Germline genome stability is regulated by the chromatin remodeler SMARCAD1 in *C. elegans*

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The reproductive success of a specie is critically influenced by its ability to protect the genetic information that will be transmitted across generations. As a consequence, it is not surprising the low tolerance germ cells have to the defective function of key genes involved in zygotic development. Among those genes are those directly involved in the maintenance of germline genome stability, that guard the genome from endogenous and exogenous insults through their involvement in DNA repair mechanisms. Consequently, due to this tight link between reproduction and DNA damage repair, the effects on fertility can be used as a readout of defects in the DNA repair machinery. One of those proteins is the chromatin remodeler SMARCAD1, which is involved in the homologous recombination pathway and has been involved in mice fertility. So far, an integrated understanding of SMARCAD1 functions remained to be addressed because of the lack of a suitable whole organism model in which to perform the studies. Here, we introduce a new player that will aid studies that allow the link of specific cellular functions with their effect on reproduction and development, the nematode *Caenorhabditis elegans*. We hypothesize that *C. elegans* SMARCAD1 ortholog promotes fertility by aiding in the maintenance of germline genome stability. Through the creation of the null allele of SMARCAD1 ortholog, *smrd-1*, we show that its function in HR is conserved and test that this function contributes to its role in genome stability. We also present for the first time studies on *smrd-1* function in meiosis, showing loss of *smrd-1* elicits a differential response to DNA damage in meiotic versus mitotic

nuclei. Based on phenotypical and experimental results, we show that *smrd-1* does not confer a mutator phenotype and that the decrease in fertility of this mutant may be associated with changes in epigenetic modifications. Overall, this work provides a new model that expands the understanding of *smrd-1* functions which encompasses its effect on reproduction.

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Abbreviations

BER	base excision repair
BIR	break-induced replication
DSB	double-strand break
dsDNA	double-stranded DNA
HR	homologous recombination
ICL	inter-strand crosslink
IR	irradiation
MEG	maternal effector gene
MEL	maternal effect lethal
MES	maternal effect sterile
MMEJ (alt-NHEJ)	microhomology-mediated end-joining (alternative NHEJ)
MMR	mismatch repair pathway
Mrt	mortal germline
MZT	maternal to zygotic transition
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
PGC	primordial germ cell
ROS	reactive oxygen species
SDSA	synthesis-dependent strand annealing
SMARCAD1	SWI/SNF-related, Matrix-Associated actin-dependent Regulator of Chromatin, subfamily A, ontaining DEAD/H Box 1
SSA	single-strand annealing
SSB	single-strand break

ssDNA	single-stranded DNA
TLS	trans-lesion synthesis
UV	ultraviolet light

Preface

To my mentor Judy. For her support, trust and confidence in me. For the opportunity to learn from her as a scientist and person, that I will keep as an internal guide from now on. For the understanding during my first experience as a mother and for being there during the most important moments of my life. Thank you.

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To my husband, Braulio. Without his love and support this wouldn't have been possible.

And to our best accomplishment, our son Octavio.

Thank you.

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1.0 INTRODUCTION

1.1 DNA REPAIR

1.1.1 Protecting the genome

DNA is constantly subject to exogenous and endogenous sources that damage and threaten the integrity of DNA. Exogenous forms of damage include UV light, X-rays, γ-irradiation (IR), as well as chemicals that originate from cigarette smoke (benzo[a]pyrene). They can also include agents that induce crosslinks whose removal induces DSBs (double-strand breaks) or indirectly through the generation of reactive oxygen species (ROS) (De Falco and De Felice, 2021). Intrinsic errors generated during metabolic processes are a natural source of ROS, as well as defects arising from DNA replication, recombination, and chromosome segregation, which induce hydrolysis, deamination, and torsional stress of DNA helix, and incorporation of mismatch errors. All of them, represent eventual sources of DSBs (Scully et al., 2019). If left unrepaired, their effects on somatic cells can become the source of diseases (Knoch et al., 2012), some of which could be fatal, like cancer, or if affecting germ cells, could cause sterility and could even represent a threat to the species (Bloom et al., 2019). That is why an intricate battery of DNA damage repair mechanisms is responsible for protecting the genome from both types of cells. Understanding the nuances involved in each repair mechanism not only helps in the design of drugs and therapies for the treatment of already known diseases (Helleday et al., 2008)(Maginn et al., 2014) but will aid in deciphering the causes of infertility (Agarwal et al., 2019)(Zini and Libman, 2006).

The selection of a DNA damage response is based on the type of damage generated, which can be divided into two general classes: one includes base damage that does not alter the phosphodiester backbone, and usually affects one or two bases/nucleotides; the other involves single-strand and double-strand DNA breaks (SSBs and DSBs, respectively). In many cases, including during DNA replication, SSBs can be converted into DSBs (Kuzminov, 2001). Indeed, DNA replication is a specific source of DNA damage when the progression of the replication fork is altered, generating what is known as replication stress (Zeman and Cimprich, 2014). Besides the previously known exogenous and endogenous sources of DNA damage, endogenous factors are particularly overrepresented in this case due to the ability of DNA to naturally form secondary structures such as secondary hairpin loops, G-quadruplexes or R-loops, topological stress, and proteins bound to DNA, among others (Muñoz and Méndez, 2017).

The nature of the DNA break is the main determinant of the DNA repair pathway to be activated. The presence of damaged bases leads to the excision by the NER (nucleotide excision repair), BER (base excision repair), and MMR (mis-match repair) pathways, while the generation of DSBs as a convergence point of different types of damages involves NHEJ (non-homologous end-joining), MMEJ (microhomology-mediated end-joining), SSA (single-strand annealing), and HR (homologous recombination). It is not uncommon for pathways to share many of their components with each other. For example, ICLs (inter-strand crosslinks), comprised of covalently bound DNA strands that are repaired by the Fanconi anemia pathway, also use factors known to be part of the NER, TLS (trans-lesion synthesis), and HR pathways (Deans and West, 2011).

Gametogenesis is unique in that it employs DNA damage in the form of DSBs during meiosis to assure the correct passage of information into gametes while generating a level of genetic variation. For example, mammalian oocytes in meiosis I use HR, while in meiosis II DNA repair is halted and DNA damage awaits to be repaired in the embryo before the zygote starts its transcription (Khokhlova et al., 2020). Below, we will discuss the factors and the mechanisms involved in the different DNA repair pathways, with a special emphasis on HR and NHEJ.

