.D and the Kinase CHK-2 in the Caenorhabditis elegans germline

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Declaration of Honor

I hereby declare that I have authored the present master thesis independently, did not use any sources other than those indicated, and did not receive any unauthorized assistance, that I have not previously submitted this master thesis topic in any form, in this country or any other, for academic credit, and that this work is identical to the one assessed by the registered supervisor.

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Straßer, Julia

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Finally, I would like to thank the great number of worms that let their lives for the sake of research and discoveries made by their usage as a model organism.

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Abbreviation

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
Bp	base pair
Cas9	CRISPR Associated
CHK-2	Checkpoint Kinase 2
CO	Cross Overs
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
C.elegans	Caenorhabditis elegans
DAPI	4',6-Diamidino-2-phenylindol
DBS	Double Strand Break
DNA	Deoxyribonucleic acid
PP2C	Type 2C Protein Phosphatases
FHA	Forkhead Associated Domain
Glp4	Gliptine
O/n	overnight
PAM	Protospacer Adjacent Motif
PBST	Phosphate Buffer Saline Tween
PC	Pairing Center
PCR	polymerase chain reaction
PPM-1.D	protein phosphatase, Mg2+/Mn2+ dependent 1D
PPM-1.G	protein phosphatase, Mg2+/Mn2+ dependent 1G
RAD1	DNA repair protein RAD51 homolog 1
RT	Room Temperature
SC	Synaptonemal Complex

Abstract

Meiosis is a form of cell division to produce gametes containing only one copy of each chromosome. In meiosis two rounds of cell divisions follow one round of DNA duplication. *C.elegans* is an excellent model organism to study meiosis as the gonad contains all the stages of prophase I as a spacio-temporal gradient.

This thesis evolves around CHK-2 and its interplay with PPM-1.D during the regulation of meiotic entry. It was shown in my hosting lab that the key meiotic regulatory kinase CHK-2 is regulated through the scheduled degradation of PPM-1.D by PROM-1-mediated proteasome degradation. A model that was under investigation was that PPM-1.D regulates CHK-2 by direct binding. For this, three truncations of CHK-2 were constructed, their phenotype in *C. elegans* was described and the consequences of the deletions of the domains concluded for their role in meiosis. Two of the truncations were identified to be essential for the proteins' stability whereas one was found highly interesting as, although the protein levels were decreased the organisms remained intact and no embryonic lethality was observed.

Furthermore, I addressed a possible involvement of the CHK-2 paralogue T08D2.7 I found that it is likely not involved in the regulation of meiotic entry and no redundant activity with CHK-2 could be observed.

Zusammenfassung

Die Meiose ist eine Form der Zellteilung, bei der Gameten erzeugt werden, die jeweils nur eine Kopie jedes Chromosoms enthalten. Bei der Meiose folgen zwei Runden der Zellteilung auf eine Runde der DNA-Duplikation. *C. elegans* ist ein hervorragendes Modellorganismus, um die Meiose zu untersuchen, da die Gonaden alle Stadien der Prophase I als räumlich-zeitlicher Gradient zeigen.

Diese Arbeit dreht sich um CHK-2 und dessen Wechselwirkung mit PPM-1.D während der Regulation des Startes in die Meiose. In dem Labor in der die praktische Arbeit durchgeführt wurde konnte gezeigt werden, dass die Schlüsselkinase CHK-2 der Meiose durch den geplanten Abbau von PPM-1.D mittels PROM-1-vermittelter Proteasomen-Degradation reguliert wird. Ein untersuchtes Modell war, dass PPM-1.D CHK-2 durch direkte Bindung reguliert. Dafür wurden drei Trunkationen von CHK-2 konstruiert, deren Phänotyp in *C. elegans* beschrieben und die Konsequenzen der Deletionen der Domänen für ihre Rolle in der Meiose analysiert. Zwei der Trunkationen wurden als essentiell für die Stabilität der Proteine identifiziert, während eine als hochinteressant befunden wurde, da die Organismen trotz verringerten Proteinspiegels intakt blieben und keine embryonale Letalität beobachtet wurde.
Darüber hinaus untersuchte ich eine mögliche Beteiligung des CHK-2-Paralogen T08D2.7 und fand heraus, dass es wahrscheinlich nicht an der Regulation des Eintritts in die Meiose beteiligt ist und keine redundante Aktivität mit CHK-2 beobachtet werden konnte.

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Introduction

Meiosis

Meiosis is a highly specialized type of cell division to produce cells containing half the number of chromosomes to become a germ cell like the sperm and oocyte.
This fundamental process was first described by Oscar Hertwig in 1875 by recognizing that fertilization proceeds into the nucleus and thereby must contain the chromosomes. (Oscar Hertwig, 1909)
Edouard van Beneden then showed that for successful fertilization, two half nuclei are essential to fuse to one cell containing a full set of chromosomes. (Hamoir, 1992)
As a conclusion of this, August Weismann predicted in 1886 that the chromosome number in germ cells had to be systematically reduced and are kept from increasing at fertilization. (Churchill, 1968) From this conclusion, the term Meiosis originates from the Greek word for reduction. In 1911 Thomas Hunt Morgan was then able to show the first evidence that the chromosomes cross over during recombination in drosophila. (Ernst Hadorn, 1946) Since then, the process has been described in more detail however there is a wild field of research questions that are yet waiting to be answered. The known process is described in the following chapter.

Meiosis progression

The whole process starts with one round of DNA replication followed by two rounds of chromosome segregation. (J. Watson, 2008)
To undergo meiosis, two homologs, of which one is inherited paternally and the other one maternally, duplicate by DNA replication like in mitosis for cell division. The duplicated chromosome pairs then together and form a structure, called bivalent. This pairing is part of the meiotic prophase I in which the neighbouring maternal and paternal homologs genetically recombine by exchanging corresponding fragments.

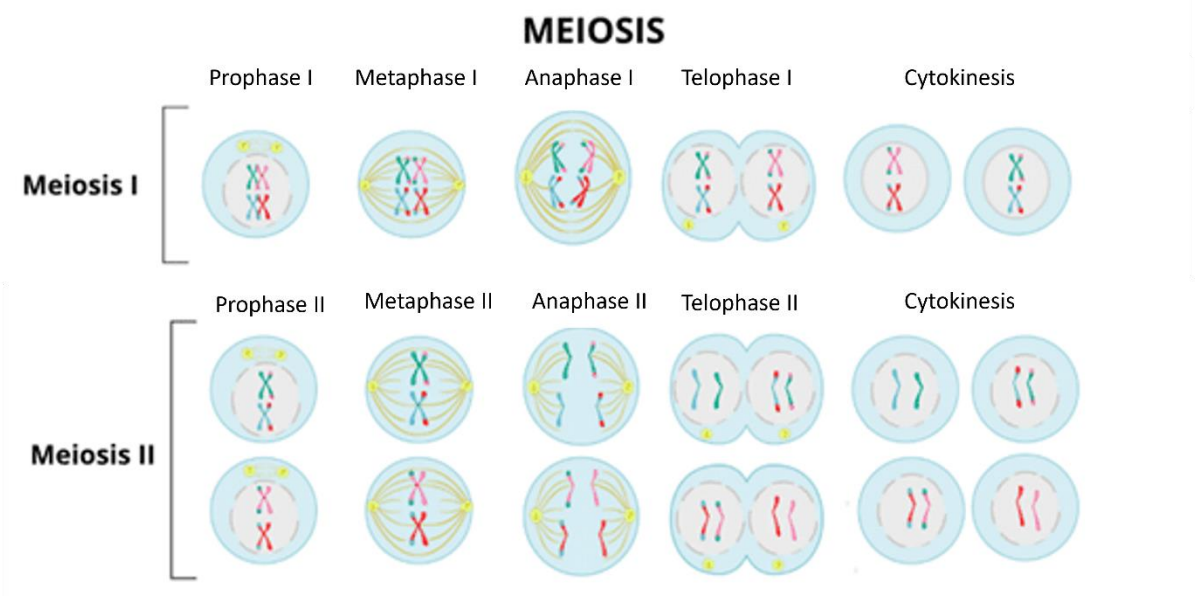

Figure 1 ***Meiosis progression*** *from Meiosis I and II starting at Prophase until Cytokinesis modified from* (Sandra Ferreiro, 2023)(Sandra Ferreiro, 2023)

In metaphase, the chromosomal complex aligns at the equator of the spindle apparatus and bivalents get again separated into two pairs of sister chromatids in anaphase. In telophase, the sister chromatids are then also separated by a physical barrier. This finishes meiosis I to produce haploid cells another round of cell division termed meiosis II is subsequent. The two daughter cells, now contain each one chromosome pair which are separated without preceded DNA replication. The sister chromatids align along the spindle and the homologs are separated forming a total of four haploid cells. Due to the genetic recombination and random assortment of chromatids from the parents, all four daughter cells vary in their genetic makeup. (Alberts et al., 2007)

Genetic recombination

In prophase I homologous sister chromatids pair up in during synapsis for them to be in close proximity. Through this the exchange of genetic material the process of crossing over is enabled. (Alberts et al., 2007a) The points of crossing over are called chiasmata that physically link the chromosomes together which is essential when movement takes place.(Page & Hawley, 2004) The cohesion complex forms a ring around the sister chromatids to ensure they stay linked together. This ensures the

correct alignment on the metaphase plate and the homologues are oriented the correct way on the spindle apparatus(Petronczki et al., 2003) In anaphase I the cohesion is degraded and allows the homologues to be separated. The sister chromatids are however still connected by the cohesin to prevent the separation prematurely. (Kleckner, 2006; Watanabe, 2005)

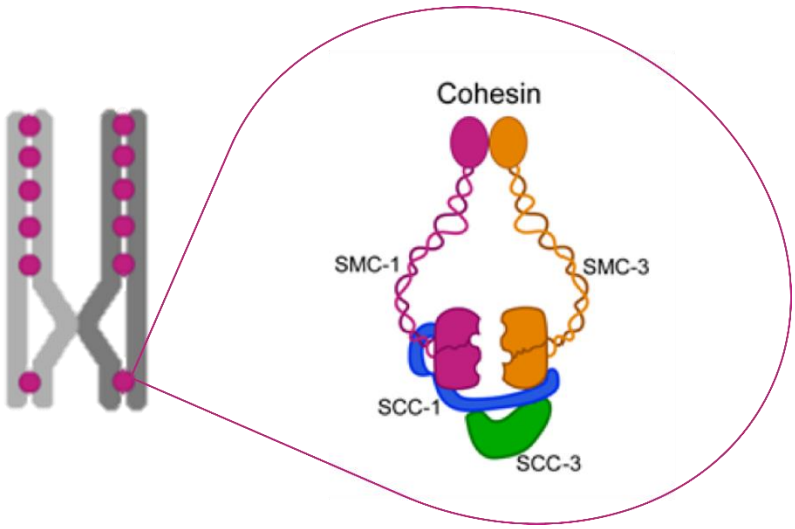

Figure 2 This picture shows a depiction of cohesin holding the sister chromatids together during prophase. modiefied from(Chawla & Csankovszki, 2024)

Genetic recombination is essential for evolutional purposes. Through genetic variation is established. One part of it is general or homologous recombination in which bits of genetic information are exchanged for the creation of new combinations of DNA in each chromosome. The recombination is guided by base pair interaction with essential steps. The cross-over can only take place at the interaction site of non-sister chromatids, induced by a double-strand break or short DBSThis break is introduced by the topoisomerase-like protein SPO11, which is a special endonuclease that cuts both helix strands.(Keeney et al., 1997) At the 5' end, the nucleotides are chewed back so a 3' overhang is formed, as well as creating a complete break in the DNA molecule. The 3' ends are then able to search for another DNA helix, which has to be a homologous sequence. For accurate formation, the helix has to be in its relaxed and unfolded conformation. For general recombination various proteins are needed such as the E.coli protein RecA. A homolog of Rad51 which is found in yeast, mice, and *C.elegans* amongst other organisms. Rad51 forms a nucleoprotein filament along the DNA helix and holds the strands together allowing the DNA synapsis reaction.(Sung, 1994) The single strand of the cleaved helix gets integrated into the homologues DNA helix forming a three-stranded intermediate in

which the single strand forms bands with those of the opposite strand and switches into the major groove. When the synapsis is taking place the branches can migrate, in which single strands displaces a paired region of another single strand without changing the total number of nucleotides. The reaction can be further catalysed by hydrolysed ATP which affects the tightness of the protein binding. (Alberts et al., 2007)

Homologous recombination

Homologous alignment is a process in which identical DNA sequences are aligned along each other. Although mostly identical there might be small regions that differ in some base pairs and therefore carry a different variant known as alleles. Before the steps of recombination can occur, strand breaks are introduced. This allows one strand to align with a complementary strand that originates from the other parental chromosome.(San Filippo et al., 2008) With this strand invasion the strands pair or strand pairing accompanied by strand exchange proteins that work as enzymes to catalyse the process of recombination. A Holliday junction is established and is now able to migrate along the DNA strand thereby increasing the branch that is to be exchanged. (J. D. Watson et al., 2015) The recombination strands are held together doing a reciprocal exchange with two of the four strands creating two pairing and two non-pairing DNA strands. These strands can then isomerase into a more open structure and with ATP gained from hydrolysis and the cross-over point can then be moved along the axis for the extension of the heteroduplex DNA region. The cutting process, to regain two separate helices, is called resolution, where either the crossing strands are cut which would result in an almost unaltered sequence, or the non-crossing strands are cut resulting in two recombined chromosomes with exchanged DNA fragments. (Alberts et al., 2007) The double-strand break-repair pathway is introduced by the break of a double strand with a second double strand remaining intact creating an asymmetry. The broken DNA strands are then further cleaved into single-stranded DNA and terminate at the 3’ ends while the 5’end is degraded. this facilitates the strand invasion and the formation of a double Holliday junction and repair the damage caused by the double-strand break. If the repair strand is not identical to the damaged strand information can get lost and is then replaced with the

genetic information of the repairing strand. This non-reciprocal process gives rise to a gene-conversion event. The resolution of the recombination event depends on the way the strands are cleaved and determining, whether a crossover or non-crossover product is formed. (J. D. Watson et al., 2015)

Each meiotic step is critical and could result in major consequences impacting the fertility, survival, and possible physiological impairment of the progeny. However, due to its importance a short note on the mechanisms taking place during Prophase I.
After the interphase in the leptonema, the chromosomes condense which makes them visible under the microscope. Further, between the homologous chromosomes, the synaptonemal complex is formed to ensure the correct alignment. In the next step, in the zygonema, the homologs align along their entire length and the synapsis continues. Here, the bivalents are formed and the synaptonemal complex stabilizes the pairing. In pachynema, the synapsis is then completed, and the homologous chromosomes are strongly associated together which is essential for the genetic recombination by crossing over. The next step in prophase I is diplotema, where the synaptonemal complex is disassembled, however, the chromosomes are still connected at the sites of crossing over by the chiasmata. In the last phase the diakinesis the chromosomes condense even further so bivalents can be observed. At this point the cell is prepared for metaphase I.(Bolcun-Filas & Schimenti, 2012a)

Often genetic modifications can cause a lack of needed proteins which could lead to a lethal outcome for the next generation, however, multiple protein functions are yet unknown and need further investigation.

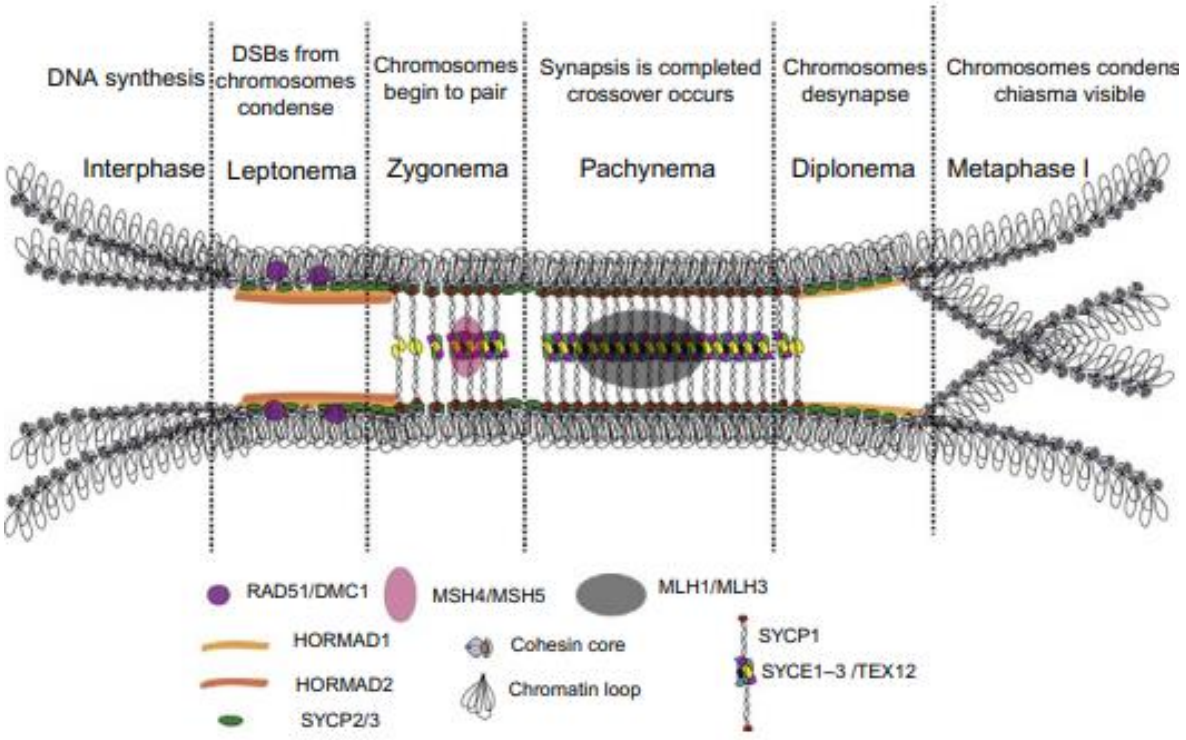

Figure 3 ***Representation of Prophase*** */*(Bolcun-Filas & Schimenti, 2012b

Model organisms used in Meiosis

Arabidopsis

Plants are a widely used model organism and are the base for fundamental research. The life cycle of Arabidopsis consists, like all plants, have of a haploid, and a diploid phase. The plant is considered hermaphrodites as female and male meiosis occurs in differentiated flowers or floral parts. (Watson, 2008)

To enter meiosis in Arabidopsis, similar to most organisms that have a diploid set of chromosomes, the first step is a replication step in the pre-meiotic region to ensure to have two sister chromatids before entering meiosis. To ensure their correct distribution, a high emphasis is placed on the specific organization of the chromosomes. The individual homologs associate into bivalents which then lead up to the segregation during the first meiotic division. Importantly, the sister chromatids only separate during the second meiotic division. During prophase, the chromosome pairing and the formation of the synaptonemal complex is formed which results in homologous recombination followed by the segregation of the chromosomes. During

the leptonema and zygonema stages the chromosomes condense gradually until the pachytema stage. In meiosis II the sister chromatids separate after the cohesion is lost leading to the partition into four haploid genomes (Horlow & Doutriaux, 2003)

Drosophila Melanogaster

Drosophila is a popular model organism used in a variety of research projects. The insect is about 3mm in size and is especially favored due to its easy handling and relatively short life cycle lasting 15 days at 20°C or 10 days at 25°C. (Watson, 2008) The genome consists of 165 million bases building up 14 000 genes on four chromosome pairs. Similar to humans, the flies have either two X chromosomes or one X and one Y chromosome. It is known that the Y chromosome is not necessarily needed for survival, however, a fly having X/0 is no longer fertile.(Kaufman, 2017)

Drosophila is a rather prominent model organism of choice when it comes to meiosis research. With the help of electron microscopy, the early stages including homolog pairing, the formation of the synaptonemal complex, and the recombination could be observed in the development of oocytes. Interestingly, the fourth chromosome is able to segregate without undergoing an exchange. In males, the separation of the chromosomes differs in its system of homolog segregation as there is no synaptonemal complex formed which leads to the absence of recombination. (Orr-Weaver, 1995)

Before undergoing meiosis the stem cell destined for the germline develops into a cytoplast followed by four mitotic divisions. For the formation of a 16-cell cyst, it is necessary that these divisions are incomplete to result in an overall interconnection. In the following step, two-cell cysts are formed by components of the synaptonemal complex being loaded next to centromeres which then are paired in four-cell cysts and consequently clustering in eight cells. By the initiation of the euchromatic synaptonemal complex or short SC, prophase I of meiosis is started leading up to the formation of double-strand breaks. The repair of these double-strand breaks happens in early to mid-prophase. By the end of the mid-prophase one of the nuclei develops into the nucleus of the oocyte whereas the other cells of the cysts will further serve as nursing cells. In the following stages, the chromosomes are newly organized and condense to a karyosome. Additionally, the euchromatic synaptonemal complex

starts to disassemble and transition into late prophase. Importantly, the centromeres and the centromeric synaptonemal complex remain in their formation and only condense in late prophase after the transcription is upregulated. (Hughes et al., 2018)

Mouse

Mice as model organisms are also widely used in meiosis research due to their physiological similarity to humans. However, this also means that their genome is 20 times bigger than the one from Drosophila for example. As mice are breeding rather fast and having multiple offspring they are commonly used in studies of vertebrate molecular genetics. A majority of commonly occurring mutations are known and are rather similar to those of humans. After years of research, the techniques to modify the genetics of mice made it easier to create so-called knockout mice. Here embryonic stem cells modified in the desired gene region are then transferred into an isolated early embryo. This embryo will then grow, and the somatic cells of the grown mouse are then tested for the presence of the altered gene and are then selected for further breeding.(Alberts et al., 2007)

Mice undergo meiosis during gametogenesis, which occurs in both males (spermatogenesis) and females (oogenesis). The ability to study meiosis at different developmental stages in both sexes provides valuable insights into the molecular mechanisms and regulation of meiosis. To study the mechanism and the importance of meiotic regulators fertility studies are regularly carried out to investigate their impact on prospective progeny. Additionally, due to the elevated complexity of mice, studies are often carried out first in other model organisms such as Drosophila melanogaster or *C.elegans*. Specific functions of proteins that are highly conserved such as small ubiquitin-related modifications or short SUMO which is known to be peptide modifying and highly involved in meiotic regulations.(Bhagwat et al., 2021)

C.elegans

Since the experimental work for this thesis was carried out in *Caenorhabditis elegans* this model organism will be discussed a bit more in detail before describing its role in meiosis.