1.1.1.1 Homologous recombination (HR)

Homologous recombination is restricted to the S and G2 phases of the cell cycle because of its requirement for having long homologous sequences for template repair (Scully et al., 2019). The first event in HR involves the recognition of the DSB by the MRN complex, which in mammals is comprised of its namesake, the subunits MRE11, RAD50, and Nbs1 (Figure 1). ATM (ataxia-telangiectasia mutated kinase) is then recruited by interaction with Nbs1 to the DSB (You et al., 2005)·(Falck et al., 2005) where it phosphorylates H2AX on Ser139 (γH2AX). This phosphorylation is then recognized by MDC1 (mediator of DNA damage checkpoint 1) (via its BRCT domain) and recruits more ATM (via its FHA domain) to phosphorylate additional H2AX molecules, amplifying the γH2AX signal up to 2 Mega bases from the DSB site (Liu et al., 2012). It also phosphorylates CtIP (CtBP-interacting protein) that is recruited by MRE11(Cannavo et al., 2018) with the aid of the chromatin remodeler CHD1 (chromodomain helicase DNA binding protein) (Gnugnoli et al., 2021), being the CtIP-MRE11 interaction required for the activation of MRE11 to start resection through its endonuclease activity (Liu and Huang, 2016). At the same time, in order for CtIP to be fully functional, it also has to be phosphorylated by CDK2 (cyclin-

dependent kinase 2) that is recruited by interacting with MRE11 and will allow the recruitment of BRCA1 (breast cancer gene 1) by CtIP (Buis et al., 2012). Formation of the complex MRN-CtIP-BRCA1 is only found in S and G2 phases, making it one of the requirements to limit HR during these cell cycle phases (Chen et al., 2008).

MRE11 activity consists of the nicking of the DNA strand that has up to a 300 nt 5' overhang (De Falco and De Felice, 2021). In addition to this, MRE11 activity is also stimulated by the presence of a block in the site of the DSB, which can consist of nucleosomes, Ku70-Ku80 complex or RPA (replication protein A) (Reginato et al., 2017). This first resection step, known as "short-range" resection, is followed by the bidirectional generation of long stretches of ssDNA (single-strand DNA) in a process known as "long-range" resection. At this point, the HR repair mechanism cannot be directed to the NHEJ, since the Ku complex has a low affinity for single-strand ends (Gnügge and Symington, 2021). The enzyme EXO1 (exonuclease 1), with 3'-5' exonuclease activity, is recruited by the MRN complex to start the long-range-resection generation long ssDNA tails. Additionally, DNA2 (DNA replication helicase/nuclease 2) is a DNA helicase activity that has ssDNA endonuclease activity that can act on free ssDNA ends. This activity is mediated by the RecQ helicases BLM (Bloom syndrome protein) and WRN (Werner syndrome ATP-dependent helicase), as well as the accessory factors TOP3 (topoisomerase 3), RMI1 and RMI2 (RecQ-mediated genome instability protein 1 and 2) (Pinto et al., 2016)(Wang et al., 2018c). The RPA protein coats the generated ssDNA after resection and its presence is also required for the promotion of the BLM and DNA2 activities (Zhou et al., 2015)(Qin et al., 2020). The chromatin remodeler SMARCAD1 (SWI/SNF-related, Matrix-Associated actin-dependent Regulator of Chromatin, subfamily A, containing DEAD/H Box 1) recognizes ubiquitinated

H2AK125/7/129 by the BRCA1-BARD1 complex and binds also to TOPBP1 (DNA topoisomerase II binding protein 1) (Tong et al., 2020). ATM phosphorylates SMARCAD1, which will allow RING1 (ring finger protein 1) to bind and ubiquitinate SMARCAD1. This chromatin remodeler then stimulates EXO1 and DNA2 long-range resection (Chakraborty et al., 2018). RPA-coated ssDNA is also involved in the recruitment of ATR (ataxia telangiectasia and Rad3 related) by the action of its interactor ATRIP (ATR-interacting protein) (Zou and Elledge, 2003), happening at this point the exchange of ATM for ATR activities. Interestingly, both enzymes were shown to phosphorylate EXO1 to target it for its degradation as part of the mechanisms involved in long-range inhibition (Bolderson et al., 2010). Similarly, these same phosphorylation processes on RPA are responsible for long-range inhibition as well (Zhao et al., 2020). To stimulate ATR activity, this kinase needs to interact with TOPBP1, which is recruited by interaction with the 9-1-1 (RAD9-RAD1-HUS1) complex (Mordes et al., 2008). In order for recombination to take place, RPA is replaced by RAD51 by the action of DSS1 (Zhao et al., 2015), functional homologs of the yeast RAD52 epistasis group and the BRCA2 (breast cancer type 2) protein (Symington, 2002)·(Liu et al., 2010), to form a nucleoprotein filament that has the ability to search for homologous sequences throughout the genome with the aid of the BRCA1-BARD1 complex, recruited by PALB2 (partner and localizer of BRCA2) protein. The nucleoprotein filament is stabilized by the aforementioned proteins, in addition to the RAD51 paralogue complexes BCDX2 (RAD51B-RAD51C-RAD51D-XRCC2) and CX3(RAD51C-XRCC3) (Masson et al., 2001), as well as the Shu complex (SWS1 and SWSAP1) (Zhang et al., 2017)(Martino and Bernstein, 2016). The stabilized filament promotes then the formation of a displacement loop (D-loop) (Andriuskevicius et al., 2018).

At this stage, HR can proceed through different sub-pathways that will depend on the characteristics of the D-loop formed and how it will be processed, influenced by the stability of the loop and helicases involved in its processing. One of the sub-pathways involves the formation of a Holliday junction (HJ), in which after the DNA synthesis using the complementary strand of the RAD51 coated filament, a second-end capture takes place by the annealing of the second ssDNA overhang of the damaged sequence with its complementary strand in the DNA used as a template for DNA synthesis (Elbakry and Löbrich, 2021). The way this structure is resolved will promote different outcomes, depending on the helicases and topoisomerases involved in the process, giving rise to crossover (CO) or non-crossover products happening with the same frequency (Elbakry and Löbrich, 2021). A predominant HR sub-pathway is called synthesis-dependent strand annealing (SDSA) and involves the annealing to its non-invading complementary sequence of the invading strand after repair synthesis completion (Sarbajna and West, 2014). If there is no formation of HJ because there is only invasion by one RAD51-coated strand, then the sub-pathway is termed break-induced replication (BIR), and the probability of genomic rearrangements and point mutations taking place increases (Chandramouly et al., 2013).