C.elegans is a nematode worm that is found in free-living soil and broadly used in various fields of molecular biology as a model organism. *C.elegans* have various benefits in comparison to other model organisms such as a rapid generation time facilitating genetic screens. The animal has two sexes which are male and hermaphrodite enabling sexual reproduction as well as self-fertilization. (Brenner, 1974) This comes in very beneficial as a homozygous mutagenized hermaphrodite will carry on the mutation to the next generation without the need of being mated which would result in a thinning out of carrying progeny. (Girard et al., 2007) Due to its simplicity, transparency, and easy cultivation, it is a convenient model organism for studies in developmental biology. (Wood, 1988) The life cycle is very rapid and the developmental time can be easily influenced by altering the incubation temperature. (J. D. Watson et al., 2015)

Growing from 250 µm as a freshly hatched larvae to an approximately 1mm big adult worm the nematode undergoes several developmental stages which time points can vary depending on the incubation temperature. The first in-cell cleavage takes place at approximately 40 minutes and a total of 150 minutes of in-utero development before the eggs are laid at the gastrula stage with around 30 cells in the developing embryo. At 22°C the ex-utero development takes around nine hours before the larvae hatch. With sufficient nutrients, the development from an L1-L2 stage larvae takes around 12 hours in which they grow to 380 µm in size. After eight hours the nematode will grow into the L3 stage with a size of half a millimetre before additional eight hours the L4 stage with 650 µm is reached. This stage is very distinctively looking having a patch in the middle of the body showing the position where the vulva will be formed in the adult stage. After approximately ten hours the worm grows into a young adult and finally, after eight further hours, the matured adult with the size of 1mm is formed. This adult is fully developed and is capable of reproducing and laying up to 300 eggs over their adult life which lasts around 15 days. In case of a lack of nutrition, overcrowding, or too high temperatures the L1 larvae can go into an L1 arrest or develop into a dauer larvae which can live up to 4 months in that stage before growing directly into an L4 once better living environment is given. (Hall et al., 2002)

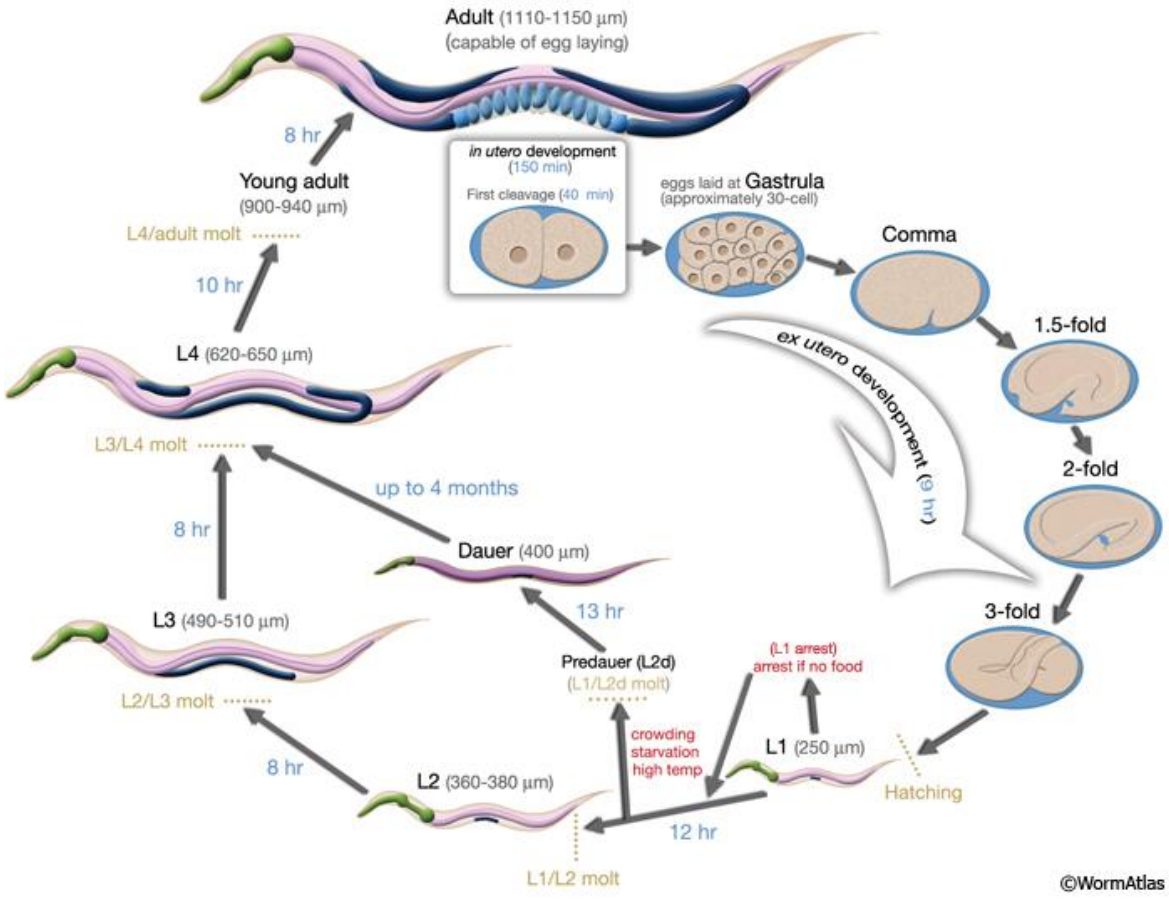

Figure 4 ***C.elegans life cycle*** *at 22°C. The picture above depicts all stages of the development of C.elegans, starting from in utero development up until the fully grown adult* (Hall et al., 2002)

The life cycle above is the ideal one documented in the Bristol strain or so-called N2 strain which is commonly used in most laboratories. The strain was first isolated in 1951 and introduced into the research community in 1974. Since then, the strain has spread all over the world, however as the technique of cryogenic preservation the strain most likely accumulated multiple silent mutations over the years. Unfortunately, the original isolated strain is no longer available so the exact knowledge of how many mutations accumulated over the decades remains unknown. (Hodgkin & Brenner, 1977) Wild *C.elegans* also have a great variety of genotypes and phenotypes which were also found to be contaminated by the N2 strain. By taking the nematode from the wild and transferring them into the laboratory the animal is also required to adjust to the new environment which would also lead to artificial evolution being forced on them. Nevertheless, the N2 strain is a very powerful tool and should not be denigrated as major research results came from this particular strain. (Sterken et al.,2015)

Meiosis in *C.elegans*

The advantage of studying meiosis in *C.elegans* is that the meiotic prophase I can be directly observed in the gonad, as depicted in the figure 5 below.

To start meiosis the DNA has to be replicated which starts in the progenitor zone of the gonad or the mitotic region of the gonad and is finished in the meiotic S-phase just at the beginning of the transition zone. In the transition zone, the homologs start to pair with their corresponding partner and align which is accompanied by a typical half-moon shape of the chromosomes, easily detectable by DAPI staining. During this process, the chromosomes can distinguish between self-versus non-self for proper alignment with its parental homolog. During the phase of side-by-side alignment rapid chromosome movements occur and those are driven by cytoplasmic motor proteins via SUN/KASH domain protein complexes. The pairing is completed by the end of the transition zone. (Hillers et al., 2012) The homologs are then held together after the formation of the synaptonemal complex (SC) to enable the cross-over formation which takes place in mid-pachynema. This process is started by the formation of double-strand breaks of the DNA and has to be finished before the synaptonemal complex again disassembles in late prophase. For the chiasmata formation, the crossing over is essential to ensure that each homolog is recombining. The correct repair to establish the obligate crossover can be observed in the formation of 6 bivalents in diakinesis and is a clear indication of meiotic failure which could have occurred before. For the segregation of the homologs to undergo the first meiotic division a bipolar attachment is formed which promotes the spindle apparatus to separate the sisters following the second round of meiotic divisions. (Hillers et al., 2012)

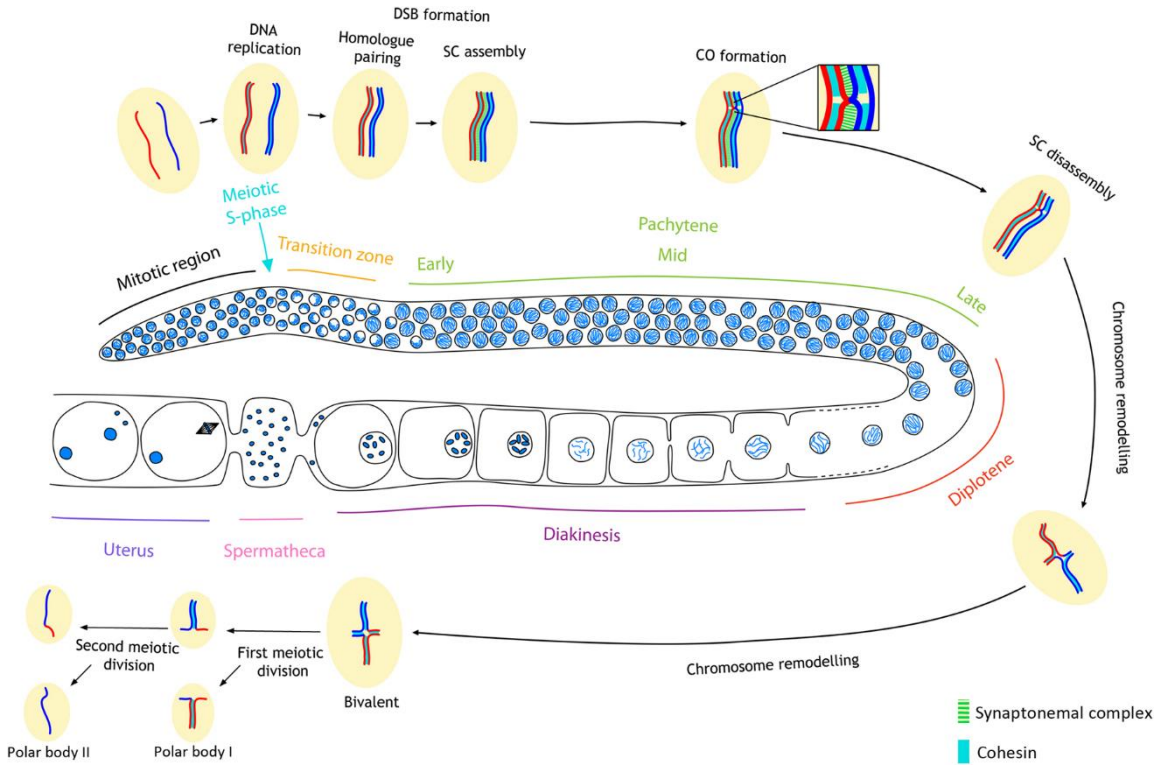

Figure 5 ***Prophase I along the C.elegans gonad.*** *The picture above indicates a dissected gonad and showing the nuclei's as they travel and develop.* (Girard et al., 2007)

In conclusion, *C.elegans* are often favoured in comparison to other model organisms, especially in meiosis research a special emphasis goes on their transparent bodies which makes it possible to observe chromosome dynamics directly under the microscope without complicated staining methods. The model organism is so well established in research laboratories that conserved molecular mechanisms are well known and genetic modifications can easily be performed due to the well-annotated genome. Due to the hermaphroditism, *C.elegans* do not need to be fertilized by another individuum which ensures the progeny will have the same genotype and can be easily kept this way without having to cross the worm with a male. (Girard et al., 2007)

CHK-2

CHK-2 is a checkpoint kinase homologous to the human CHEK2 kinase, which is a highly conserved protein in multiple organisms. Since the thesis is focused on *C. elegans*, where the *chk-2* gene is located on chromosome V, this section will mainly describe the function of the protein in this model organism. The similarity to the human homolog is supported by a blast analysis done by Higashitani et al., where it was found that both homologs have forkhead-associated or FHA domain as well as a protein kinase domain. It was shown that the CHK-2 expression was highest in the germ layer of adult staged worms. (Higashitani et al., 2000) .

CHK-2 is also essential for correct meiotic chromosome dynamics and is needed for the initiation and maintenance of chromosome pairing and synapsis during early meiosis.

When *chk-2* is mutated, the phosphorylation of key synapsis-related proteins is impaired, leading to a failure in the formation of the synaptonemal complex. As a result of these defects, the homologous chromosomes are unable to undergo the precise movements necessary for proper alignment on the meiotic spindle. This misalignment disrupts the normal sequence of meiotic events, leading to a block in the progression of meiosis, often at the prophase I stage. The inability to correctly pair and synapse chromosomes triggers the meiotic checkpoint mechanisms, which halt the cell cycle to prevent the propagation of errors. However, if the checkpoint fails to restore normal function, the cell either undergoes meiotic arrest or proceeds with abnormal chromosome segregation. (Woglar & Villeneuve, 2018)

This failure in chromosome movement and synapsis due to *chk-2* mutations has severe downstream effects on fertility in *C. elegans*. The arrest or improper progression of meiosis results in the production of defective gametes, which are either inviable or give rise to embryos with chromosomal abnormalities. Consequently, the fertility of *C. elegans* is significantly compromised. (MacQueen & Villeneuve, 2001)

In further experiments done by Higashitani et al., it was shown that by repression of CHK-2, the lethality increased highly by having 95% of eggs dying in early embryogenesis. (Higashitani et al., 2000) A similar result with a lethality of 95.7% is shown in the paper of Amy MacQueen and Anne Villeneuve from 2001 (MacQueen & Villeneuve, 2001). Organisms carrying a mutation in *chk-2* are defective in meiotic

chromosome segregation based on observations of chiasmata lacking between the homologs at the first meiotic division. This could be from an original defect in recombination or a defect in the numerous other landmark events that occur during meiotic prophase because in a worm carrying a *chk-2* null mutation about 12 univalents are seen in diakinesis oocytes instead of 6 bivalents that can be seen in the wild-type strain N2. (MacQueen & Villeneuve, 2001) In a healthy wild-type worm, the bivalents correspond to a pair of homologous chromosomes that are linked to each other by the chiasmata.

When analyzing the gonad of *chk-2* mutants by looking at DAPI stained chromosomes the chromosomes appear strongly disorganized in the nuclei encompassing the entire pachytene stage. In comparison to wild-type nuclei where the chromosomes are highly organized in parallel tracks. The mutant chromosomes are not organized in parallel indicating a failure in alignment and synapsis between the corresponding homologs (MacQueen & Villeneuve, 2001). CHK-2 is known to be needed for a spatial reorganization of the meiotic prophase I nucleus, happening in the transition zone which corresponds to the leptotene/zygotene. In the absence of the chiasmata, the recombination machinery is highly impacted and has a leading defect displaying in the establishment and maintenance of stable chromosome pairing.(Dernburg et al., 1998)

In the paper of Amy MacQueen and Anne Villeneuve (MacQueen & Villeneuve, 2001) they induced a mutation, *me64*, which carries a nonsense mutation that terminated the translation after residue 305. This is the location in the middle of the kinase domain making it a loss-of-function allele and is considered the null mutant. Another allele, the *me18* carries a missense mutation on the 21st residue altering an alanine to a valine giving a similar result as the *me64*.

Figure 6 chk-2 and the locations of the induced truncations from the paper MacQueen & Villeneuve

From observing the DAPI-stained gonads of the *me64* it was found that the mutants are lacking the spatial organization to form the half-moon shape typical for the transition zone. The nuclei are not clustering when entering meiosis but instead show

a peripheral distribution that can be seen until the end of the pachytene stage. This suggests that CHK-2 is not only needed for the homolog pairing but also for the spatial organization of nuclear components involved in the pairing.(MacQueen & Villeneuve, 2001)

In the transition zone, CHK-2 is located at the pairing centre and colocalizes with phosphorylated SUN-1 (S8-PI) and stays colocalized until mid-pachytene. However, this is no longer true in end-pachytene. (Rosu et al., 2013) The localization of CHK-2 is consistent not only with homolog pairing but also with its mechanism of triggering the nuclear reorganization. (Kim et al., 2015) HIM-8, which is a PC protein, located on the X-chromosome, found to be phosphorylated by CHK-2 in wild type, HIM-8 was found to be directly recruited by CHK-2. In a mutated CHK-2 protein carrying a point mutation in the N-terminus of HIM-8, as in the *him-8(me4)* allele, it was found that the interaction point of the two proteins is located in the FHA binding motif of CHK-2. By using this knowledge, that the phosphorylated HIM-8 is a read-out for active CHK-2, the activity of CHK-2 can be observed. In the wild type, CHK-2 activity was shown to cover up to 48% of the leptotene, zygotene, and pachytene stages. However, mutants that are found lacking to form crossovers as a cause of absent synapsis show an increase in CHK-2 activity up to 90% in the defined zone. An increase to 64% of CHK-2 activity was also found in mutations that are blocking the formation of DSBs into crossover formations such as MSH-5 or interfere with DSB in general such as SPO-11 with an increase to 62% of activity. This results in the conclusion that a defect in synapsis and cross-over formation leads to a feedback loop that keeps CHK-2 active indicating a delay in meiotic progression. (Kim et al., 2015)

Chk-2 has a closely related gene the encoded by the open reading frame T08D2.7 also a group intern called *chek-2* which has a 95% similarity in coding sequence and is located on the X chromosome. *Chek-2* is also an orthologue of human CHEK-2.