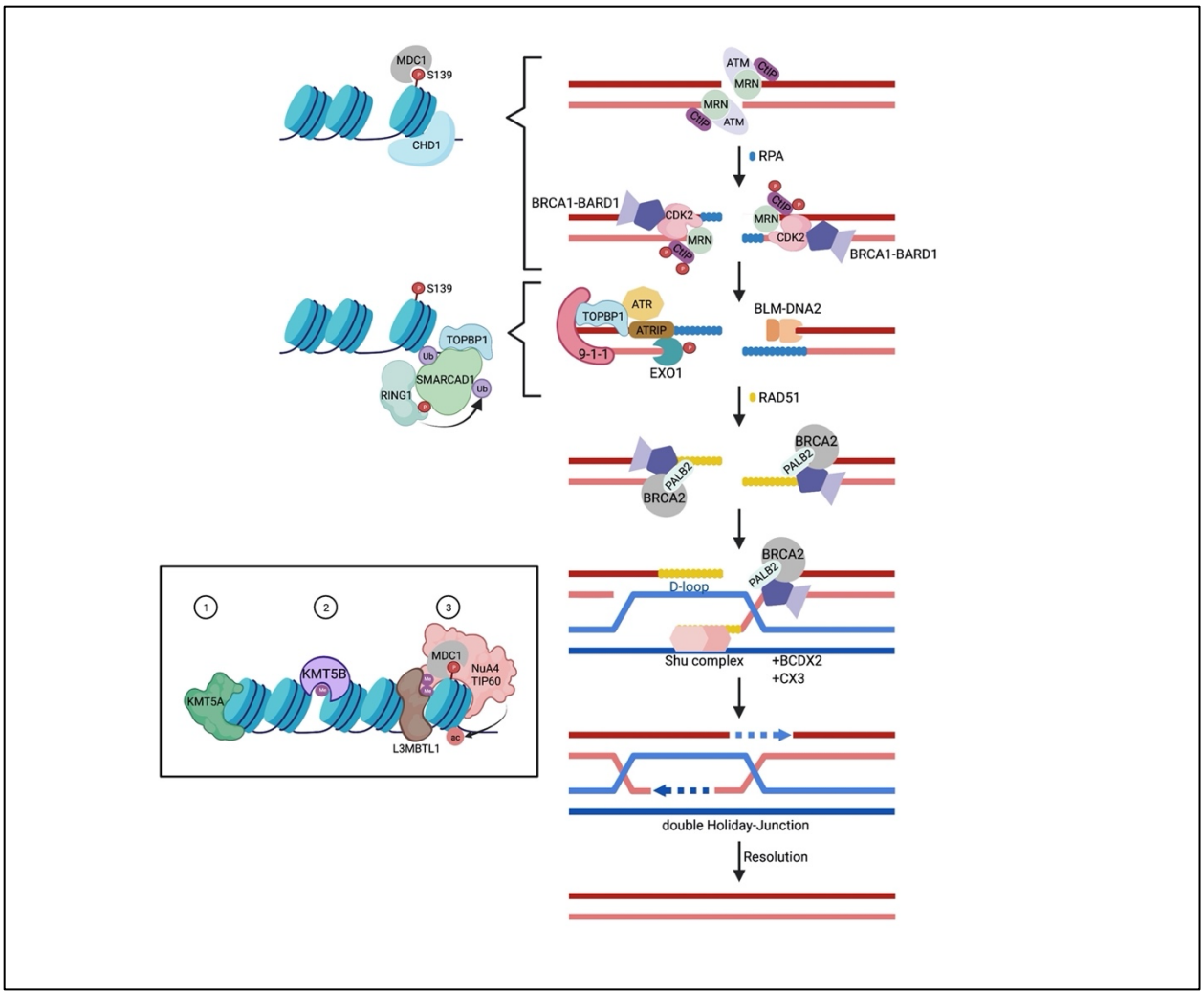


Figure 1: Homologous recombination (HR) pathway. Representation of the steps involved in human and yeast HR, including most of the factors. Top left, the events happening at the chromatin level for the indicated steps by brackets. Bottom left, initial chromatin modifications that determine the preference of HR over c-NHEJ. For a detailed description of the interaction between the different factors shown in this figure, refer to sections 1.1.1.1 and 1.1.1.3. Created with Biorender.com.

1.1.1.2 Classical Non-homologous end-joining (c-NHEJ)

Classical non-homologous end-joining (c-NHEJ) repairs DSBs outside the S and G2 cell cycle phases and even around 80% of the DSBs that are not near the replication forks are repaired by this pathway (De Falco and De Felice, 2021). Although it does not require a template, it usually requires ≤ 4 bp of micro-homology and the DNA ends often need to be processed whether it is for the lack of homology or for the presence of modified nucleotides that generate "dirty" ends. As a consequence, micro-deletions or micro-insertions can be created (Chang et al., 2017), as can large deletions or chromosomal rearrangements if multiple DSBs happen at the same time and nearby in the genome (Schimmel et al., 2017).

The first step in this pathway is the recognition of the DSB by the nuclear complex Ku70-Ku80 that binds to blunt ends or ends that have small ssDNA overhangs (Mimori and Hardin, 1986) (Figure 2). This complex acts then as a scaffold to recruit the other components of the pathway like DNA-PKcs (DNA protein kinase catalytic subunit), LIG4 (DNA ligase 4), XRCC4 (X-Ray Repair Cross Complementing 4), XLF (XRCC4-Like Factor) and PAXX (Blackford and Jackson, 2017). It also antagonizes end-resection (Mimitou and Symington, 2010). The binding of DNA-PKcs to Ku70-Ku80 is required for the phosphorylation of the chromatin in the vicinity of DSBs and downstream factors of the pathway, including the disassembly of the complex at the last step during ligation (Chang et al., 2017). DNA-PKcs also recruit the endonuclease Artemis, which eliminates short overhangs or hairpins (Goodarzi et al., 2006) to make the ends suitable for ligation. This, together with the action of DNA polymerase λ and DNA polymerase μ that add nucleotides to the 3'-ends, facilitates the formation of ends that can be ligated (Nick McElhinny et al., 2005). In the ligation step, the complex LIG4-XRCC4 seals the double ends by stimulation of

LIG4 enzymatic activity by XRCC4. The scaffolding factors XLF and PAXX take part in this process by interacting with the LIG4-XRCC4 complex and positioning the DNA ends for the ligation to take place (Lisby and Rothstein, 2015).