PPM-1.D/Wip1

The protein phosphatase Mg2+/Mn2+-dependent 1D or in short PPM-1.D is a wildly studied phosphatase, especially in cancer research.(Pecháčková et al., 2017) Although the thesis focuses mainly on meiosis, the protein's involvement in cancer

gives a fundamental knowledge of its background and to be able to understand its function a small side note will be given in the following paragraph.

PPM-1.D is part of the protein phosphatase 2C, PP2C, family which is highly involved in stress response pathways. Its role in cancer has its background in the regulation of the p38 MARK/p53 pathway and functions as an oncogene. By inhibiting the expression of PPM-1.D by chemotherapeutic agents, the p38 MARK/p53 signalling cascade can be activated which ultimately would lead to cell apoptosis. (Yoda et al., 2006) According to various studies, it is suggested that active PPM-1.D is involved in the initiation of the development of malignancies. (Hsu et al., 2018)(Kamada et al., 2017) (Zane et al., 2012)

The PPM.1-D phosphatase is also named wild type p53 induced phosphatase 1, in short WIP1, and is known to be involved in the negative regulation of the DNA damage response. (Husby et al., 2021) When induced in a p53-dependent manner, the activation of p38 MAPK is inhibited by the dephosphorylation of Thr180 in p38 which ultimately leads to the inhibition of the p53 function. (Takekawa et al., 2000) CHK-2, as a checkpoint kinase, is also highly involved in cancer and was shown to be inactivated by PPM-1.D by the dephosphorylation of threonine 68 of active CHK-2, thus leading to the suppression of CHK-2 mediated apoptosis.(Fujimoto et al., 2006) When DNA damage response pathways are active, CHK-2 gets activated to trigger cell cycle arrest as long as PPM-1.D does not keep CHK-2 inactive. (Nahta & Castellino, 2021)

In vitro studies PPM-1.D was identified as a strong interactant of CHK-2, which is also resistant to washes including high salt concentrations or detergent. This was also confirmed in a pull-down using HA-tagged CHK-2 and showed that by inhibiting Wip1 phosphatase activity, the interaction is almost completely disappearing. This suggests that either the catalytic activity of CHK-2 or a certain conformation is needed for this specific interaction. It was found that CHK-2 has to be phosphorylated to be able to interact with PPM-1.D. In further experiments, it was shown that fully active CHK-2 is not sensitive to PPM-1.D phosphatase and when PPM-1.D is present in excess it can reduce the kinase activity of CHK-2 to 50%. (Oliva-Trastoy et al., 2007) When looking at the specific case of meiosis mice oocytes were used as a model. In the paper of Leem et al., it was found that PPM-1.D suppressed the DNA damage response in prophase to ensure the process of homologous recombination is not

disturbed by DNA repair mechanisms that would inhibit proper crossover formations. (Leem et al., 2018)

PPM-1.D degradation

At the beginning of my master thesis it was found in the lab that at the meiotic entry PPM-1.D gets degraded, giving the now CHK-2 the ability to regulate the start of meiosis. This degradation was found to be due to the SCF^{PROM-1} complex, now published in (Baudrimont et al., 2022) *prom-1* was identified in a *C.elegans* screen selecting for mutants with increased uptake of the dye acridine orange by apoptotic corpses, which is a result of meiotic recombination failure. It was found that by creating a null genotype of *prom-1*, the brood size highly decreased showing a strong embryonic lethality, however with a high variability suggesting that the lethality was the effect of chromosomal defects in the beginning of meiosis. In the absence of the formation of double-strand breaks, the *prom-1* mutant displaced a drop of apoptosis which led to the suggestion that by deleting prom-1 cell cycle checkpoints can be bypassed. In cytology, it was shown that the meiotic entry zone, a zone where meiotic markers get expressed and which encompasses around 2-3 cell rows in the wild type, as well as the transition zone are prolonged in the *prom-1* mutant, however, the half-moon shape is almost completely absent. PROM-1 contains an F-box-like motif which suggests it is involved in ubiquitination. A pairing defect seen in the *prom-1* phenotype suggests that by its absence and therefore resulting inability to degrade specific proteins that would need to be degraded to ensure proper pairing. (Jantsch et al., 2007) In fact, one of PROM-1's targets was found to be PPM-1.D. This was shown by tagging PPM-1.D in a *prom-1* depleted strain and co-expression experiments in yeast cells. In contrast, to the wild type where PPM-1.D gets degraded before entering meiosis, the protein is still present and stays at its rim localization until late pachytene. Additionally, by deleting PPM-1.D in *prom-1* mutants the phenotype can be rescued resulting in the formation of 6 bivalents instead of 12 univalent in the single mutant.. (Jantsch et al., 2007)

Research question

Mutual dependent for the co-localization of PPM-1.D and CHK-2 in the mitotic zone of the *C.elegans* gonads can be easily detected by cytologic staining using tagged

CHK-2 and PPM-1.D strains. By experiments performed previously in the lab, it was found that the c-terminal tail of PPM-1.D might play a role for the colocalization of the two proteins. Therefore, the main research question was to find the specific protein domain on CHK-2 which is presumably interacting with PPM-1.D. To achieve this, three specific truncations were designed and created with CRISPR/Cas9. Additionally, the stability of CHK-2, its ability to be correctly translated into protein, and its activity to regulate meiotic entry was examined. Additionally, we wanted to find out whether persistent activity of CHK-2 which is triggered until a certain recombination intermediate has been established depended on a domain of CHK-2's carboxy terminus. The feedback activity of CHK-2 can be followed by using CHK-2-dependent targets as a monitor, such as SUN-1 (S8-PI) and DSB-2 loaded onto chromatin.

DSB-2 is a protein needed for the formation of a double-strand break that is concentrated on chromatin. DSB-2 disappears when the levels of RAD-51 foci weaken which marks early recombination intermediates. The location and detectability of DSB-2 on the chromatin are elongated in strains carrying mutations that are not able to form CO-intermediates or double-strand breaks.(Rosu et al., 2013)

Furthermore, I was interested to find out whether the paralogue CHEK-2 can compensate CHK-2 activity. The last question that was investigated was raised due to the fact that during mitosis the nucleic pores are degraded during the cell division process. It was found that this is not the case in meiosis, which raised the hypothesis that PPM-1.D would be priming the pore to block its degradation during meiosis.

Materials and methods

CRISPR/Cas9

CRISPR/Cas9 is a widely known methodology to target specific sites in the genome for editing. There are three types of CRISPR systems whereas Cas9 is type II. In the presence of the Cas9 protein the double-stranded RNA-specific ribonuclease RNase III is triggered by a trans-activation crRNA or in short tracrRNA which is complementary to the sequences in pre-crRNA. It is believed that Cas9 is the only protein that is able the silence foreign DNA by crRNA-guiding.(Deveau et al., 2008) Cas9 is involved in the maturation of crRNA (deltcheva et al., 2011) which is not solely capable of guiding plasmid DNA cleavage catalysed by Cas9. Thus, a tracrRNA for the pairing with the repeat sequence is needed to achieve the proper maturation of the crRNA to ultimately lead to the cleavage of plasmid DNA. For the complete reaction magnesium and a complementary crRNA to the desired DNA strand are needed. However, it is required that the crRNA does not contain a noncognate target DNA-binding which would not enable plasmid cleavage. Importantly, a protospacer adjacent motif or short PAM sequence is needed for a GG dinucleotide alongside the crRNA. For the specific targeting of a unique sequence of interest in any dsDNA a guide RNA is additionally needed. (Jinek et al., 2012)

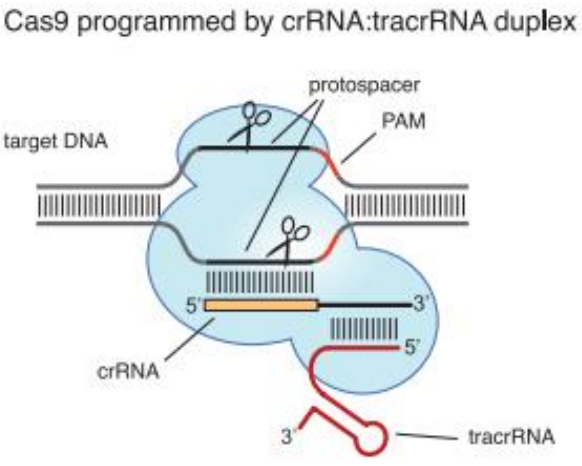

Figure 7. ***A schematic visualization of the CRISPR/CAS9 system*** *whereas by activation the tracrRNA and a Cas9 are led by a two-RNA structure is formed leading to the cleavage of a dsDNA specifically targeted by a crRNA.* (Jinek et al., 2012)

To use the technique in *C.elegans* the specific guide RNA was designed by using Genious software which can identify sites in the target area.

The design has to be in a genomic context and not in the spliced version of the gene. To find the position of gRNA an NGG is searched for on the 5'-3' or the complementary strand. After identifying the position 20nt are marked upstream form the NGG which are then marked as the guide RNA. To order from Dhaermacon the NGG is not included and ordered form the 5'-3' direction. The repair template has to be designed on that specific strand, never the reverse. Fort the construction it is important to keep in mind that the repair template should not be cut by the Cas9. To avoid this either the NGG is changed, or 4-5 silent mutations have to be added. A special emphasis is to not change the reading frame. To finish up the design homology regions flanking the last point mutation consisting of 35bp or longer are added.

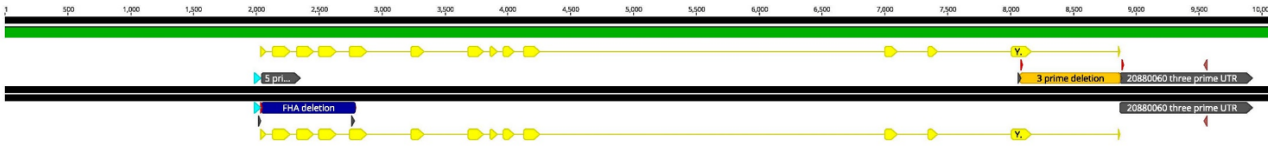

Figure 8 ***Deletions along the chk-2 sequence.*** *The figure above shows the 5'Δ, the FHA Δ and the 3' Δ along the chk-2 sequence.*

The following strains were constructed with the help of CRISPR/Cas9
The 5'Δ having 312bp deleted spanning from position 2038-2351. The FHA Δ is overlapping with the 5'Δ but spanning from position 2038-2784 and therefore 747bp. The longest truncation is the one on the 3'end having a deletion of 792bp from position 8078-8869 which is just were the 3'UTR starts.

Overall the following strains were constructed.
chk-2 $^{5'\Delta}$::3xFLAG (jf191) ;ha::ppm1d(jf183)
chk-2 $^{3'\Delta}$(jf177); ha::ppm1d (j183)
chk-2 $^{fha\ \Delta}$::3xFLAG (jf173);ha::ppm1d(jf183)

A full list of all used strains can be found in the annex.

The repair sequence of the 5’ Δ

5’ - TTA GTT GTT TTT AAT CGA ATT TTC GCG CAA TTT TCT TCG TTT TTT TCT CGA TTT TCT CGA TTT CTC CGA AAT TTT CTC CAT TTT TTC AGC GAT TTC CAT GCG CCG TTC GGA TGC TGC TGC GAA GGA TTT CAA CTT TTC TCA AGT CGC CAA AGA CGT GCG GCT CTA CAG ATT TAT CTC GAA AAT TCA ATT TTC CAT TGA - 3'

repair sequence of the FHA

5' - GTG GTT TTT AAT CGA ATT TTC GCG CAA TTT TCT TCG TTT TTT TCT CGA TTT TCT CGA TTT CTC CGA AAT TTT CTC CAT TTT TTC AGC GAT TTC CAT GCG CAA AAA ATA CGA CGT GAC CAA TCG CCT GGG AAA AGC GCG TTT TGG CAA AGT TTT GCT GCG ATA CAA GAA ATC CGA TTC CGA ATC CGT GGT ACG GG - 3'

repair sequence of the 3’Δ

5'- AAA ATT TCC TCA ATT TTC AGC CAT CAA ACC GCC GCT CTG CCG TCG AAC TAA TGA GTA CTC AAT GGA TGA AAT GTG CGG ATT GGA CTG CGA CTG CGG GAG GAG GAG GAG GAG ATT ACA AAG ATC ATG ACG GTG ATT ATA AAG ATC ATA TCG ATT ACA AGG ATG ACA AGT GAA ACA CGT TTC ACG TGG GTC C -3

The injection mix was prepared according to the paper of Paix et al. from 2015. For an easier finding of edited genomes, a co-CRISPR was used which targets the dpy-10 region on chromosome II. This results in the worms developing a dpy or rol phenotype which facilitates the identification of those worms in which the CRISPR gene edition was successful. The dpy-10 mutation has no impact on the other chromosomes or the development of the worms and will be lost eventually in the next generations. (Paix et al., 2015)

Experimental design

Injection

Worms at the stage of L4 have to be pre-selected the day before and kept at 16°C for 24 hours. On the day of injection, the mix is prepared as follows: 1.4μl H_2O, 0.75 μl Hepes, 0.5μl KCl, 2.2μl repair template, 0.75μl gRNA 1, 0.75μl gRNA 2, 0.5μl dpy-10 repair template, 0.4μl dpy-10 gRNA, 6.7μl tracR, 6μl Cas9. This mix is then centrifuged at 13000 rpm for 2 minutes followed by 15 minutes of incubation at 37°C. The injection is done according to the protocol of Paix et al from 2015 After injection the worms are singled out and kept at 20°C. After 24 hours they are then moved to fresh plates and incubated at 16°C overnight (o/n) then the next day moms are burned off. If injected on Tuesday at 14°C until next Monday. If injected on Wednesday at 16°C until Monday then the selection on Monday plates are screened for worms having a dpy and rollers phenotype(Paix et al., 2015)

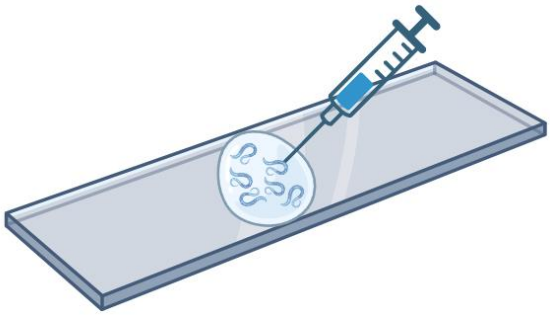

Figure 9 Depiction of the injection of the solution into the gonad of C.elegans Created with BioRender.com

Genotyping

The basis of working in genetics is genotyping as it is essential to determine the genotype of the *C.elegans* worm that is worked with. This is done by PCR where a single adult worm which already produced progeny on the plate are lysed. The lysis buffer mixture is made out of 5mL of 1M KCl, 1mL 1M Tris of pH 8.0, 250µL 1M MgCl2, 450 µLTween 20 and 450 µL NP-40. Then it is filled up to 100mL with sterile water and aliquoted a 1mL per aliquot. Additionally, right before the usage 10µL of proteinase K is added in each aliquot. Proteinase K aliquots are made from 20mg Prot. K, 1mL 50mM Tris pH 8.0 and 5mM CaCl2x2H2o.

Since Proteinase K is temperature sensitive, working on ice is essential.

For the lysis of a single worm 10µl of the mixture is pipetted into PCR tubes.
To lyse the worms the tubes are put at -80°C for at least 10 minutes, however, it is also possible to keep it at this temperature for a longer time for storage. After freezing the tubes are put into the thermocycler with the following incubation steps. The tubes are left at 60°C for one hour, followed by an increase to 95°C for 15 minutes. Afterwards, the lysates should be stored at 4°C and worked within the following 3 days.

These lysates are then used as the DNA template when making a master mix for PCR. Depending on the characteristics of the expected result the corresponding polymerase was used which was either the Dream Tag Polymerase or the Phusion polymerase. The constitution of the mastermixes are shown in the table below.

Table 1 This table shows the master mix used for the Dream Tag Polymerase and the Phusion Polymerase

Dream tag polymerase	
H20	16.8 µL
10x buffer	2.5 µL
dNTPs	2.5 µL
Primer 1	1 µL
Primer 2	1 µL
DNA Template	1 µL
Polymerase	0.2 µL
Total Volume	25 µL

Phusion polymerase	
H20	14.8 µL
5 x buffer	5 µL
dNTPs	2 µL
Primer 1	1 µL
Primer 2	1 µL
DNA Template	1 µL
Polymerase	0.2 µL
Total Volume	25 µL

The polymerase chain reaction starts with initialization at 95°C for 3min followed by the denaturation also at 95°C for 30 seconds. The annealing temperature depends on the primer and has to be adjusted in each PCR and lasts also approximately 30 seconds. The last step, the elongation, was done at 72°C and the time depends on the size of the DNA which is to be amplified and also has to be adjusted individually. To make sure that no contamination happened, at least on reaction control without any DNA template is used. The amplified DNA is visualized by gel electrophoreses. The gel is made from agarose in TAE buffer and ethidium bromide. The percentage of the agarose depends on the size and size difference of the DNA pieces. To load

the samples, 5µL of loading dye is added to each sample tube and the gel is run at 180Volt. To take pictures a GelDoc form Bio-Rad is used.
When a worm with the wanted genotype is identified the progenies that are genetically identical are then used for staining.

For genotyping the 3'Δ the following primes were used:
Forward ATGGAAATTACGGGGAATTGGGG
Reverse CCCGTCTGCCGTCGAACT

For genotyping the 5'Δ the following primes were used:
Forward TTCCTCCGGATGATGGTCGG
Reverse ACTTGGCGCGTTAGATGACTG

For genotyping the FHAΔ the following primes were used:
Forward GCTGCATCTTGTCGAAAATTGTG
Reverse TTTTTCAGCGATTTCCATGCTGACG

For the genotyping of the *chek-2* the following primes were used:
Forward AGTGTGCTGCTTTAAAACCCTTC
Reverse ACCATATACGAGTACTTGGCGAC

And to ensure that the HA-tag on *ppm-1.d* is still present in the strains after crossing the following primers were used:
Forward TGATTTCAGTGGCTTTCAGACG
Reverse TTCCCCAAATTGTATGGGTGTTCG

The full list of primers is documented in the annex.

Staining

The staining of dissected gonads is used to visualize the phenotype and the protein localization.
To prepare, one day before the glass slides used for the dissection are coated with 10µL of Poly-L-Lysine and put at 65°C overnight. On the same day *C. elegans* in the

L4 stage are selected and put at 20°C overnight. Importantly, to obtain comparable results also wild type (N2) worms and when possible, a wild type which carries a tag on the same protein as the mutated worm are also picked at the same developmental stage and incubated at 20°C. In the case of the stainings done for this thesis, additionally to the mutated worms, also N2 and the strain *chk-2::3xFLAG;ha::ppm-1.D* were used. These two strains are genetically identical with the only change being that *chk-2* is tagged with a 3x Flag tag at its N-terminus and *ppm1d* is tagged with ha at its C-terminus.

The cutting buffer, in which 5-10 worms are cut is made from 100µl 10x PBS, 10µl Levamisol, and 890uL H_2O. After the worms are put into a 10µL droplet of the buffer the worms will stop moving after a few seconds due to the Levamisol. Then the worms are cut open, on the tail and head and the gonad is released from its body. Afterwards, 10µL of the fixing buffer consisting of 100µL of 10x PBS, 125uL of 16% Formaldehyde and 775µL of H_2O is added. A cover glass is added and incubated for 5 minutes at RT before dropping the slide into liquid nitrogen. When taking out the liquid nitrogen the coverslip is removed with one quick movement and directly put into ice-cold methanol and incubated for 2 minutes at -20°C. Afterwards, the slides are washed 3x in PBST for 10 minutes before blocking the slides in 1% BSA in PBST for 20-60 minutes in a moisturized chamber. The antibody solution is prepared according to their recommended dilution in 500µL 1xPBST and put on the samples with coverslips. For incubation, the antibody samples are put at 4°C overnight. The following day the slides are washed 3x in PBST for 10 minutes and the secondary antibody is added for 1-2 hours at room temperature. To finish the staining the slides are again washed 3x for 10 minutes in PBST and DAPI is applied for 1 minute before a last wash of 20 minutes in PBST. Vecta shield is added and covered with a coverslip before sealing the slide with transparent nail polish.