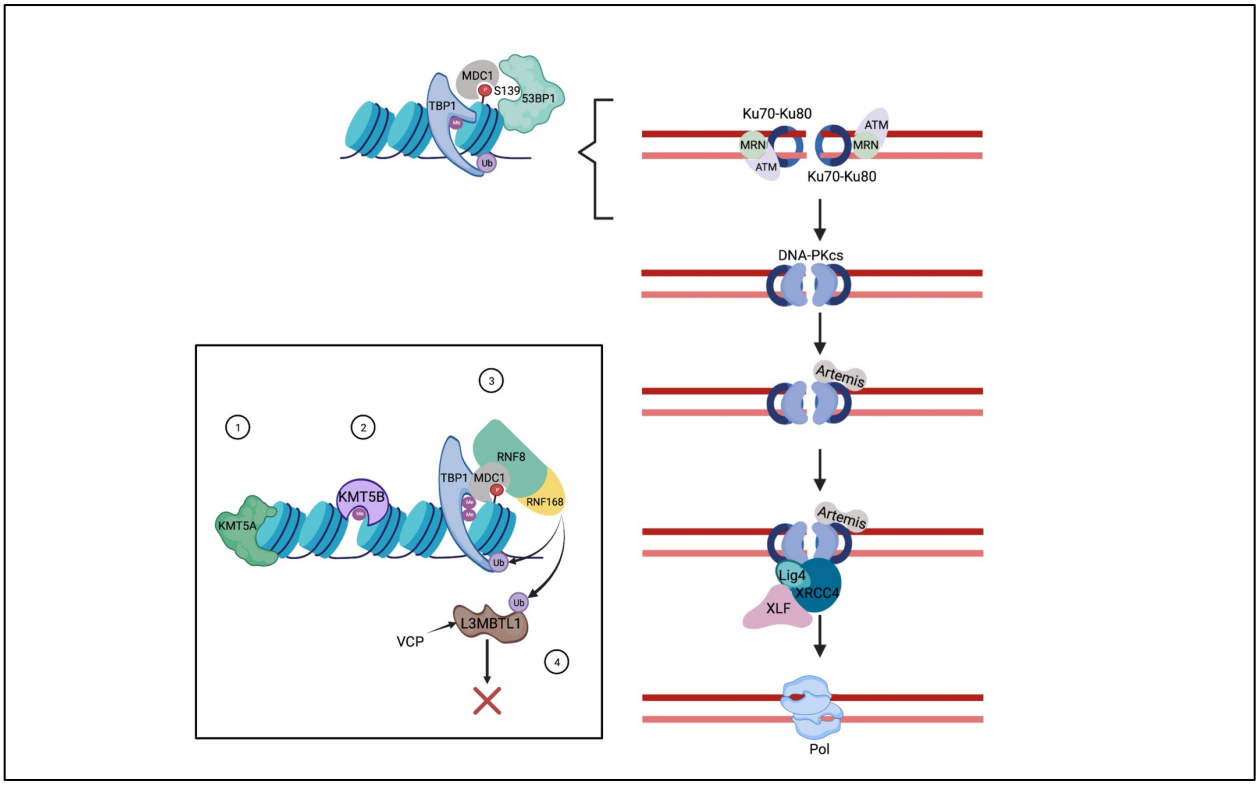


Figure 2: c-Non-homologous end joining (c-NHEJ) pathway. Representation of the steps involved in c-NHEJ, including most of the factors. Top left, the events happening at the chromatin level for the indicated steps by brackets. Bottom left, initial chromatin modifications that determine the preference of c-NHEJ over HR. For a detailed description of the interaction between the different factors shown in this figure, refer to sections 1.1.1.2 and 1.1.1.3. Created with Biorender.com.

1.1.1.3 HR or c-NHEJ?

Key to the decision between c-NHEJ or HR is the earliest changes that occur at the break site and the proteins that first associate there. At any given time, approximately 80% of Histone H4 is in the form of H4K20me2 (Histone H4 lysine 20 dimethylation) (Hartlerode et al., 2012). This modification is done by the KMT5A (Lysine Methyltransferase 5A) enzyme that interacts with the histones H2A and H2B to form H4K20me (Girish et al., 2016), followed by the KMT5B (Lysine Methyltransferase 5B) enzyme that uses as substrate H4K20me to form H4K20me2 (Weirich et al., 2016). This mark is important, since it will be recognized by 53BP1 to favor the c-NHEJ pathway or MBTD1 (Mbt Domain Containing 1) to favor HR pathway (Paquin and Howlett, 2018). Which one of those will bind will depend on other factors that are present in the context of the DSB.

In order for c-NHEJ to be favored, 53BP1 binds via its BRCT domains to γH2AX, via its Tudor domain H4K20me2, and via its UDR domain to H2AK15Ub (histone H2A lysine 15 ubiquitination) (Botuyan et al., 2006)(Fradet-Turcotte et al., 2013). H2AK15Ub is created by RNF8/RNF168 (Mattiroli et al., 2012)(Gatti et al., 2012). RNF8 (Ring Finger protein 8) recognizes MDC1 and polyubiquitinates histone H1 (Huen et al., 2007), which allows the binding of RNF168 (Ring Finger protein 168) and the subsequent mono-ubiquitination of H2AK15. It also mono-ubiquitinates L3MBTL1 (L3MBTL Histone Methyl-lysine Binding Protein 1), which marks it to be degraded by VCP (Valosin-Containing Protein) (Acs et al., 2011). So, besides occupying the same binding sites as for HR (H4K20me2 and H2AK15), it also has to remove a factor involved in HR (L3MBTL1) and the complex NuA4/TIP60 has to be absent (Jacquet et al., 2016).

For HR to be favored, L3MBTL1 and/or JMJD2A (JmjC histone demethylase) bind to H4K20me2. This PTM is also recognized by the MBTD1 domain of the NuA4/TIP60 complex, which will acetylate H2AK15. In order to prevent binding of the Ku complex and activation of NHEJ during S phase, RAD18 and PARP (Poly (ADP-ribose) polymerase) also act to decrease the affinity of Ku for the DSB (Saberi et al., 2007).