The slides are now first checked at the Zeiss Axio Imager M2 to confirm that the staining worked successfully.

The antibodies used are listed in the annex.

Delta Vision

The pictures that will be used for quantification are taken in a higher resolution with the Delta Vision. To obtain the correct settings for picture taking the tagged wild type

is used first and the correct exposure for all wanted wavelengths was determined by letting the software find the optimal exposure time. This is done on multiple gonads on more than one slide. When three replicates are given a similar time, the average is used to take the pictures on all slides and genotypes. The pictures are then deconvolved with a conservative enhance ratio with 15 cycles and noise filtering at 200nm.
The final pictures are then processed with Image J to find the ideal stack and stitched together in Photoshop.

Picture quantification

To quantify the signal of a protein the obtained pictures from the Deltavision are first projected using Image J. The exact script that was used can be found in the annex. The settings are chosen by adjusting them according to the signals in the wild type. The gonad pictures taken from the upper and lower part of the gonad are used to see the nuclei travelling through the phases of meiotic prophase I.
When the pictures are fully projected the gonad is then segmented into seven parts starting from the distal tip to the phase of diplotene. Each of these sections is analysed in ImageJ by putting an oval over each nuclei which was not overlapping with others to determine only the signal from one single nuclei. From each of the ovals, the area and the integrated density are measured and used to calculate the normalized intensity of the protein signal. To find out the intensity also a tagged wild type is used to see the normal signal intensity and additionally, a non-tagged wild type is used to subtract the background noise.

Cell row count

The cell row count was done on the finalized gonad projections. Thereby, the gonad was segmented into single-cell rows and the rows with at least 50% of positive cells for the corresponding signal are considered as active protein signals.

Viability

The viability is analysed by singling out 10 worms at the L4 stage and incubated at 16°C. The worms are then daily transferred onto a new plate after 24h over a time course of four days. After each day the plates are checked and the progeny, which includes eggs and larvae, are counted. By recounting the plates, the number of hatching progenies can be observed and differentiated from the nonviable eggs laid by the hermaphrodite. With this data, the total brood size, the viability of the total brood size, as well as the lethality, is then calculated. Importantly in addition to the desired strain also 10 N2 worms are analysed to do an adequate comparison. To facilitate the task of counting the moving larvae it is best to draw a grit onto the plate and place the plates on ice a few minutes before counting.

Setting up crosses

For constructing a strain with an additional genotype that has already been established in the laboratory a cross is set up. Therefore, males from one of the strains are needed. To archive this L4 are picked, and heat shocked at 30°C for 4-6 hours and then incubated at 25°C overnight. The progeny of these worms will have a higher percentage of being male. These males are then set up with L4 on special plates that have a lower amount of E. coli on them to make sure all worms meet up on the plates. The ideal number of worms would be a ratio of 5 males per one hermaphrodite. Whereas up to three hermaphrodites are placed on one plate. The worms are incubated at 20°C overnight and the hermaphrodites are then singled out the next day. To make sure that the worms did indeed cross, the progeny should be around 50% male. When the F2 is in the L4 stage they are again singled out to ensure they do not mate with their brothers but self-fertilize. When those worms produce enough eggs, the mothers will be used for genotyping.

In case of a lethal phenotype when a mutation is homozygous a balancing strain is crossed in. The decision on which balancer is used depends on the exact location of the desired variant. Balancer strains are well established and to be used to maintain a strain even though they would be non-viable when homozygous. When a strain is left in non-balanced the lethal mutation is likely lost over the next generations. To

avoid this happening genetic balancers were established. For each chromosome, a map was created. Some of the balancers even resemble other chromosomes in an altered form. These inversions are lethal when homozygous which ensures that the surviving progeny is heterozygous for the desired variant and the balancer.
The SC4 balancer is in its original state heterozygous with an unc, rol phenotype. Meaning that when crossed with a non-viable mutation of CHK-2 those worms showing a normal phenotype are used to single onto smaller plates.

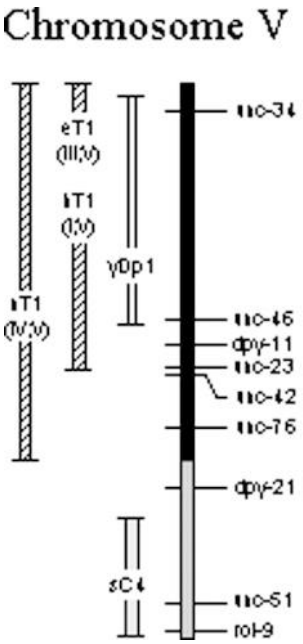

Figure 10 ***Map of genetic balancers for chromosome V.*** *This graph helps choosing the correct balancer for the desired gene.* (Girard et al., 2007)

In case of chk-2 it was found best to use the SC4/UR. To see how a balancer strain is crossed in an example is shown below.

$$\frac{\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}^{V}}{+} \; x\; N2$$

Table 2 In the first step to cross a non-viable strain into a balancer is to create males. To avoid having to heat shock them N2 males are used. And crossed with a heterocygous hermaphrodite. The outcome from this are 50% N2 and 50% heterocygous worms

FO	$\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}^{V}$	$+^{V}$
$+^{V}$	$\frac{\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}^{V}}{+}$	$\frac{+^{V}}{+}$
$+^{V}$	$\frac{\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}^{V}}{+}$	$\frac{+^{V}}{+}$

$$\frac{\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}}{+}^{V} \times \frac{UR}{SC4}$$

Table 3 When the desired males are obtained they are crossed with the balancer strain. The outcome would be that 25% have the combination of the unc rol phenotype with the variant, 25% are unc, rol with the wild type, 25% have the balancer strain in combination with the wild type and 25% have the desired combination of the variant and the balancer.

	$\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}^{V}$	$+^{V}$
UR^{V}	$\frac{\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}}{\text{UR}}^{V}$	$\frac{+}{\text{UR}}^{V}$
$SC4^{V}$	$\frac{\text{chk} - 2\text{fha'}\Delta :: 3\text{xFLAG}}{\text{SC4}}^{V}$	$\frac{+}{\text{SC4}}^{V}$

To ensure that the correct genotype is used those worms with a wild-type phenotype are singled out as L4 to ensure there is no cross happening with any potential males on the plate. All worms having the unc,rol phenotype are excluded. When multiple progenies are present on the plates those worms can be used for genotyping to ensure that the wanted strain is identified.

The following strains were constructed by crossing pre-existing strains:
chk-2 3'Δ(jf177); chek-2 Δ (ok431); ha::ppm1d (j183)
sGFP::npp-7 (2230) ; ppm1d(jf120)
sGFP::npp-7 (2230) ; ppm1d(jf120) ppm1g (jf157)
ha::ppm1d; spo-11/nT1[qIs51] (IV); nT1[qIs51] /+ (V);chk-2 3'Δ::3xFLAG (jf177);
ha::ppm1d; spo-11/nT1[qIs51] (IV); nT1[qIs51] /+ (V) ;chk-2 3'Δ::3xFLAG (jf177); chek-2 (ok341)

A full list of all used strains can be found in the annex.

DAPI body count

The DAPI body count was done on every strain by looking at the slides under the Zeiss microscope. For each gonad, the DAPI bodies in diakinesis are looked at and counted.

Western blot

A western blot is another possibility to determine the amount of protein present based on the tag of the protein. To obtain the sample 200 worms at the L4 stage have to be selected for each genotype the day before. The next day, one tablet of protease inhibitor (PI) is dissolved in 1 mL H_2O which is then a 10x solution. For the buffer 100µl of the PI, 100µL of 10x TE and 800µL of H_2O are mixed to get 1mL. For each sample 35µL is used. Then 8µL of 5x leammli buffer is added to the samples, then the samples are boiled at 99°C and put into liquid nitrogen 3 times and then stored overnight at -20°C. the next day the samples are again boiled at 99°C for 10 minutes. To prepare 1L of SDS buffer/Running buffer 100mL of 10x SDS and 900mL of H_2O were used the samples are then loaded onto the gel and run at 150 Volt for 30-45 minutes. Afterwards, the protein from the gel is transferred to a cellulose membrane. For the transfer a sandwich is assembled with three Whatman papers then the cellulose membrane, which was first activated by methanol and quickly washed in TBST. The gel is put on top of the membrane and 3 more Whatmann papers are placed on top. Importantly, the whole sandwich should not dry out and the air bubbles are then removed before the whole sandwich is put into the transfer chamber. This is then run at 100V mA at 4°C in the cold room for 1 hour. The membrane is then blocked in blocking buffer made out of 5% dried milk powder in TBST for 1 hour. Afterwards, the primary antibody is applied overnight at 4°C on a moving platform. The next day, the membrane is washed 3x for 10 minutes in TBST the secondary antibody is applied for another hour and the washing step is repeated. To develop the membrane, the WesternBright Chemilumineszenz Substrat Sirius from Biozym was used. The two solutions are mixed in a 1:1 ratio and put on the membrane for 1 minute. The picture is then developed in the ChemiDoc from BioRad over 10 minutes with increasing exposure. This way the right exposure can be easily found, and the correct time determined. In case another protein detection from the same samples is done, the stripping buffer was put on the membrane for 10 minutes and washed in TBST afterwards. Then the next primary antibody was put on the membrane.

Freezing stains

To store strains for a longer time and to conserve them freezing is the method of choice. For this, a freezing solution is used made from 60 mL M9, 18 mL Glycerol and 42 mL of S-Buffer. The S-Buffer stock is made from 129 mL 0,05M K_2HPO_4, 871mL 0,05M KH_2PO_4 and 5,85g NaCl. The freezing solution is filtered sterile and stored at -20°C and placed at -4°C for defrosting. To prepare the worms for freezing they need to be chunked on 4 medium-sized plates and incubated until the agar is mostly gone and the worm stages are L1/L2. To ensure the survival of the strain in case of a mishap during the freezing one plate is kept at 14°C. Then 5 mL of the freezing solution is used per plate to wash down the worms from the plates which are then aliquoted into 5 microcentrifuge tubes. Everything is then stored at -80°C and after a couple of days one tube is checked if the worms do wake up and so see if there are some bad contaminations on the plates.

Bleaching stains

In case of bad contamination on the plates and rechunking does not work, the worms are bleached to ensure clean working.
The bleaching solution is made out of 1 part “Danchlor”, 2 parts 5M NaOH and 7 parts dH_2O. For the bleaching 4 medium plates need to be full of adult staged worms. Then 12 mL of the bleaching solution is put on the plates and used to wash down the worms by pipetting gently. The worms in the solution are then transferred in a 15 mL falcon tube and need to be shaken for 7 minutes by hand. They need to be checked regularly and when only eggs are left in the tube, it is centrifuged at 3000 rpm for 4 minutes at room temperature. Then the supernatant has to be removed and the pellet has to be washed with M9 three times to remove all the bleaching solution. After each washing step the tube has to be centrifuged again for 4 minutes at 3000 rpm. The eggs are then pipetted on fresh plates around the E.coli with a little bit of M9 and are then put at the desired temperature.

Results

A *chk-2* mutant with a deletion of the first 42 amino acids revealed that this region is essential for the protein's stability

To investigate the three versions of CHK-2 truncation, the strains were constructed by CRISPR/Cas9 as described in the methods section.
The first strain to be shown in these result sections is *ha::ppm-1.d;chk-2$^{5'\Delta}$::3xFLAG.* For this strain the first 42 amino acids from the 5' end were deleted, however, leaving the start codon intact to ensure the protein would still be translated.

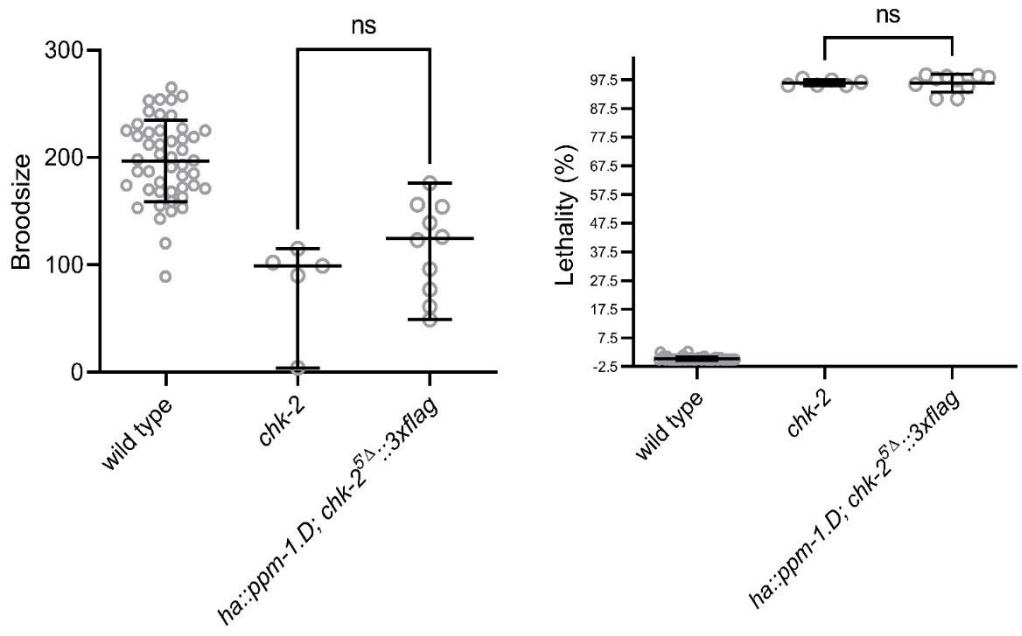

Figure 11 ***Broodsize and Lethality of strain ha::ppm-1.d;chk-2$^{5'\Delta}$::3xFLAG****. The graph on the left depicts the brood size of the wild type n=48, the chk-2 null mutant (me64) n=5 and the 5'Δ mutant of chk-2 (jf191) n=10. The graph on the right shows the lethality in % of the wild type n=48, the chk-2 null mutant (me64) n=6 and the 5'Δ mutant of chk-2 (jf191) n=10.*

The broodsize and lethality were counted as described in the method section and analysed with Prism. This is to find our whether the change in the *chk-2* gene has a direct impact on the fertility. The strain turned out to be non-viable which made it necessary to balance it with the SC4 balancer to maintain the strain. Accordingly, the lethality was analysed showing a similar result to the *me64* allele, which is a the *chk-2* null allele due to an early stop codon with 96% lethality and a broodsize of 115 of

total eggs and larvae on average. However, most of the eggs laid by the F0 hermaphrodite never hatched and are accordingly embryonic dead. The average brood size of the wild type is around 200 with 99% alive progeny. The data shown in the graph of chk-2 are obtained by counting and not from the original paper.

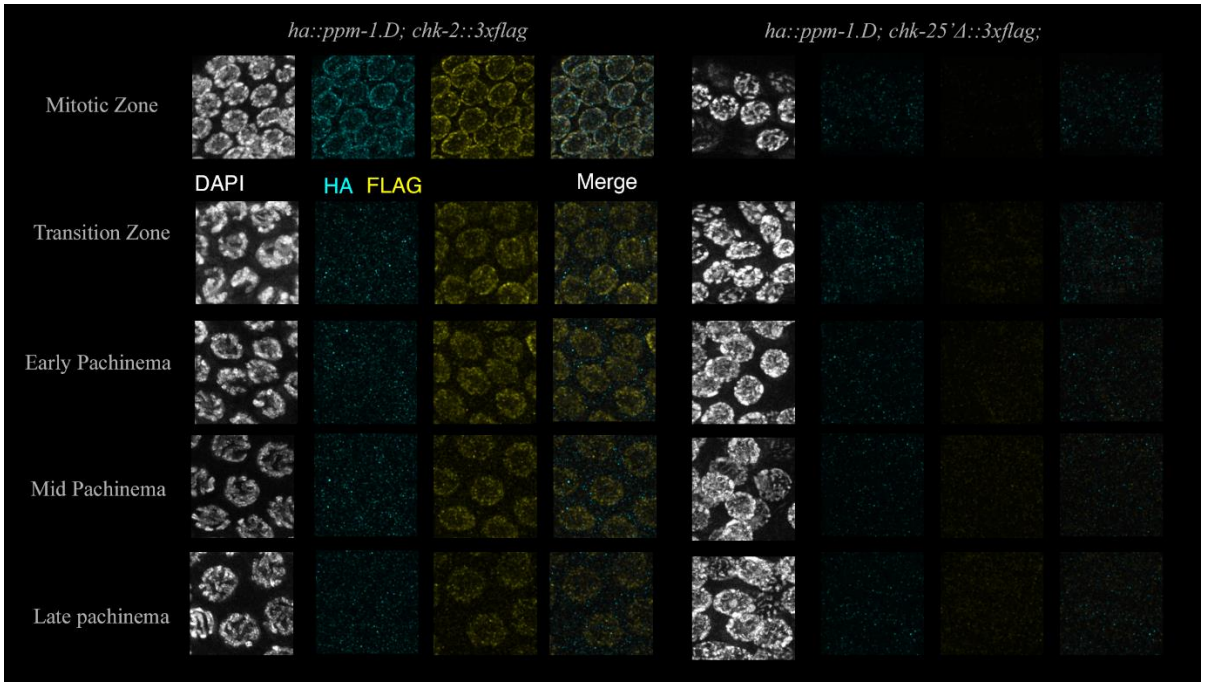

Figure 12 ***Image of the CHK-2 and PPM-1.D activity in the strain ha::ppm-1.d;chk-2$^{5'\Delta}$::3xFLAG****.the picture shows the nucleic staining inside the gonad with closer depictions of the mitotic zone, transition zone, and early, mid and late pachynema. Thereby the tagged wild type is shown on the left and ha::ppm-1.d; chk-2$^{5'\Delta}$::3xFLAG; is on the right*

The picture quantification was done staining the gonads by antibody staining and taking the pictures with the Deltavision. The exact procedure is described in the methods.

In the pictures, the HA::PPM-1.D is shown in cyan and CHK-2::3xFLAG is coloured in yellow. In the wild type, colocalization at the rim of the two proteins can be nicely seen in the mitotic zone. Further, the HA::PPM-1.D is decreasing starting from the transition zone, the CHK-2::3xFLAG can be nicely seen throughout the gonad steadily decreasing but clearly visible. In contrast in the pictures taken from the *ha::ppm-1.d;chk-2$^{5'\Delta}$::3xFLAG* the staining is almost gone and hardly visible. The HA::PPM-1.D can still be seen along the rim, however in a very decreased amount. The staining of CHK-2::3xFLAG is no longer visible in any of the different zones. In the DAPI

staining it can be clearly seen that the half-moon shape in the transition zone is not visible in the gonad of the *ha::ppm-1.d;chk-2$^{5'\Delta}$::3xFLAG* strain.