Chromatin remodelers have also been involved in the preference of pathways. For example, SWR1 (Swi2/Snf2-Related 1) binds to the Ku70-Ku80 complex, was shown to facilitate c-NHEJ; INO80 and RSC recognize γH2AX and facilitate both, c-NHEJ and HR by increasing the access to broken DNA in a chromatin context, as well as INO80 being involved in the efficient activity of MRE11 (van Attikum et al., 2007)(Tsukuda et al., 2009)(Kent et al., 2007).

1.1.1.4 Micro-homology mediated end-joining (MMEJ)

The main difference between c-NHEJ and MMEJ (also known in the literature as alternative NHEJ (alt-NHEJ), or theta-mediated end joining (TMEJ)) is that the latter requires short microhomology sequences between 4 to 20 bp that arise by a 5'-DNA end-resection of 15 to 100 nt from the site of the DSB. These homologous sequences will be annealed and used for the ligation of both ends (Seol et al., 2018). This repair mechanism starts with the recognition of the DSB by PARP1, which competes with the Ku70-Ku80 complex to prevent c-NHEJ from happening. After binding to the DNA, PARP1 forms long negatively-charged poly(ADP-ribose) (PAR) chains on itself and on the chromatin proteins surrounding the break (PARylation), which will serve as a platform for the recruitment of downstream DNA repair factors (Gibson and Kraus, 2012). Then, MRE11 endonuclease activity of the MRN complex, just like in HR, is stimulated by

phosphorylated CtIP to generate 3'-overhangs of 15-100 nt. PARP1 then recruits DNA polymerase θ, which binds to the 3' ends of the annealed micro-homology sequences and mediates the fill-in synthesis of the annealed region, which will lead to LIG1 (DNA ligase 1) or the LIG3–XRCC1 complex to seal the break (Masani et al., 2016). An additional function of this polymerase is the addition of nucleotides to increase the micro-homology in the case that is not already present (Chang et al., 2017).

1.1.1.5 Single-strand annealing (SSA)

Single-strand annealing requires extensive 5'end-resection between 25 and several hundred of nucleotides (Symington, 2016). This is because, unlike alt-NHEJ, the annealing, in this case, is between homologous sequences along the 3'-ssDNA generated that are longer than in alt-NHEJ. The resection is initiated by the MRN complex and CtIP, which is followed by a long-range resection like in HR by EXO1, DNA2 or BLM (Zhu et al., 2008). The 3' ssDNA overhangs are coated with RPA to prevent the formation of secondary structures. In this case, the annealing of homolog sequences is mediated by RAD52, which has the ability to displace the RPA molecules from the filament (Symington, 2002). The annealing of the homologous sequences will generate un-annealed regions that have to be removed before ligation by the complex XPF-ERCC1 (Pâques and Haber, 1997). As a consequence, tracts of sequences are lost, making SSA a mutagenic DNA repair pathway.

1.1.1.6 Replication fork meets DNA damage

One of the main sources of endogenous DNA damage results from obstacles encountered by replication forks, including inter-strand crosslinks, protein-DNA crosslinks, SSB, DSB, nucleotide depletion and simultaneous RNA transcription (Rickman and Smogorzewska, 2019)(Li et al., 2021)(Duxin et al., 2014)(Brambati et al., 2015)(Arnaudeau et al., 2001)(Buss et al., 2008). As a consequence, cells have developed four distinct mechanisms to overcome the stress resulting from these obstacles: the damage bypass mechanisms trans-lesion synthesis (TLS) and template switching (TS), as well as fork reversal and fork breakage (Figure 3).

TLS and TS can be used to overcome the stall of replication forks induced by the formation of bulky lesions, generated by UV, alkylating agents, hydrolysis and reactive oxygen species (Sancar et al., 2004). TLS uses specialized DNA polymerases that allow continuing with the replication across the lesion. The error-free TS uses the sister duplex as an alternative template to realign the nascent strand and restart replication (Lehmann et al., 2007). In order to gain access to the alternative template strand, this bypass mechanism can involve replication fork regression or HR-mediated strand invasion of the homologous duplex. Replication fork regression requires HLTF (Helicase Like Transcription Factor) and RAD5 to facilitate dsDNA translocase activity, while HR-mediated strand invasion involves RAD51 nucleofilament formation to perform homology search and generation of the displacement loop (D-loop) (Burkovics et al., 2014).