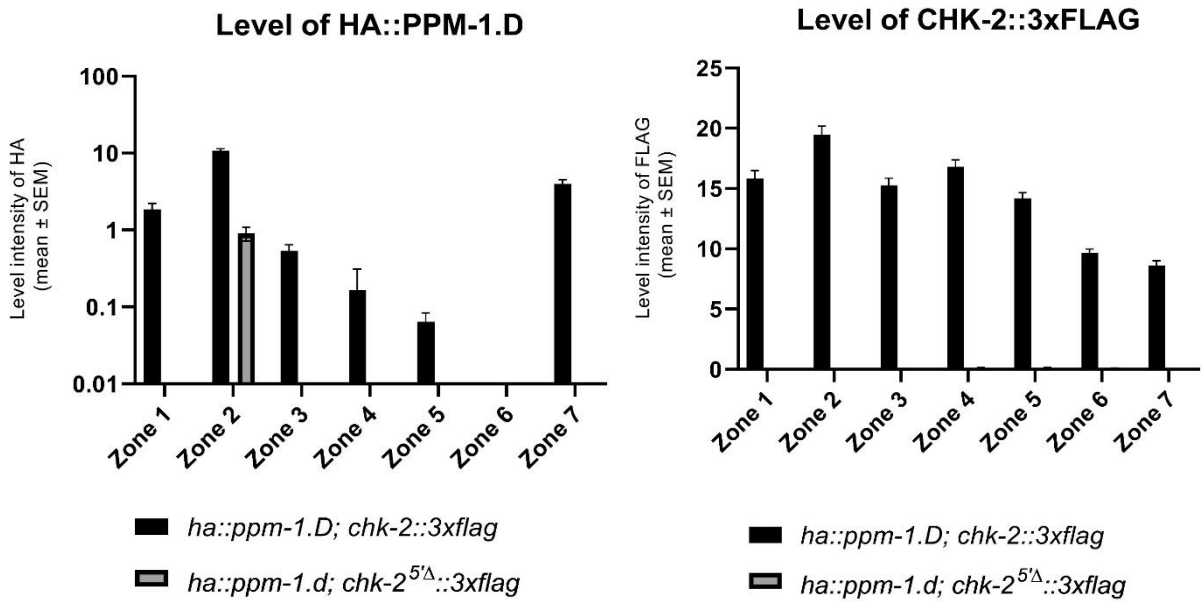

Figure 13 ***Intensity measurement of the gonads from the strain ha::ppm-1.d;chk-2$^{5'\Delta}$::3xFLAG in comparison to the wild type****. The graph on the left depicts the quantification of HA::PPM-1.D done on gonads of the tagged wild type (n= 5)* ha::ppm-1.d; chk-2::3xFLAG and *ha::ppm-1.d; chk-2$^{5'\Delta}$::3xFLAG (n=3). The graph on the right shows the protein quantification of CHK-2::FLAG measured on the gonads of* ha::ppm-1.d; chk-2::3xFLAG *(n=5)*and *ha::ppm-1.d; chk-2$^{5'\Delta}$::3xFLAG (n=3)*

To investigate the protein intensity the gonad is divided into seven zones starting at the very distal tip until the start of diakinesis. The measurements were taken from the pictures of the gonads that can be seen in Figure 12. In Figure 13 a tagged wild type is used as well as the *ha::ppm-1.d; chk-2$^{5'\Delta}$::3xFLAG*. Both measurements have the values of the non-tagged wild-type intensity measurements subtracted from them, meaning no background was put into the final result. It can be seen that the FLAG signal and correspondingly the chk-2 present is strongly reduced until to a point where detection was no longer possible. This is true in all seven zones equally.

While the result of the FLAG seems to be rather straightforward, the HA-tagged PPM-1.D showed a contradicting result. In the wild type the signal is rather strong and

although it is known to be degraded in the transition zone the intensity analysis shows that the signal degrades much slower and makes a reappearance in zone 7.
The mutant however shows only one zone with a strong signal which is the end of the mitotic zone and the beginning of the transition zone.
By deleting the 5' end of CHK-2, the protein is no longer present, and the worms show a strongly decreased viability.

The deletion of the FHA domain of CHK-2 leads to a similar phenotype as the null mutation

The second *chk-2* truncation was done by deleting the FHA domain of the protein which is a highly conserved domain. With the correct naming: *ha::ppm-1.d; chk-2$^{FHA\Delta}$::3xFLAG*

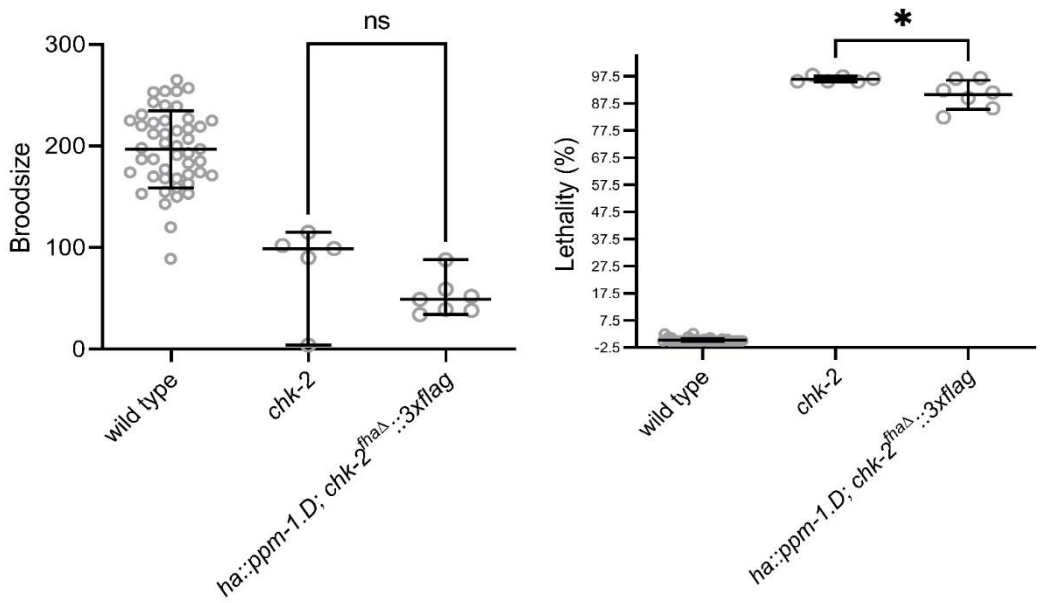

Figure 14 ***Broodsize and Lethality of strain ha::ppm-1.d;chk-2$^{FHA\Delta}$::3xFLAG*** *The graph on the left depicts the brood size of the wild type n=48, the chk-2 null mutant (me64) n=5, and the FHA'Δ mutant of chk-2 (jf191) n=7. The graph on the right shows the lethality in % of the wild type n=48, the chk-2 null mutant (me64) n=6, and the FHAΔ mutant of chk-2 (jf191) n=7.*

This analysis was done by counting the number of eggs and larvae coming from singled mothers and seeing how many of those eggs would eventually hatch. The protocol is listed in the methods section.

The phenotype observed from this mutation is comparable to the 5'Δ and the *me64* mutant allele. The brood size is strongly reduced compared to the wild type and shows an average of 51 laid eggs per singled hermaphrodite and accordingly, the lethality of the progeny is at 90% meaning only 10% of the already reduced progeny survives.

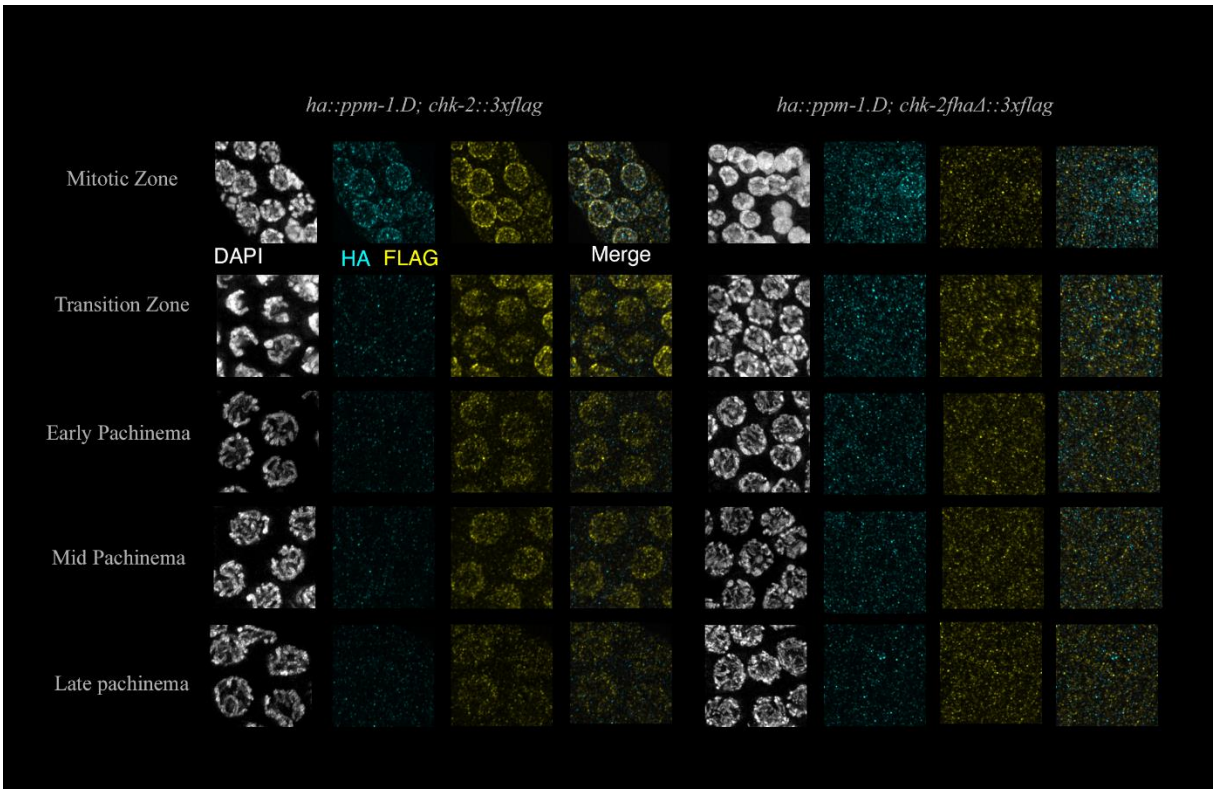

Figure 15 ***Image of the CHK-2 and PPM-1.D activity in the strain ha::ppm-1.d;chk-2^{FHAΔ}::3xFLAG*** *the picture shows the gonad staining with an example of the mitotic zone, transition zone, and early, mid, and late pachynema. The tagged wild type is shown on the left and ha::ppm-1.d; chk-2^{fhaΔ}::3xFLAG; is on the right*

In Figure 15 the DAPI staining of the gonads carrying the 5' deletion mutating of chk-2 shows in the mitotic zone a high number of non-viable nuclei that can only very rarely be observed in the wild type. In the transition zone, the typical half-moon shape that occurs during the recognition of two homologs is not present which can then be further seen in all time points of pachynema. Normally in that stage, the paired-up homologues can be observed together whereas in the mutant single floating chromosomes can be seen.

Looking at the HA tag of the PPM-1.D protein a strong signal can be observed however the recognition of the rim location is much less present than in the tagged

wild type. A clear difference that also can be seen on the pictures is that the signal does not go down as much as in the wild type where only a few dots can be observed. Both pictures were taken with the same settings and stained in the same procedure. Meaning the background is present in both genotypes equally.

The FLAG tag representing CHK-2 also shows a clear difference in comparison to the wild type. The strength of the signal is unambiguously weaker throughout the gonad and only a slight rim localization can be observed in the transition zone but not in the mitotic zone where the rim staining is the strongest in the wild type.

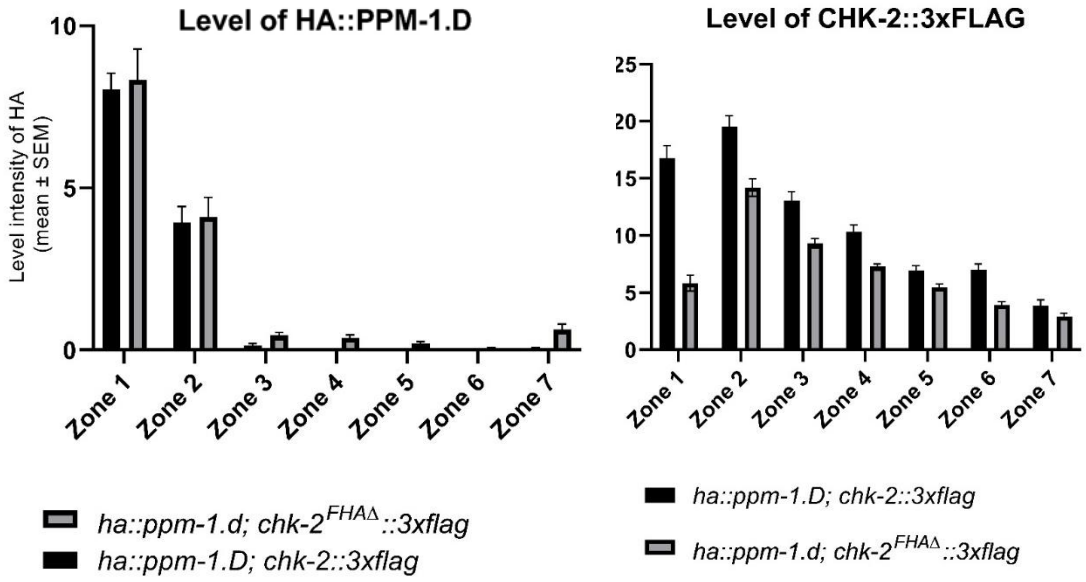

Figure 16 ***Intensity measurement of the gonads from the strain ha::ppm-1.d;chk-2$^{FHA\Delta}$::3xFLAG in comparison to the wild type*** *The graph on the left depicts the quantification of HA::PPM-1.D done on gonads of the tagged wild type ha::ppm-1.d; chk-2::3xFLAG* (n=10))and *ha::ppm-1.d; chk-2$^{fha\Delta}$::3xFLAG (n=8). The graph on the right shows the protein quantification of CHK-2::FLAG measured on the gonads of ha::ppm-1.d; chk-2::3xFLAG* and *ha::ppm-1.d; chk-2$^{fha\Delta}$::3xFLAG n=8*

When looking at the intensity levels, obtained from the pictures taken that can be observed in figure 14. The proteins in Figure 16 it can be seen that there is a strong reduction of the CHK-2::3xFLAG in zone 1 which equals the mitotic tip of the gonad. There is also a decrease in protein intensity in the following zones 6 however the further along the gonad the difference of intensity shrinks to be almost the same by

zone 7. When looking at the intensity measurement of the HA::PPM-1.D the zones of activity are similar without any significant change in comparison to the FHA deletion mutant. However, in zones 3 to 7 indicating the remaining part of the gonad until diplotene HA::PPM-1.D is not as low as in the wild type.

The amount of HA::PPM-1.D in zone 1 does not seem to be affected by the deletion of the FHA domain of CHK-2 when looking at zones 1 and 2 which represent the mitotic tip and the transition zone. In the wild type, it can be observed that the protein levels decrease to almost zero after the transition zone, however, in the mutant, the levels are still present and are higher in comparison to the wild type carrying the same tag.
The quantification of the CHK-2 carrying the 3xFLAG tag with a clear reduction is shown on the graph. The strongest drop in protein intensity can be seen in the first zone which corresponds to the mitotic zone. The remains are lower than the wild type throughout the gonad but have a peak in the second zone and flatten over the remaining zones 3 to 7. In the last zone, there is no significant difference in intensity levels between the wild type and the mutant.
Deleting the FHA domain of CHK-2 shows a similar phenotype as the 5' deletion, having a decreased protein activity and high lethality.

Removal of the 264 last amino acids of CHK-2 does not compromise viability of the organism

The third mutation of CHK-2 was designed to have a deletion of 792 bp right before the start of the 3' UTR. However, it was made sure that the FLAG tag remained intact to observe the location of the protein. The construction of this strain was again done by using CRISPR/Cas9 as described in the methods section.

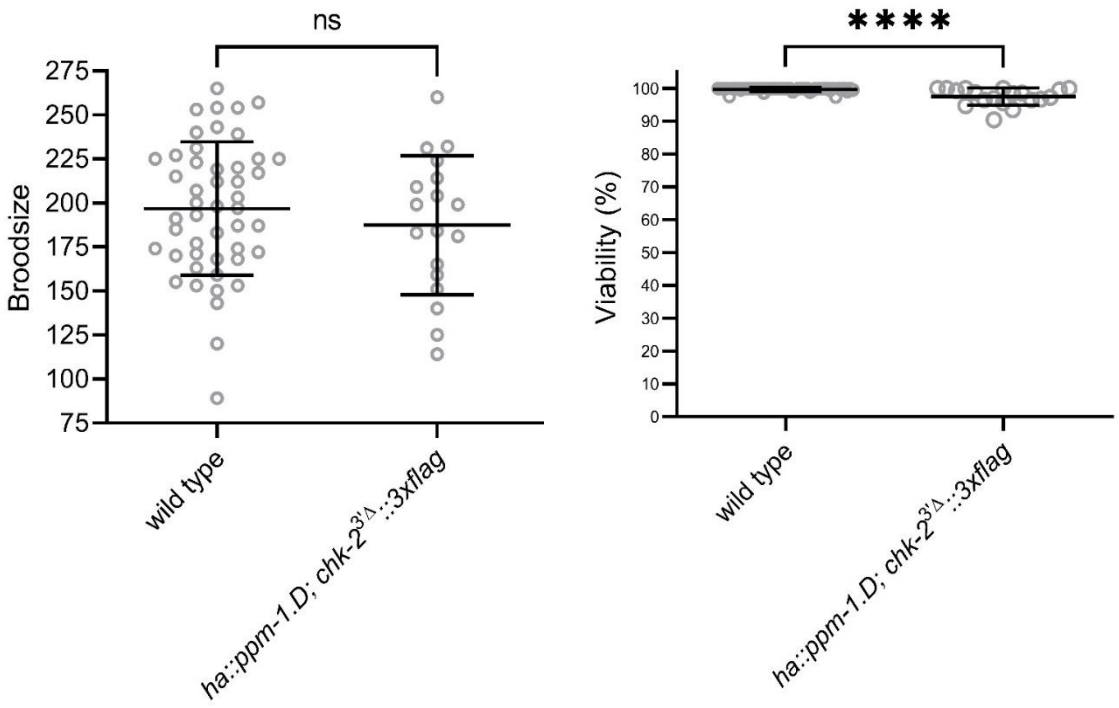

Figure 17 ***Broodsize and Viability of the ha::ppm-1.d; chk-2$^{3'\Delta}$::3xflag strain****. The graph on the left depicts the brood size of the wild type n=48 and the 3'Δ mutant of chk-2 (jf177) n=19. The graph on the right shows the viability in % of the wild type n=48 and 3'Δ mutant of chk-2 (jf177) n=19.*

The analysis of *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xflag* strain was done the same way as on the other strains. Since this stain is viable it was decided that showing the viability instead of the lethality was more suitable. The strain shows a high viability of 97,7% making it the only strain constructed for this thesis which did not need to be balanced. Despite the high viability of this mutant there is a significant difference to the wild type. When counting the brood size an average progeny of 187 per single hermaphrodite could be observed showing no significant difference to the wild type.