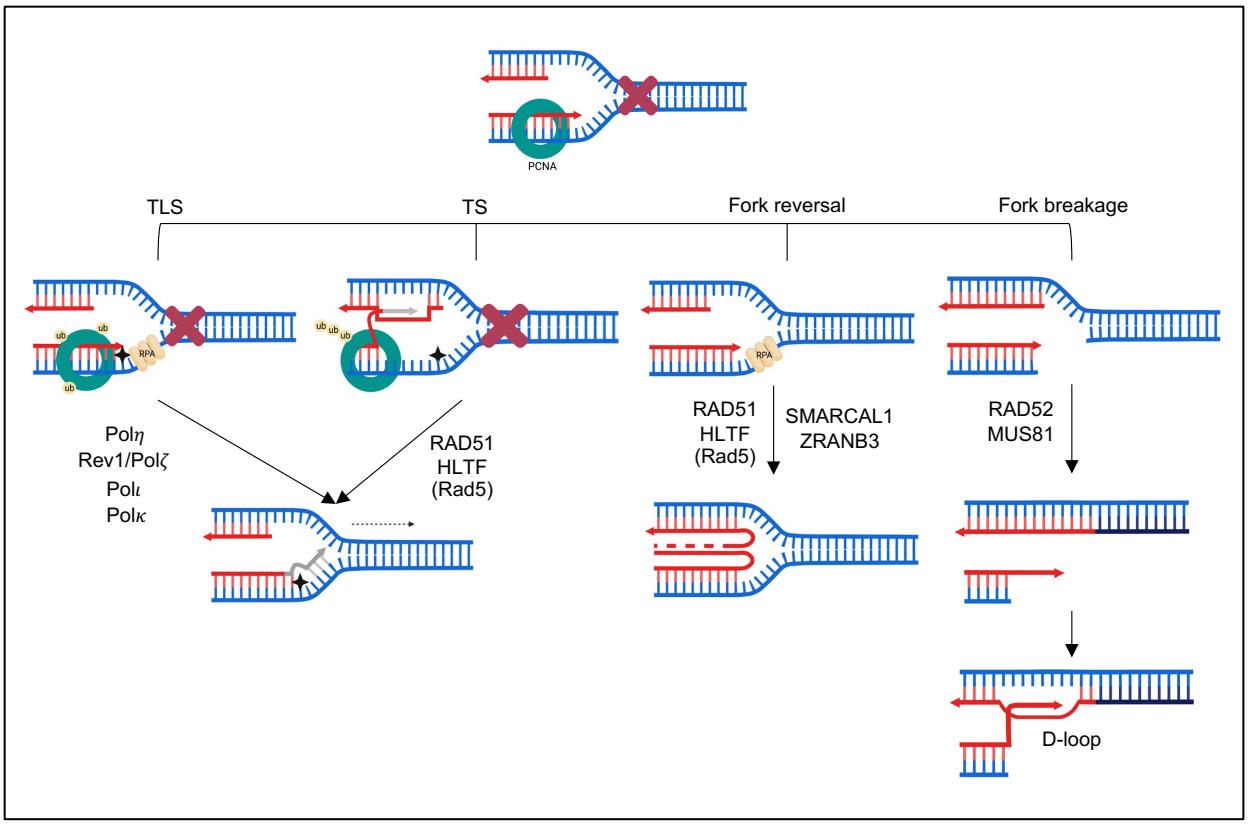


Figure 3: Mechanisms to resolve stalled replication forks. Translesion synthesis (TLS) is started by the ubiquitination of PCNA and polymerization by alternative polymerases. Template switch (TS) requires the polyubiquitination of PCNA that is mediated by the Rad5 homologo HLTF, as well as the formation of a RAD51 nucleofilament In fork reversal, in addition to RAD51 and HLTF, as well as the chromatin remodeler SMARCAL1 and DNA annealing helicase and endonuclease ZRANB3. In fork breakage, the activities of RAD52 and MUS81 are required to generate the DSB that will be repaired by HR. Based on *Kondratick et. al.* 2021(Kondratick et al. 2020). Created with Biorender.com.

If during replication the leading strand is perturbed, the polymerase will dissociate causing fork un-coupling. In the meantime, the helicases ahead of the replication fork will continue with the un-winding of DNA generating extended regions of ssDNA (Atkinson and McGlynn, 2009), that are protected by RPA. The first step of replication fork reversal is accomplished by the SNF2 family of chromatin remodelers SMARCAL1, ZRANB3, and HLTF (Qiu et al., 2021).

SMARCAL1 fork reversal activity is catalyzed by its interaction with RPA bound to the leading strand (Bétous et al., 2013). However, in the case of ZRANB3, RPA has an inhibitory effect on this chromatin remodeler to reverse the replication fork in the leading strand while HLTF, just like SMARCAL1, needs to interact with RPA in order to induce fork reversal. These three proteins appear to act coordinately as the triple knock-outs do not display additivity (Taglialatela et al., 2017). Another consequence of RPA binding to the ssDNA is the initiation of a checkpoint response which occurs through the binding of ATR via the ATRIP bound to RPA-ssDNA (Zou and Elledge, 2003). Activation of ATR is then facilitated by recruitment and interaction of 9-1-1 complex by TOPBP1 (Yan and Michael, 2009). One of the substrates of ATR is CHK1 (Checkpoint Kinase 1), which prevents the initiation of replication at origins near the stalled fork (Ge and Blow, 2010). Contrary to the BRCA2 requirement in HR for stabilization of RAD51 filaments, in this case, the exchange of RPA for RAD51 and subsequent roles in fork reversal involves alternative factors that remain to be studied in detail (Berti et al., 2020). What has been already determined, though, is that the presence of the RAD51 filament is required for the inhibition of the activities of MRE11, EXO1, DNA2 and MUS81 (Methyl methanesulfonate Ultraviolet Sensitive gene clone 81), which could recognize the nascent DNA as a single-ended DSB and promote its degradation (Mijic et al., 2017). Finally, by the action of TOP2A (DNA Topoisomerase 2 Alpha), extensive fork reversal is promoted with the aid of the DNA translocase PICH (Plk1-Interacting Checkpoint Helicase) (Qiu et al., 2021). Fork reversal happens under normal conditions, for example when replicating trinucleotide repeats, revealing that these repair mechanisms may have evolved to facilitate the replication of complex genomic sequences (Follonier et al., 2013).

Fork breakage involves detachment of one of the fork arms leaving a one-ended DSB, that is resolved by HR (Petermann et al., 2010)(Lettier et al., 2006) or BIR (break-induced replication) (Sotiriou et al., 2016)(Sakofsky and Malkova, 2017). It is generated after the replication fork meets ssDNA nicks or gaps that were not repaired on the template DNA. However, it can also be induced by the action of the nuclease MUS81, whose recruitment is itself dependent on RAD52 to induce the formation of DSBs (Murfuni et al., 2013). This mechanism of fork breakage has been shown as a response to the replication stress caused by HU treatment (discussed in more detail below) (Hengel et al., 2016).

A DNA repair pathway tightly associated with S phase is the mismatch repair pathway, MMR (Huang and Li, 2018). It is involved in the repair of base-base mismatches, as well as small insertions and/or deletions loops generated during replication. MMR recognizes altered structures in the DNA helix and proceeds to remove fragments of sequences around the damage. The generated gap is then filled by DNA synthesis (Liu et al., 2017). Different types of damage are recognized by distinct heterodimeric complexes: MSH2-MSH6 (MutSα) recognizes mis-paired bases; MSH2-MSH3 (MutSβ) recognizes insertion and deletion loops (Jiricny, 2013). Both complexes have the ability to slide over the DNA until a lesion is found (Brown et al., 2016). After lesion recognition, the binding of the MLH1-PMS2 complex leads to the degradation of the mutated sequence and DNA synthesis restart to fill the gap. The MLH1-PMS2 complex can interact with both MutSα and MutSβ complexes, and it is this interaction that activates the endonuclease activity of PMS2 to incorporate single-strand breaks that will allow the action of the exonuclease EXO1 (Jeon et al., 2016). After resection by EXO1, DNA Polδ bound to the PCNA (Proliferating Cell Nuclear Antigen) complex will fill the gap and LIG1 will ligate the ends.