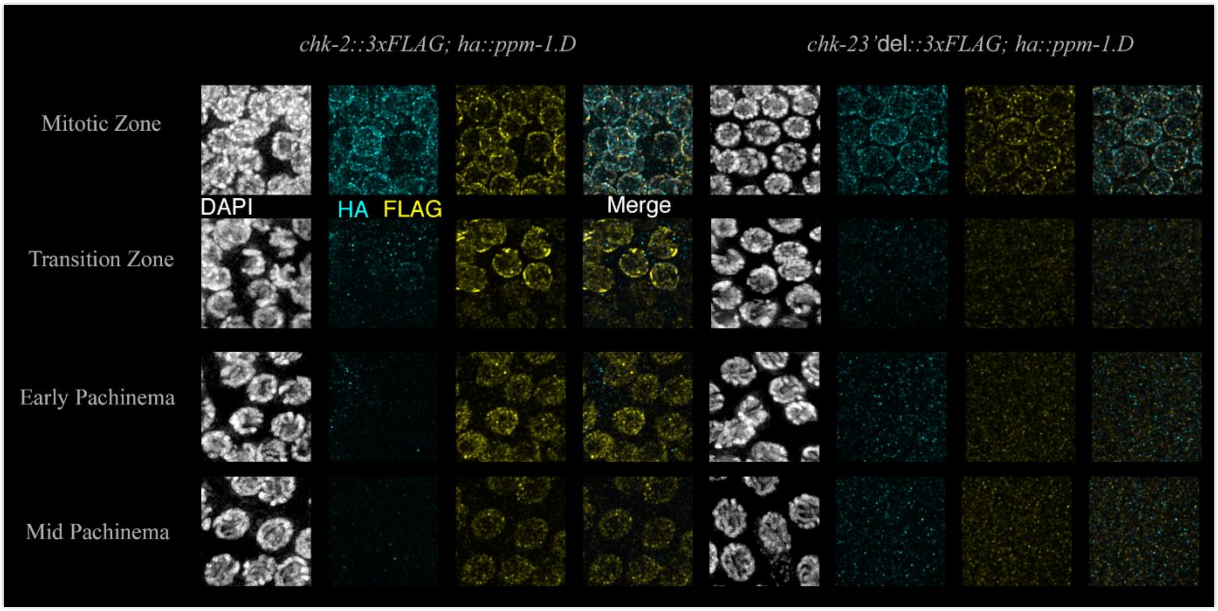

Figure 18 ***Picture analysis of ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG in comparison to the wild type****. The picture shows the gonad staining with an example of the mitotic zone, transition zone, and early, mid, and late pachynema. the tagged wild type is shown on the left and ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG; is on the right*

The staining of the FLAG antibody revealed that the rim staining on the mitotic zone was still very much present, but the intensity levels dropped drastically already starting in the transition zone. When looking at the pictures from the staining, the nuclei can no longer be recognized based on the FLAG staining. Additionally, aggregates formed by CHK-2 that can be seen in the stained wild type are no longer be seen in the stained mutant.

The HA staining follows a similar pattern in the mutant as in the wild type, however, in the mutant, the staining looks slightly less intense than in the wild type. The signal of the HA::PPM-1.D seems to be marginally stronger in the wild type yet the extent cannot be determined by the pictures only.

In the pictures taken a certain background staining is still present, however by doing the picture quantification the background was removed in the analysis.

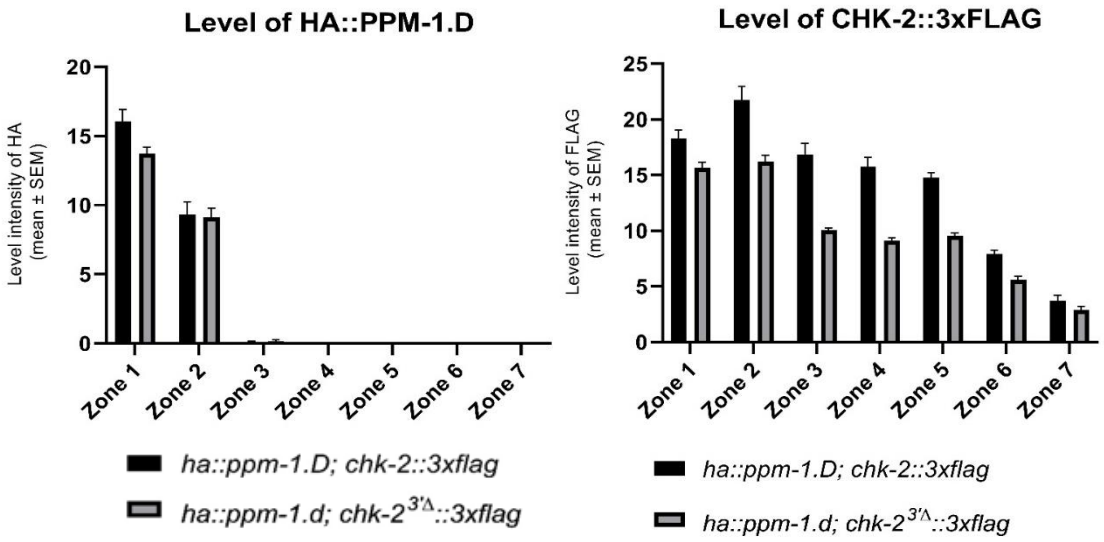

Figure 19 ***Intensity measurement of the gonads from the strain ha::ppm-1.d;chk-2$^{3'\Delta}$::3xFLAG in comparison to the wild type*** *The graph on the left depicts the quantification of HA::PPM-1.D done on gonads of the tagged wild type* ha::ppm-1.d; chk-2::3xFLAG and *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG. The graph on the right shows the protein quantification of CHK-2::FLAG measured on the gonads of* ha::ppm-1.d; chk-2::3xFLAG, *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG*

The HA::PPM-1D quantification on the left shows that in zone 1 the protein levels are slightly reduced however this is not significant and is therefore neglectable. In zone 2 the quantified level of protein is highly similar and in the following zones, the amount of protein is so low that neither in the mutant nor in the wild type the levels are high enough to be shown in the graph.

In Figure 18 the right graph shows a bit of a discrepancy when comparing the wild type to the mutant. While in zone 1 the amount of protein is somewhat similar, in zone 2 a decrease in the mutant can be observed. this distinction even increases more in zones 3 and 4. The level of protein seems to be constant in these zones until zone 5 in the mutant gonads while the levels in the wild type slowly decrease. Solely in zone 6 the CHK-2::3xFLAG levels are again decreasing in the last two zones 6 and 7 and have again a similar level as the wild type.

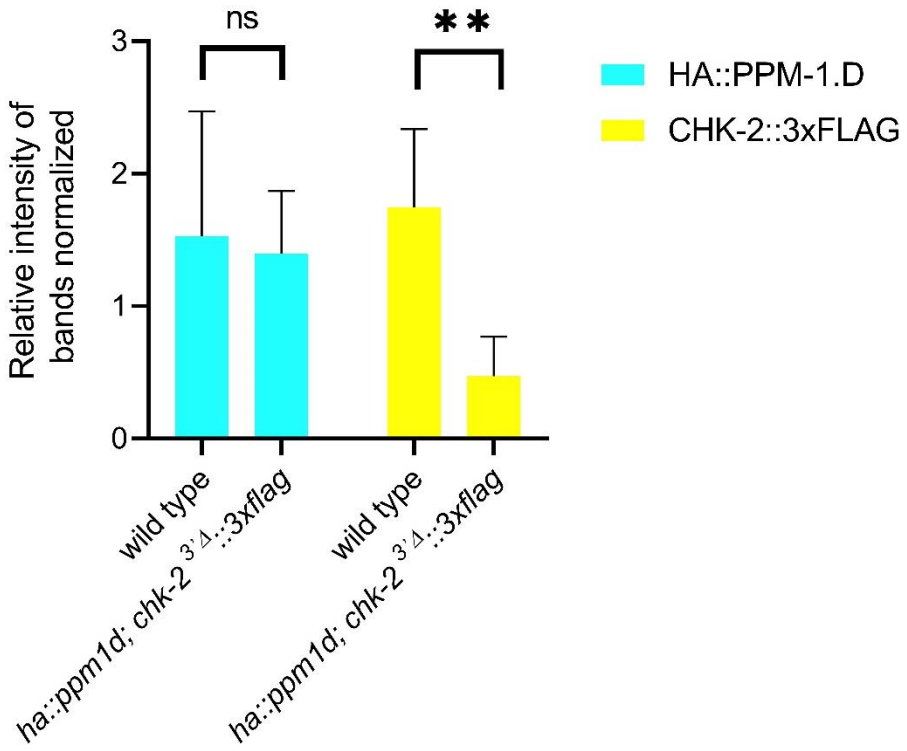

Figure 20 ***Western blot analysis****. The graph shows the normalized relative band intensity of the genotypes* ha::ppm-1.d; chk-2::3xFLAG and *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG. Thereby HA::PPM-1.D is on the left in cyan and CHK-2::3xFLAG is shown on the right in yellow*

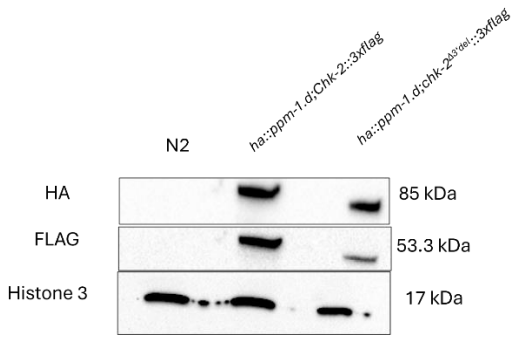

Figure 21 ***Western blot of ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG****. In the figure above the protein bands of the western blot is shown of the constructed strain, the tagged wild type and N2*

Due to the viability of the mutant, it was possible to measure the relative intensity of the proteins based on the tags. This was done by Western blot with the exact procedure described in the methods section. The intensity of the HA-tagged PPM-1.D shows in the mutant and the wild type a similar result with a rather high standard deviation. In the protein levels observed in the CHK-2::3xFLAG, a significant

difference can be observed. The amount present in the wild type is considerably higher than in the mutant.
Overall with the 3' truncation a clear decrease in CHK-2 activity could be observed while the organism remain viable.

The CHK-2 paralog CHEK-2 does not act redundantly with CHK-2

Given the mild phenotype observed in the *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xflag* mutant, I wanted to test whether the paralog CHEK-2 was having a compensatory function. For better understanding the results of the single mutant *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xflag* are repeatedly shown in the quantifications in the following section. This is for easier comparison; however, the results will not be described again in detail in the text.

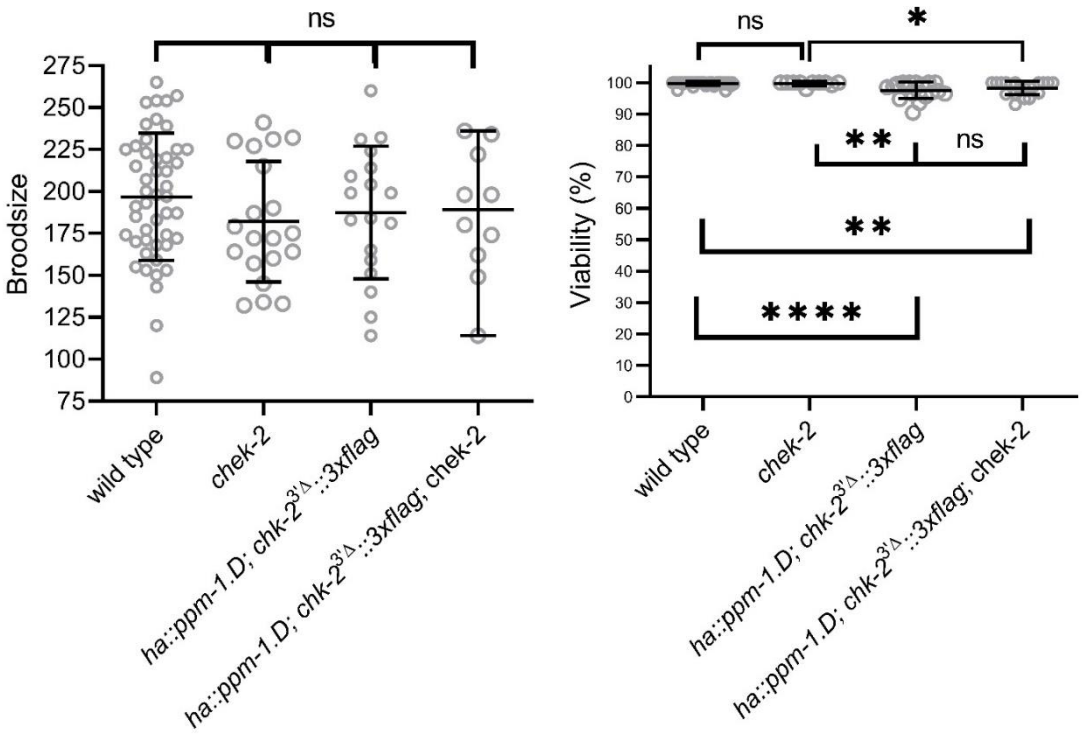

Figure 22 ***Broodsize and Viability of ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG; chek-2****. The graph on the left depicts the brood size of the wild type n=48, the chek-2 null mutant (ok431) n=10, the 3'Δ mutant of chk-2 (jf177) n=18 and the double mutant chk-2$^{3'\Delta}$::3xflag; chek-2 n= 20. The graph on the right shows the viability in % of the wild type n=48, the chek-2 null mutant (ok431) n=10, the 3'Δ mutant of chk-2 (jf177) n=18 and the double mutant ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG 2 n= 20*

After the crossing of the *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xflag*; with the *chek-2(ok431)* strain it was found that the new strain was still viable and found to have a similar viability as the N2. The number of progenies laid per F0 hermaphrodite is 186,7 on average and is not significantly different from the wild type, and both strains that were crossed. The viability of the progeny lay at 98,25% Though still very high it is significantly lower than the wild type and the *chek-2* at 99.71% strain. However, the double mutant is just as viable as the single mutant.

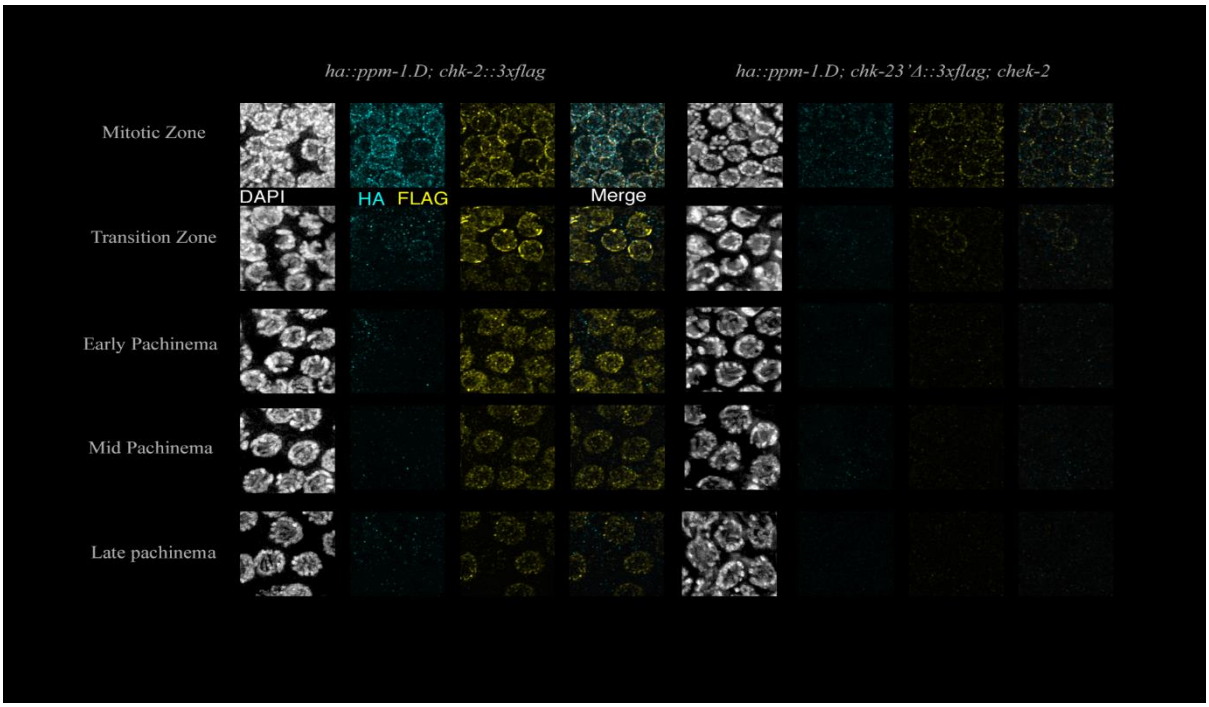

Figure 23 ***Staining comparison of the ha::ppm-1.d; chk-23'Δ::3xFLAG; chek-2 to the wild type****. The picture shows examples of the gonad staining with an example of the mitotic zone, transition zone, early, mid, and late pachynema. Thereby the ha::ppm-1.d; chk-2::3xFLAG is shown on the left and ha::ppm-1.d; chk-23'Δ::3xFLAG; chek-2 is on the right*

To have a more specific look in the protein activity the gonads were dissected and stained by antibody staining. This was done in order to find out if the presence or absence of CHEK-2 is influencing the CHK-2 activity. The protocol used, is written in the methods section.

In the pictures taken, it can be seen that PPM-1.D and CHK-2 are located at the nuclear rim and colocalized there however, the level is present to a much lesser extent. In the transition zone, the protein signals are no longer visible and besides some background, the staining is completely gone in early, mid, and end pachynema.

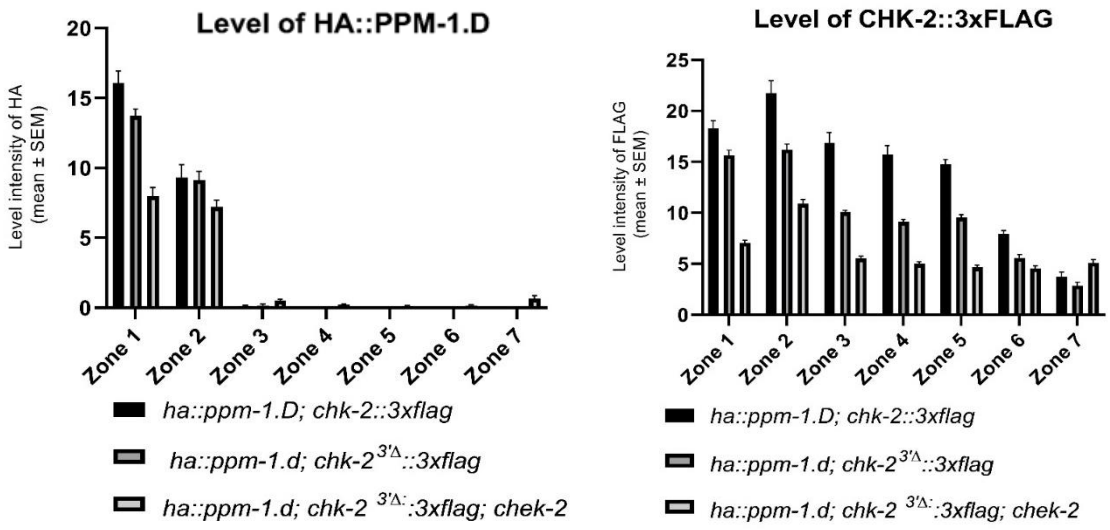

Figure 24 ***Intensity measurement of the gonads from the strain ha::ppm-1.d;chk-2$^{3'\Delta}$::3xFLAG;chek-2.*** *The graph on the left depicts the quantification of HA::PPM-1.D done on gonads of the tagged wild type* ha::ppm-1.d; chk-2::3xFLAG, *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG and ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG; chek-2. The graph on the right shows the protein quantification of CHK-2::FLAG measured on the gonads of* ha::ppm-1.d; chk-2::3xFLAG, *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG and ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG; chek-2*

To gain more detailed data from the pictures in figure 23 the intensity level of the protein signals were measured. This was done with the same strategy as the previous strains.

In the protein level quantification, it can be seen that the amount of HA::PPM-1.D is strongly decreased in zone 1 between the wild type and the *chek-2* mutant, however, the level almost equals out in zone 2. In the following zones the levels of the protein drop to around zero and are approximately zero. On the protein level of CHK-2 there is also a clear drop of intensity present in all zones along the gonad but are getting more similar towards zone 7. The overall drop is even higher in comparison from the single mutant to the double mutant.