1.1.2 Genotoxins in the study of DNA repair

One of the foremost strategies to elucidate the role of the proteins involved in DNA repair has been the evaluation of the response that mutants have upon treatment with genotoxic agents. Below, I will focus specifically on those agents that will be used in this study and the specific mechanism of action as well as the consequences generated in cellular responses.

1.1.2.1 Hydroxyurea

Hydroxyurea (HU) is a non-alkylating antineoplastic and antiviral drug, that has been used as an inhibitor of DNA synthesis. Consequently, it targets S phase of the cell cycle and has a reversible mechanism of action on DNA replication, depending on its concentration and duration of exposure (Timson, 1975). The main cellular target of HU is the enzyme RNR (Ribonucleotide Reductase), which catalyzes the reduction of ribonucleoside diphosphates to deoxyribonucleotides that will be used for DNA replication and repair (Nordlund and Reichard, 2006). As a consequence, HU's main cytotoxic action lies in the stalling of the replication fork that eventually collapses into DSBs (Petermann et al., 2010). When the exposure is for a short period of time, a replication fork restart mechanism is activated, while under long-exposures the repair of the collapsed replication fork is by HR. In addition, HU cytotoxicity can arise from the generation of ROS, which has been proposed to be responsible for its cell-killing process (Przybyszewski and Malec, 1982)(Huang et al., 2016)(Davies et al., 2009).

One of the main drawbacks of using HU as a therapeutic agent is its side effects, especially when the targets are the germline cells, which are transduced as a decrease in fertility (Lanzkron

et al., 2008). Indeed, previous studies showed that pre-meiotic spermatogonia and stem cells turned out to be more sensitive to HU (Arlt et al., 2018). Based on those results, an in-depth study on the effect of HU treatment in mice testes showed the increased presence of SC (Synaptonemal Complex) gaps in pachytene nuclei (Bolcun-Filas et al., 2009). Additionally, unrepaired DSBs also affect the SC assembly in mice (de Vries et al., 2005), contributing to the observed defects. These results are supported by the previous findings that the increased presence of gaps in the SC is linked to male infertility (Codina-Pascual et al., 2006).

1.1.2.2 Camptothecin

One of the advantages of using camptothecin (CPT) for therapeutic and research purposes is the fact that it specifically and reversibly targets the TOP1 cleavage complex (TOP1cc). This transient intermediate complex is generated as a consequence of the nicking of DNA by TOP1, in order to allow the broken strand to rotate around the TOP1-bound DNA, as part of the TOP1 activity of DNA supercoiling relaxation. The binding of CPT to TOP1cc slows down its activity, which gives enough time for the replication and transcription complexes to collide with the TOP1cc. This collision is the one responsible for the now irreversible entrapment of TOP1 to DNA (Pommier, 2006), leading to the formation of DSBs.

Since TOP1 tends to be concentrated in supercoiled chromatin regions, particularly involved in replication and transcription, the main consequences of CPT treatment are connected to this two processes. Replication fork collision is the main cytotoxic mechanism of CPT in dividing cells. The blocking of transcription complex by TOP1cc bound to DNA makes CPT a potent inhibitor of transcription elongation (Collins et al., 2001), but also, as a consequence of this,

there can be a downstream accumulation of negative supercoiling that could favor the formation of cytotoxic and mutagenic R-loop structures (DNA:RNA hybrids) (Pommier, 2006).

To date, three repair mechanisms have been identified, which can be grouped into the TDP1 (Tyrosyl-DNA Phosphodiesterase 1) excision pathway involving XRCC1 and BER; the endonuclease pathway involving MRE11, MUS81, RAD1, and HR; and the fork-regression pathway involving the BLM helicase and TOP3 (Pommier et al., 2006). The action of any of them will be determined by the type of lesion, whether it is replication or transcription-associated. For example, the preferred repair mechanism involved in transcription is the TDP1-BER pathway (Miao et al., 2006), while the mechanisms involved in replication will be HR and fork regression pathway.

1.1.2.3 Ionizing radiation

Ionizing radiation (IR) directly induces DNA damage, which includes DSBs, SSBs and abasic sites that arise as a consequence of the generation of ROS (Borrego-Soto et al., 2015). It has recently become recognized, that besides the aforementioned damages, the major mutagenic effects are a consequence of the induction of clustered DNA damage. This type of damage is generated as a consequence of the occurrence of two or more types of DNA damage in a small region usually within a few nucleotides. For example, the combination of a DSB and oxidized guanine, or a DSB and a closely located SSB may be seen (Mavragani et al., 2019). One of the reasons that make it resistant to repair is the fact that there is no availability of an undamaged strand that could help in the repair of the lesion, since the damage can be on both strands, being the safest option to kill the cell (Eccles et al., 2011).

Since the type of damage generated is wide, the specific repair mechanism called upon for repair of the lesion will depend both on the type of damage, as well as the stage of the cell cycle, as discussed at the beginning of this chapter.

The conservation of DNA repair pathways across species allowed us to benefit from the advantages provided by each one of them to study different aspects of DNA repair. One of those is the nematode *C. elegans*, which facilitates the research on DNA damage and its effects on a whole organism. Below, we will discuss in more detail the contribution of this model to the current understanding of DNA repair mechanisms.

1.2 *C. elegans* AS A MODEL FOR DNA REPAIR

1.2.1 Emerging from soil

The free-living worm *C. elegans* has become a robust model organism for the study of neurobiology, development, aging, and signal transduction, among others (Antoshechkin and Sternberg, 2007). Among the reasons that made it possible was its small size, which simplifies handling many specimens at the same time; the economic and simple maintenance helped by its feeding with *E. coli*; and its translucid body that allows the direct observation of internal processes under a dissection microscope. By 1998, the worm genome was sequenced (*C. elegans* Sequencing Consortium, 1998), corroborating that the majority of its genes retain homology with humans (Shaye and Greenwald, 2011). Also, detailed lineage analysis of the exact number and types of cells that constitute the worm are known and development has been described in detail. This has facilitated a detailed description of its anatomy through development, which can be visualized online with 3D models of its architecture (Davis et al., 2022). Another key advantage of *C. elegans* is its short hermaphrodite life cycle of ~ 3 days, going through embryogenesis and four larval stages until molting into adults at the optimal growth temperature of 20°C. Growth at different temperatures from 16ºC – 26.5°C can alter growth rates and affect the penetrance of different phenotypes. An example of this involves the study of response mechanisms to stress.