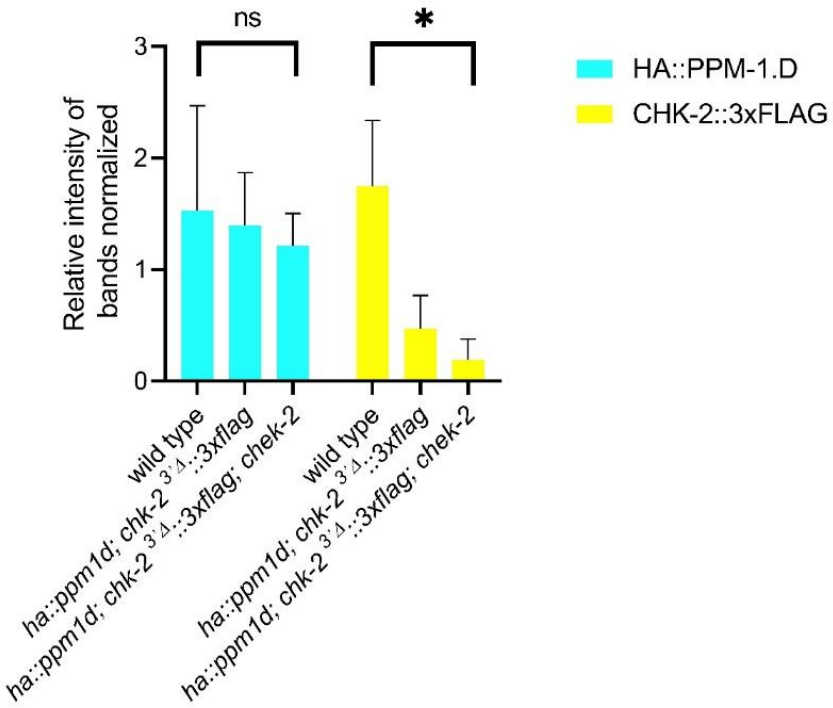

Figure 25 ***Western blot analysis of ha::ppm-1.d;chk-2$^{3'\Delta}$::3xFLAG;chek-2.*** *The graph shows the normalized relative band intensity of the genotypes* ha::ppm-1.d; chk-2::3xFLAG, (n=4) *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG (n=4))and ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG; chek-2 (n=3)). Thereby HA::PPM-1.D is on the left in cyan and CHK-2::3xFLAG is shown on the right in yellow.*

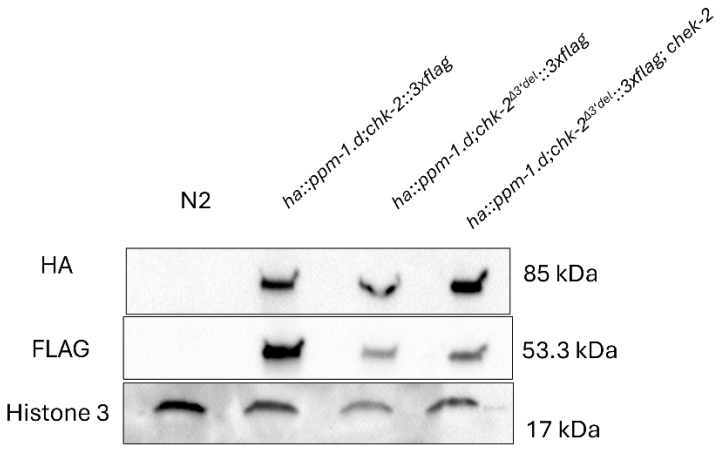

Figure 26 ***Western blot bands of ha::ppm-1.d;chk-2$^{3'\Delta}$::3xFLAG;chek-2.*** *The figure above shows the protein bands of the western blot analysis of N2, the tagged wild type, the single chk-2 $^{3'\Delta}$ mutation as well as the double mutation of chk-2 $^{3'\Delta}$;chek-2*

In the third way of measuring the amount of protein, the western blot shows a similar result to the measurement shown by the picture quantification. The levels of

HA::PPM-1.D look slightly decreased in the single mutant and even lower in the double mutant however due to the high standard deviation no significance can be measured. The level of CHK-2 however is significantly lower than the wild type in the double mutant. Whereas again due to the standard deviation, no statistical significance from the single and the double mutant can be observed.
The deletion of *chek-2* shows an even further decrease in CHK-2 activity, however, the worm remains viable.

DAPI body count of bivalents directly depicting meiotic errors: an overall comparison of constructed strains

The diakinesis gives a reliable picture of meiotic errors that occurred before. Due to that fact a count of the bivalents and univalent of this stage was done. For this measurement the gonads were dissected and the DAPI bodies were counted under the microscope.

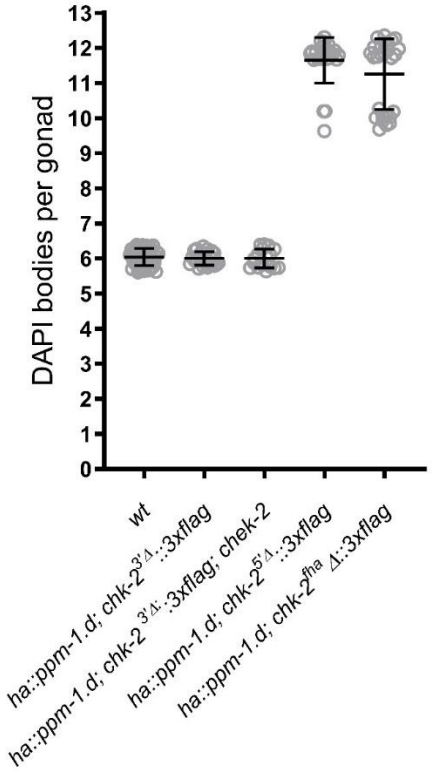

Figure 27 ***DAPI-body count of all constructed strains in comparison****. The graph above shows the DAPI body's counted on the genotypes: wild type n= 50, ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG (n=24), ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFLAG; chek-2 (n=27), ha::ppm-1.d; chk-2$^{5'\Delta}$::3xFLAG (n=25) and ha::ppm-1.d; chk-2$^{fha\Delta}$::3xFLAG (n=27)*

The amount of DAPI bodies is a good indication of meiotic failures and correlates to the condensed chromosome pairs in the diakinesis stage. In the wild type, there are 6 DAPI bodies which is the ideal number. Similar to the wild type also the *ha::ppm-1.d*; *chk-2$^{3'\Delta}$::3xflag* and the double mutant *ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFlag; chek-2;* show 6 bivalents in all gonads. In contrast, the chk-2$^{5'\Delta}$::3xFlag; ha::ppm-1.d shows an average of 11.65 DAPI bodies per gonad ranging from 9 to 12. The chk-2$^{FHA\Delta}$::3xFlag; ha::ppm-1.d also shows an increased number of DAPI bodies with an average of 11.25 with most of the gonads having either 12 or 9 univalents.
Overall number of bivalent of the strains containing the 3' truncation seem not to be affected while the FHA and 5' truncations show an increased number of univalents

By utilizing a prom-1 null strain crossed in the chk-2 feedback loop was investigated by looking at SUN-1 (S8-Pi) and DSB-2

The generation of a crossover is accompanied by feed-back control exerted through CHK-2 kinase. (Kim et al., 2015) This would lead to a longer SUN-1 (S8-Pi) or DSB-2 phosphorylation through CHK-2 when a certain recombination intermediate is not generated in a genetic background such as a spo-11 mutant. In the following experiments, it was tested whether the checkpoint kinase can do so in the genotype *ha::ppm-1.d;chk-2$^{3'\Delta}$::3xFLAG and ha::ppm-1.d;chk-2$^{3'\Delta}$::3xFLAG;chek-2*.
The *spo-11* mutation was crossed into the strains creating *ha::ppm-1.d;spo-11;chk-2$^{3'\Delta}$::3xFLAG and ha::ppm-1.d;spo-11;chk-2$^{3'\Delta}$::3xFLAG;chek-2*. In comparison to the wild type, the *spo-11* mutant shows a significantly longer phase of SUN-1 (S8-Pi) phosphorylation which can be tracked back to CHK-2 activation. The gonads carrying only the truncated version of the checkpoint kinase this phase is significantly shorter than in terms of cell rows and is equal to the wild type. A similar result is shown in the mutant only lacking *chek-2*.

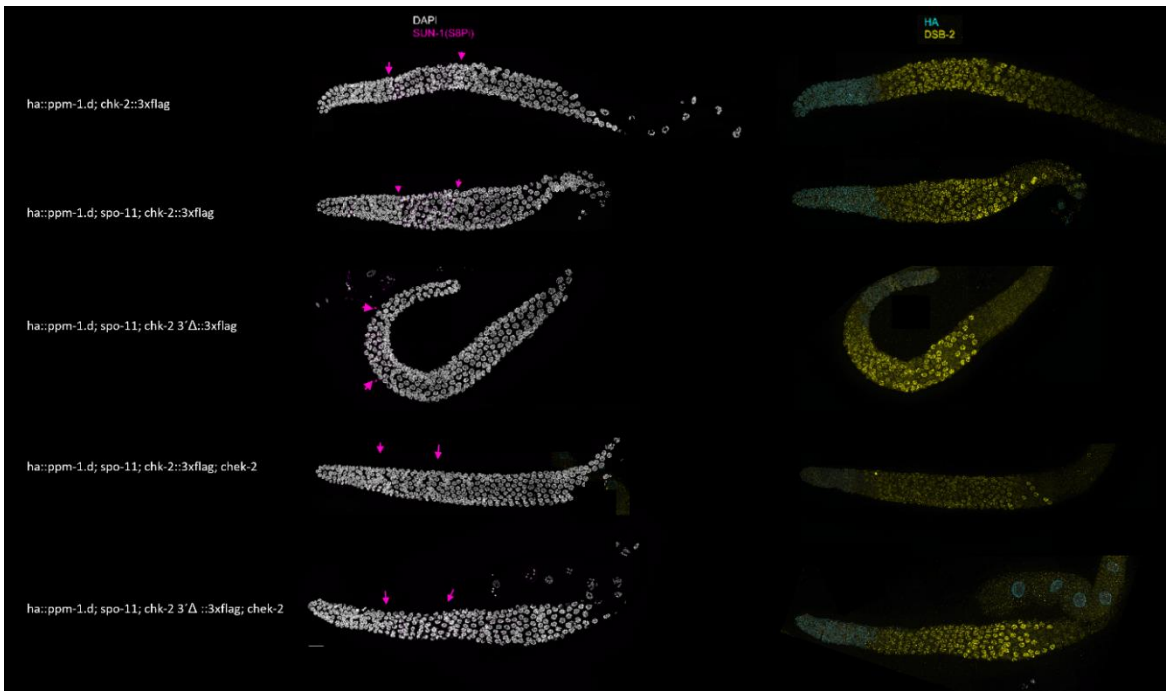

Figure 28 ***Antibody staining to obtain active cell row count analysis****. Above the gonad staining of DAPI (white), SUN-1 (S8-PI)(magenta), ha::ppm-1.D(cyan) and DSB-2(yellow)) are shown. The scalebar accounts for 10µm.*

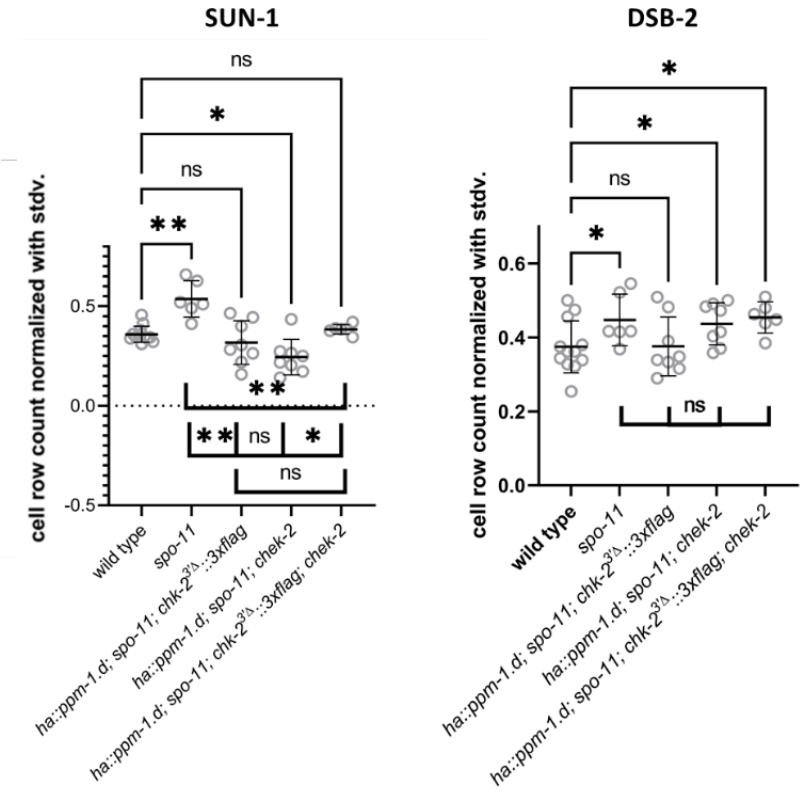

Figure 29 ***Results of the active cell row count of SUN-1 (S8-PI) and DSB-2****. The graph on the left depicts the SUN-1 (S8-PI) and on the right DSB-2 cell row count normalized to the total number of cell rows, with the standard deviation of the genotypes: wild type n=12, spo-11 n=6, ha::ppm-1.d; spo-11; chk-2$^{3'\Delta}$::3xFLAG n=8, ha::ppm-1.d; spo-11; chek-2 n=8 and ha::ppm-1.d; spo-11; chk-2$^{3'\Delta}$::3xFLAG; chek-2 n=6*

Looking at the results from the SUN-1 (S8-PI) phosphorylation between the wild type and the *spo-11* single mutant there is a significant difference in length of active SUN-1 (S8-PI) phosphorylation. The activity decreases in the 3' truncation which is no longer significantly different from the wild type with normal SPO-11 activity. A similar result is given in the worm carrying the *chek-2* mutation as in the 3' truncation of *chk-2*. The double mutation including the *chk-2* $^{3'\Delta}$ and *chek-2* seems to have again a similar SUN-1 (S8-PI) activity as the wild type meaning still decreased from the SPO-11 phenotype.

In the results coming from the cell row count of the DSB-2 activity, a similar result was observed. The DSB-2 activity was prolonged in the *spo-11* mutant in comparison to the wild type. Further, a slight increase in activity length was counted in the *chek-2* single mutant in comparison to the wild type however not different from the SPO-11 strain. The other strains have an activation length like SPO-11.

Overall in both strains the SUN-1(S8-PI) activity is decreased, while the DSB-2 activity seems not to be influenced be the absence of the 3' end of *chk-2* and *chek-2*.

The absence of PPM-1.D and PPM-1.G are not relevant for the preservation of the nucleic pore

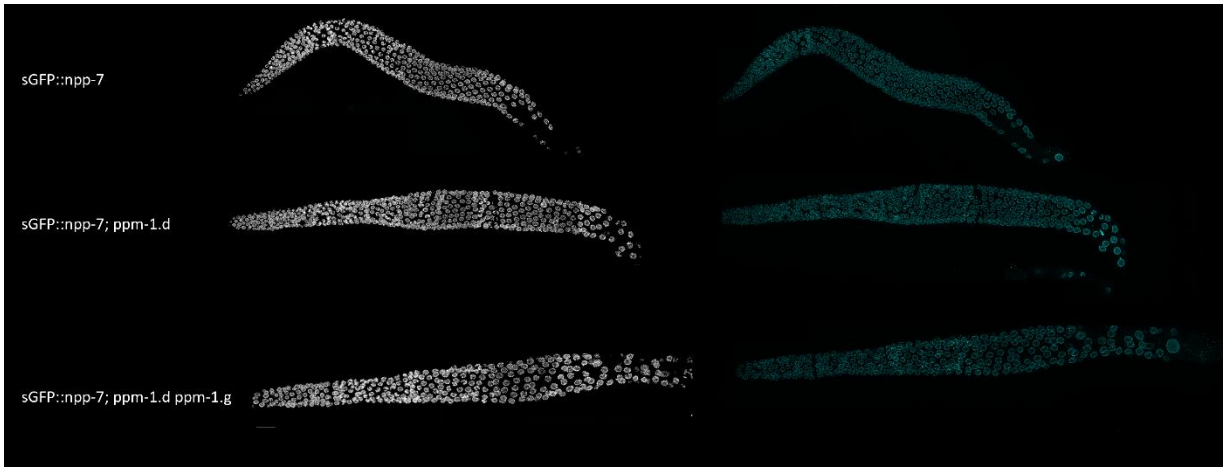

Figure 30 ***Antibody staining of NPP-7****. In the picture above three gonads are shown from the strains sGFP::npp-7, sGFP::npp-7;ppm-1.d and sGFP::npp-7;ppm-1.d ppm-1.g. The staining is against the sGFP and therefore depicting NPP-7*

This experiment was conducted to check if PPM-1.D or PPM-1.G is also play an important role in the preservation of the nucleic pore in meiosis. Therefore a strain having an sGFP tag on the *npp-7* gene was chosen and crossed into a *ppm-1.d* single and a *ppm-1.d ppm-1.g* double mutants. NPP-7 is a part of the nucleic pore. (Cohen-fix & Askjaer, 2017). The results were obtained by antibody staining according to protocol and evaluated by imaging. According to the pictures shown in figure 30 no clear difference in staining pattern is recognizable.

Discussion

Results of the chk-2$^{5'\Delta}$ show a very similar phenotype as the chk-2 null mutant

From the results from the construct *ha::ppm-1.d;chk-2$^{5'\Delta}$::3xFLAG* it is suggested that the first 42 amino acids are necessary for the stability and correspondingly the function of the protein. The nuclei show as expected a lack of chromosomal homologue pairing like in the me64 null mutant.
Further, the mutant gonads lack the transition zone and correspondingly no half-moon shape of the chromatids can be observed, which is typical for that stage. This proved to be slightly challenging, especially in the early stages where the expectations of the CHK-2 protein function were not known.

Interestingly, even though the mutant only lacks a very small portion of the protein the function is not fulfilled. Already expectant from that result, the level of CHK-2::3xFLAG measured by the picture quantification shows no signal after the background was subtracted. The little signal that can be seen in the pictures taken, is no longer localized on the nucleic rim in the distal tip just as it is no longer localizable along the chromosomes in the pachynema.
The corresponding result can be seen in the SUN-1 (S8-Pi) staining. The staining is almost non-existent as a result that CHK-2 is not active anymore and can therefore not be involved in the phosphorylation of SUN-1 (S8-Pi).

In general, the identification of the gonads coming from a homozygous mutant transpire is rather difficult. Again, since the SC4 balancer has no optical marker, the selected worms had to be cut together with their heterozygous siblings and could only be identified by the lack of a transition zone and the number of univalent in the diakinesis stage.

The measurement of PPM-1.D is slightly controversial since the it seems that in the first zone, the signal is very low to almost non-existent whereas in the second zone, the signal seems to be almost normal. This would rather be the result expected from the wild type. However, the wild type shows questionable results as it is known that

PPM-1.D gets degraded at the end of the transition zone which is rather controversial from the measurements taken.

To confirm the result of the protein expression a western blot would have been handy to evaluate the actual strength of the protein signal. However, since the strain is not viable and needs a balancer for maintenance of the strain. For a western blot 200 of a homozygous worm would have been needed which would increase the workload to at least 800 worms singled out as L4 and only picked when no progeny was observed. This would also consequently mean that the worms would be older than the state-of-the-art protocol would suggest gaining the best possible outcome. Additionally, the SC4 balancer has no optical phenotype besides being lethal when homozygous. An attempt to insert a GFP tag into the balancer was unfortunately unsuccessful. Thus, it would have been possible to get one sample for the western blot however the workload would exceed the benefit gained from it.
Regardless, the cytology staining clearly shows that when truncating CHK-2 by deleting the 5' end of the gene, the protein CHK-2 is no longer stable and consequently unstable to fulfil its function. Nevertheless, it seems not to have an impact on the expression, stability, and localization of PPM-1.D in the distal tip. Also, the degradation does not seem to be impacted by the absence of CHK-2.

The imaging process of the pictures is not an easy task as already the identification can be challenging. The strain is not viable in a homozygous state meaning it had to be balanced with the SC4 marker which however does not have a phenotypical marker. This means that when cutting and staining the gonads, distinguishing the balanced and the homozygous worms were not possible. The gonads were then identified under the microscope with the help of the SUN-1 (S8-PI) staining. This staining is present in the heterozygous but not in the homozygous. Another point of difference is the absence of the half-moon shape typical for the transition zone. The amount of on the slides that are the correct genotype is approximately 33% which makes the acquiring of the pictures more challenging. Clearly, in the pictures HA::PPM-1.D is still visible however in comparison to the wild type the levels are very low. For each genotype also some untagged wild-type gonads were stained which shows a similar level of protein. In zone 1 a very slight staining at the rim can still be observed and decreases even further as it gets degraded by the F-Box of PROM-1.

In the quantification, the intensity levels are background corrected and this leads to the result that the levels are lowered to zero. In zone 2 which equals to the end of the transition zone and the beginning of the pachynema, an increase of the protein expression can be seen. The reason for this is not known, however, it could be due to some aggregates that are formed and therefore give a stronger signal. Interestingly, in the wild type, the protein amount decreased only slowly which could be a result of staining artifacts as PPM-1.D normally gets almost fully degraded. The increase in zone 7 which would equal the end of pachynema and the start of diplonema can be explained by the role of PPM-1.D in this stage. The exact reason for this increase is not investigated yet but would be an interesting topic to study. Nevertheless, this is interestingly not the case in the wild type where the levels remain low. The question of why the protein decreases when CHK-2 is truncated at the 5'end cannot be answered directly.
Looking at the pictures acquired for CHK-2 the protein is so low that on the pictures only very light yellow dots can be seen, which indicates that the protein is not stable enough with this specific truncation. This is a similar result that was observed in the chk-2 null mutant.

The brood size shows clearly the difference between the wild type and the mutant. The mutant *ha::ppm-1.d; chk-2$^{5'\Delta}$::3xflag* shows a highly varying brood size. A difficulty here is that the mutant is phenotypically not distinguishable from the balanced wild type which led to a count of more than 10 worms and then only considering those that show a low brood size. A possibility to make sure the genotype is correct would have been genotyping the hermaphrodite after all eggs were laid. However, due to strong differential brood sizes observed on the plates, this was not done. The result from the *ha::ppm-1.d; chk-2$^{5'\Delta}$::3xflag* is not significantly different from the me43 CHK-2 null mutant. These results were counted and not taken from the original paper. The out layer was checked after the counting and it had to be taken into evaluation as the hermaphrodite did not look sick or died during the counting but simply did not produce any progeny.
For better visualization, the lethality is shown in the result section which is again not significantly different from the chk-2 null mutant.

When deleting the FHA domain a similar phenotype to the null mutant was observed.

By deleting the whole FHA domain, it was hypothesized that it would have a more severe outcome than the 5' Δ mutation which can be observed in the results of the viability and broodsize count. The amount of progeny is only around 44% of the 5' mutant and interestingly it is even reduced by 38% from the me64 null mutant. This could however come from the low number of null mutant worms counted. What can be said for sure is that this mutant is highly interfering with meiosis and resulting in a phenotype that needs to be balanced to be able to work with it. Looking at the viability it is fascinating to see that more of the progeny survive in comparison to the 5'Δ. Whereas only 4% of the progeny survive until adulthood in the 5' Δ and the null mutant alike it is 10% in the FHA deletion. This would lead to the suggestion that the 5'Δ mutant hermaphrodite can lay more eggs but have a majority of them embryonic dead or not fertilized. Unfortunately, it is often hard to distinguish fertilized and unfertilized eggs from a visual inspection of the eggs with the naked eye.

The DAPI staining in the pictures shows clearly that an increased amount of apoptosis takes place owed by the lack of the 5' end of CHK-2. This is most likely because of the important role CHK-2 plays in the cell cycle and is essential during the time of replication. Besides this stage, the gonad looks highly similar to the staining shown in picture 5 of the 5' deletion the absence of the transition zone is a clear indication that a meiotic error is occurring. The pattern that can be seen in pachynema is also very similar to the one of the chk-2 null mutant and the 5'Δ.

However, what is very interesting to see is that the HA::PPM-1D does not seem to be reduced while only the localization along the nucleic rim is not present any longer. Also, the reduction of PPM-1.D after the mitotic zone is not that strong which differs from the 5'Δ as the signal is almost gone. There is likely an increase in background noise that is seen in the pictures but also in the quantification, where the background was subtracted from the measure values, the signal of the HA::PPM-1.D stays present throughout the gonad. To reach an explanation for this, further experiments would need to be conducted, however, due to a lack of time and a different research focus this phenomenon was not further conducted.

Much more interesting for the project is the CHK-2::3xFLAG. In comparison the 5'Δ it looks like the protein is formed but is not able to execute its task. The protein does

only localize on the nucleic rim during the transition zone and the aggregates that are formed from the CHK-2 and the PPM-1.D are not colocalizing as they would normally do in the wild type. In the comparisons of protein intensity throughout the zones, although CHK-2 remains always less strong in all zones the biggest difference is found to be in the mitotic zone this corresponds to the apoptotic cells in this region of the gonad. What is important to be acknowledged is that although the 5' deletion carries more amino acids of the original protein than the FHA deletion it does seem to have a higher impact on the formation and stability of CHK-2. so it seems that CHK-2 can cope better with the loss of the FHA domain than with the 5'end of the gene.
Again, in this case a western blot was not possible because the balancer chosen does not carry any phenotypical marker so distinguishing the heterozygous and the homozygous genotype was not possible.

CHK-2$^{3'\Delta}$ shows a reduction of CHK-2 levels but remains viable

The 3' mutation is rather unexpected in comparison to the other two truncations that are described above because of its viability. This allows the working with the strain as a homozygous and does not have to be balanced. This not only facilitates the handling of the strain but also allows the possibility of making a western blot to have another look at the protein level in the worm.
As already mentioned, the strain shows a high viability which however is still significantly lower than the wild type. This is most likely caused by a certain variance that is observed in the mutant while the wild type is very stable at a higher level. Additionally, there are more single hermaphrodites counted in the wild type than in the mutant which was counted in two rounds to increase the sample size. A slight decrease in viability is however not unexpected as still a significant number of amino acids of an essential protein is reduced.
This reduction can be seen in the pictures taken by high-resolution fluorescence microscopy. In the mitotic zone, it is visible that the two proteins CHK-2 and PPM-1.D are colocalizing at the nucleic rim just as in the tagged wild type a clear difference in the two genotypes can be seen starting in the transition zone. While in the wild type, the rim staining is still visible is almost gone in the mutant. Also, the strong CHK-2 aggregates that are present in the wild type no longer exist in the mutant. This is also

reflected in the protein level quantification of CHK-2 which shows a strong drop of protein in all but the first zone.
When looking at the pictures of HA::PPM-1.D a slight reduction of protein is visible which however is not as drastic as in the CHK-2. Interestingly, when looking at the pictures only it seems like the PPM-1.D is not as much reduced throughout the pachynema, however in the quantification where the background was reduced by also measuring the intensity of an untagged wild type and reducing this in the tagged gonads, the levels of PPM-1.D is in both genotypes almost equal to zero. All gonads that were used for the quantification were stained under the same conditions at the same time point, so only small variances in staining success are possible which however should be balanced by a higher amount of gonads per genotype.

The third way of looking at the protein was to perform a western blot. This however gives the total protein present in the whole worm and not only in the gonad. Nevertheless, this method shows a similar result as observed from the image quantifications from the microscope In the bands on the western blot representing the HA::PPM-1.D no significant result could be observed. The result however shows a high standard deviation which could be caused by the varying results of the single western blot repetitions. However, when looked at individually a similar trend can be observed in each of the blots done. In contrast, the result from the CHK-2::3xFLAG is significant in the sense of protein reduction in the mutant and thereby once again confirms the results of the picture quantification.

When looking at the levels of CHK-2 in the 3'deletion it is interesting to see that even with the drop in intensity level of the protein the worm and the topological appearance of the gonad itself are phenotypically comparable with the wild type. When only looking at the DAPI staining the 3'Δetion and the wild type N2 cannot be distinguished. This led to the hypothesis that maybe the paralogue of CHK-2, CHEK-2 would take over some of the function and overcome the loss of protein. This phenomenon was already observed in other protein paralogues. This would mean that CHEK-2 would be able to recognize that CHK-2 is strongly reduced and unstable and replace it in some of its functions. To confirm this hypothesis a new cross was set up with would have the 3' deletion and a full deletion of the CHEK-2 protein.

The additional deletion of CHK-2s paralogue shows that it does not compensate the chk-2$^{3'\Delta}$

The cross of the genotypes of ha::ppm-1.d; chk-2$^{3'\Delta}$::3x*flag and chek-2* were easy to construct as all genes of interest are located on different chromosomes.

After setting up the cross and counting the viability and broodsize it was interesting to see that the worms did not show any difficulties in reproduction. This is suggested by indifferent results in brood size. Interestingly the average counted viability of the double mutant is slightly higher than the single mutant. From this result, it is suggested that the double mutant does not show any additional meiotic defects.
The first hypothesis, that CHEK-2 would take over partial function from the truncated CHK-2 could not be confirmed as the meiotic outcome did not change in the double mutant
The picture quantification, however, shows that the levels of the remaining CHK-2 decrease significantly. This suggests that the little amount of expressed CHK-2 is sufficient for meiocytes to progress through meiosis. The western blots also confirmed that the amount of the expressed 3 terminally deleted CHK-2 protein was very decreased.

The DAPI body count of the bivalent directly depicts the viability of the constructed strains

The result coming from the DAPI body count can be seen that the mutants carrying the 3'Δ and the double mutant ha::ppm-1.d; chk-2$^{3'\Delta}$::3xFlag; chek-2 are mostly unaffected and display the same number as the wild type. This is reflecting the viability and the similar brood size of these mutants as in the wild type.
In contrast, the FHA Δ and the 5'Δ of CHK-2 have a higher amount of DAPI bodies which is a clear indication of meiotic failure as a reason for loss of protein detected in the experiments before. Interestingly in the 5' mutant, the DAPI bodies are completely separated in 12 univalent whereas in the FHA mutant, either 12 or 10 DAPI bodies were counted suggesting that a slight though insufficient pairing is attempted. This could be an explanation for why the lethality is slightly higher in the FHA mutant than in the 5' Δ mutant.

The paralogue seems to be involved in the feedback loop of CHK-2 induced by the absence of a certain recombination intermediate

In the *spo-11* single mutant, where no recombination intermediates are formed, the clear prolongation of SUN-1 (S8-Pi) activity suggests persistent CHK-2 activity. However, in the 3' CHK-2 truncation mutant the number of cell rows of the CHK-2 marker SUN-1 (S8Pi) is decreased which would lead to the assumption that the 3'end would be partly responsible for the CHK-2 feedback loop. The result from the CHEK-2 mutant shows to be even lower than the 3' although not significantly different. This would lead to the suggestion that also CHEK-2 is highly involved in the feedback loop to prolong meiotic prophase in response to the absence of a certain recombination intermediate. The orthologue seems to be even more responsible for the feedback loop. When looking at the previous results one would suggest that in the double mutant of *chk-2*$^{3'\Delta}$ and *chek-2*, the SUN-1 (S8-Pi) phosphorylation would be even shorter than in both single mutants. However, the length of activity is similar to the wild type and not significantly different from the CHK-2$^{3'\Delta}$ strain. Two suggestions could be made to interpret this result. One would be that the complete absence of CHK-2 and its paralogue activity is detected and taken over by an unknown mechanism or protein. The other suggestion which would be probably more likely is that the balancer of SPO-11 broke, which was a big challenge during the construction of the strain and occurred multiple times. This would lead to a strain that did not carry the *spo-11* mutation as intended and might even be wild-type in this locus. This would suggest that this strain would not need the feedback loop as the SPO-11 normally induces the double-strand breaks, forms all recombination intermediates and no meiotic progression delay would occur.
To resolve this the strain should have been genotyped again to make sure that all wanted variants are ensured to be in the strain. However, this experiment was carried out, towards the end of the practical work of the thesis so it was no longer possible to repeat the experiments.

The result from the DSB-2 cell row count is comparable to the one from the SUN-1 (S8-PI). In the strain *ha::ppm-1.d; spo-11; chk-2*$^{3`\Delta}$*::3xFLAG* the active DSB-2 is decreased in comparison to the spo-11 single mutant which would lead to the

suggestion that the CHK-2 3' truncated protein is no longer able to keep DSB-2 active in case of the absence of SPO-11. It should be noted that *ha::ppm-1.d; spo-11; chk-2$^{3`\Delta}$::3xFlag and spo-11* each have two outliers which might alter the overall result. In this case, a repetition of the gonad staining and cell row count is suggested to increase the number of gonads observed. The strain carrying besides the *spo-11* also the *chek-2* mutation seems to be equal in their DSB-2 activity length which suggests that in this case, the CHK-2 paralogue is not so much involved in this activation mechanism as CHK-2 itself. The mutant carrying the truncated form of CHK-2 and *chek-2* null mutant is not fully credible when looking at the other strains. Again, this could be a result of a balancer breakage.

The absence of PPM-1.D and PPM-1.G are not relevant for the preservation of the nuclear pores during meiotic prophase

The quantification was only done based on the pictures taken, however, it was found that in both the single and the double mutant the NPP-7 does not degrade over the progression of prophase I. The different genotypes show a similar result where the signal given by *sGFP::npp-7* remains the same. Due to the result obtained from the experiment, it was concluded that neither PPM-1.D nor PPM-1.G is involved in preventing the nucleic pore from being degraded. To round up a single mutant of PPM-1.G should have also been included in the experimental setup, however, due to time reasons the strain was excluded. To obtain a clearer result it would have been interesting to quantify the signal intensity however as this part of the thesis was considered a side project towards the end of the laboratory work this was not carried out.

Summary

To sum up this master thesis three different truncations of CHK-2 were constructed by CRISPR/Cas9 in *C.elegans* to determine if the deleted regions were necessary for the stability and function of the protein. The 5' end and the FHA domain of CHK-2 were found to be necessary for correct meiotic entry regulation as the strain lacking these domains did have a strongly reduced protein activity as well as non-viable progeny. The phenotypes of these strains are similar to strains carrying a null mutation of CHK-2.

The third construct carried a truncation at the 3' end of CHK-2 and led to a viable strain. Although the amount of protein in the gonad is also decreased in this strain it seems that it can still promote the development of viable progeny. To check whether the paralogue CHEK-2 is taking over the activity a double mutant was constructed but also in this case the strain was viable.

Outlook

For further research, one could envision to map the interaction surfaces between CHK-2 and PPM-1.D by alternative methods, such as yeast two hybrid assays or recombinant proteins expressed in bacteria or insect cells. Once an interaction can be reconstituted it would be possible to map those via cross linking mass spectrometry. An interaction between those proteins could also be modelled via Alpha fold, which has in the meantime developed as an attractive in silico protein interaction predictor.

Overall, the practical work of this thesis was carried out during the peak of the Covid 19 pandemic which took away valuable time and resources needed.

Annex

Picture measurement script

```
run("Stack Splitter", "number=2");

// stack 1 blue
resetMinAndMax();
setMinAndMax(0,35000);
run("8-bit");
run("Subtract Background...", "rolling=50 stack");
// first part
slicenumber = nSlices/2+3;
run("Z Project...", "start=1 stop=slicenumber projection=[Max Intensity]");
run("Copy to System");
//second part
slicenumber = nSlices/2+3;
run("Z Project...", "start=slicenumber projection=[Max Intensity]");
run("Copy to System");
```

Strains

Strain number	**Genotype**
2307	chk-2 5'Δ::3xFLAG (jf191); ;ha::ppm1d(jf183)
2306	chk-2 3'Δ(jf177); ha::ppm1d (j183)
2305	chk-2 3'Δ(jf177); chek-2 Δ (ok431); ha::ppm1d (j183)
	chk-2 fha Δ::3xFLAG (jf173);ha::ppm1d(jf183)

1789	ha::ppm1d
2296	chk-2::Flag;ha:ppm-1.D (jf189)
2042	chk-2Δ; happm1d
2277	T08D2.7(ok431) X;ppm-1.D(jf183[ha::ppm-1.D])III
	T08D2.7(ok431) X;ppm-1.D(jf183[ha::ppm-1.D])III;chk-2::FLAG V
2368	sGFP::npp-7 (2230)
2369	sGFP::npp-7 (2230) ; ppm1d(jf120)
2370	sGFP::npp-7 (2230) ; ppm1d(jf120) ppm1g (jf157)
2374	spo-11(ok79) /nT1[qIs51] (IV); nT1[qIs51] /+ (V) ;ha::ppm1d
2373	spo-11/nT1[qIs51] (IV); nT1[qIs51] /+ (V) ; chek-2 (ok341); ha::ppm1d
2372	spo-11/nT1[qIs51] (IV); nT1[qIs51] /+ (V);chk-2 3'Δ::3xFLAG (jf177); ha::ppm1d
2371	spo-11/nT1[qIs51] (IV); nT1[qIs51] /+ (V) ;chk-2 3'Δ::3xFLAG (jf177); chek-2 (ok341); ha::ppm1d

Primers

Gene	Primer	Sequence
chk-2$^{5'\Delta}$::3xflag	Forward	TTCCTCCGGATGATGGTCGG
	Reverse	ACTTGGCGCGTTAGATGACTG
chk-2$^{3'\Delta}$::3x*flag*	Forward	ATGGAAATTACGGGGAATTGGGG
	Reverse	CCCGTCTGCCGTCGAACT
chk-2$^{fha\Delta}$::3x*flag*	Forward	GCTGCATCTTGTCGAAAATTGTG
	Reverse	TTTTTCAGCGATTTCCATGCTGACG
chek-2	Forward	AGTGTGCTGCTTTAAAACCCTTC
	Reverse	ACCATATACGAGTACTTGGCGAC
ha::ppm-1.d	Forward	TGATTTCAGTGGCTTTCAGACG
	Reverse	TTCCCCAAATTGTATGGGTGTTCG
chk-2::3xflag	Forward	GACGCAATTACACCCGATTTGA
	Reverse	TACACAAGCTGGACCTGTGA
spo-11	Forward	CGTGGAAACATTGCTTGAAA
	Reverse	GGACTGATGGAACCGAGAA
sGFP::npp-7	Forward	GCTTCTTGGCCTTGTAGGTG
	Reverse	ACATGGCCATCATCAAGGAGTTC
ppm-1.d	Forward	CCCCTCATCATAGTGACGTCATC
	Reverse	CTGTTTCAGCAGTCTCGTTGTG
ppm-1.g	Forward	CCTTTCCATTTCTGCAGAGCAC
	Reverse	GGTTAAAAGCGACTAGCGAGGG

Antibodies

Antibody	Host	Clonarity	Dilutionfactor	Company
HA	mouse	polyclonal	1:1000	Cell Signaling
DSB-2	rabbit	polyclonal	1:5000	
SUN-1 (S8-PI)	guinea pig	polyclonal	1:700	Eurogentec
HA	mouse	polyclonal	1:100	Cell Signaling
FLAG	mouse	polyclonal	1:250	Sigma

HA	rabbit	polyclonal	1:200	Sigma
Histone H3 (Western)	rabbit	polyclonal	1:100 000	Abcam
rabbit	goat	polyclonal	1:25000 – 1:100 000	PIERCE #31460
HA	mouse	polyclonal	1:1000	Cell Signaling
Flag	mouse	polyclonal	1:1000	Sigma
Mouse	goat	polyclonal	1:2500	Cell Signaling
Guinea pig	goat	polyclonal	1:200	A21450 Life Technologies
rabbit	goat	polyclonal	1:500	INVITROGEN
mouse	goat	polyclonal	1:500	INVITROGEN
Nuclear Pore Complex Mab414	mouse	polyclonal	1:500	Abcam

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