Having this model represents a great advantage to perform aging studies, something that would be almost impossible nowadays considering the economic, spatial, and logistics of doing it with rodents, for example. Also, the ability to study the impact of a process across generations is a helpful tool that can be used in foreseeing the long-term effect on a population (Meyer et al.,

2007). Unlike bacteria or yeast, which have also been studied extensively, the a multicellular nematode allow analysis of the consequences a defect on one type of cell can influence the response of another type of cell. Another great advantage of this organisms is the range of tools available to address the many unique lines of research. For example, using the RNAi (RNA interference) technique by feeding (Grishok, 2005) allows rapid screening of genes of interest of knock-down phenotypes. Genetic manipulations especially with CRISPR-mediated genome modification (Dickinson and Goldstein, 2016) facilitate the study of the function of different genes and their consequence by knock-out and tagged transgenes that help perform in cell localization and structural studies (Sarov et al., 2012). It's also possible to study genes by knock-out that are lethal in mice (Thijssen et al., 2021).

1.2.2 Architecture of *C. elegans* gonad

During the first embryonic divisions, the future germ cells of the worm are set apart from somatic cells, leading to the formation of two primordial germ cells (Z2 and Z3) flanked by the two somatic gonad precursors (Z1 and Z4) (Figure 4A) These are mitotically quiescent until the mid-L1 larval stage. By the time the animals are L4 larva, germ cells proliferate in the distal mitotic zone of the germ line and are undergoing meiosis as they move proximally. The L3 and L4 germline produces sperm and then as an adult, the hermaphrodite switches to producing exclusively oocytes which are fertilized by her stored sperm (Figure 4B).

Meiosis is highly conserved across species and all six chromosome pairs undergo homologous recombination to produce crossovers. The pairing of chromosomes, onset of meiotic

DSBs, and formation of the synaptonemal complex (SC) which holds homologs together occur in the transition zone- corresponding to leptotene/zygotene. Meiotic crossovers are designated during pachytene with most chromosomes in the worm receiving only one crossover. As cells exit pachytene, there is an asymmetric disassembly of the SC and when progressing from diplotene to diakinesis, chromosomes become highly condensed, forming the six bivalents. In the laboratory, the 6 bivalents and the stereotypical structure of the germ line can easily be visualized by staining the DNA with DAPI, and as a consequence, they can be referred to as "DAPI bodies". Meiotic maturation is defined by the transition between diakinesis to metaphase of meiosis I, preparing the oocyte for fertilization. In *C. elegans*, meiotic maturation, ovulation, and fertilization are temporally coupled.

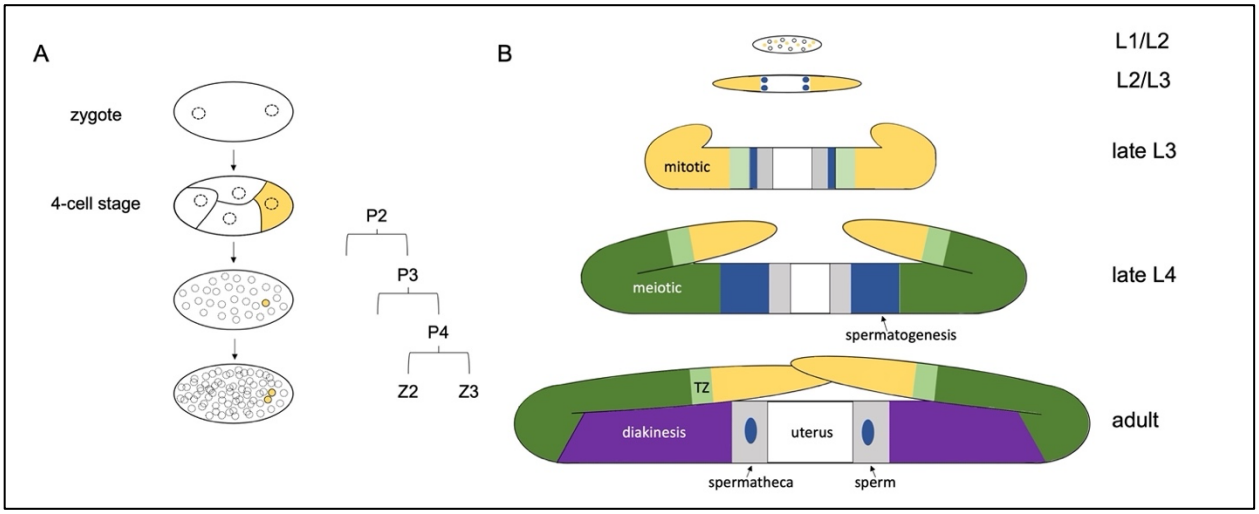


Figure 4: Representation of gonadogenesis. A- Germline lineage is shown in yellow inside the zygote. B- Hermaphrodite gonad development showing mitotic region (yellow), meiotic region (green), diakinesis (purple), spermatogenesis/sperm (blue) and spermatheca (green). Representations based on Hubbard, E.J.A., and Greenstein, D. Introduction to the germ line (September 1, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook.

C. elegans is also a great model for the study of DNA repair and that is mainly because of the structure of its gonad (Figure 5). The gonad can be easily discerned from somatic tissues and is made up of two "arms" that have germ cells organized in a stereotypical fashion. The mitotic divisions happen in the distal region with ~150 cells and then meiotic prophase I starts in a region called the "transition zone" which corresponds to leptotene/ zygotene. The largest region of the germ line is occupied by pachytene nuclei which is followed at the bend of the germ line by the diplotene stage and then diakinesis. This organization allows the study of mechanisms involved in DNA damage responses that participate in different cell cycles—the mitotic and meiotic cell divisions. This facilitated the discovery that the response to genotoxins is different between germline and somatic cells (Boyd et al., 2010). DNA damage checkpoint responses differ in the mitotic and meiotic nuclei, with DNA damage-induced cell cycle arrest only in the mitotically proliferating cells, and checkpoint-induced apoptosis at the last steps of pachytene (Gartner et al., 2000). However, the meiotic pachytene checkpoint can also be induced when there is a compromised repair in mitotic germ cells and the damage is carried over into meiosis (Stevens et al., 2013).

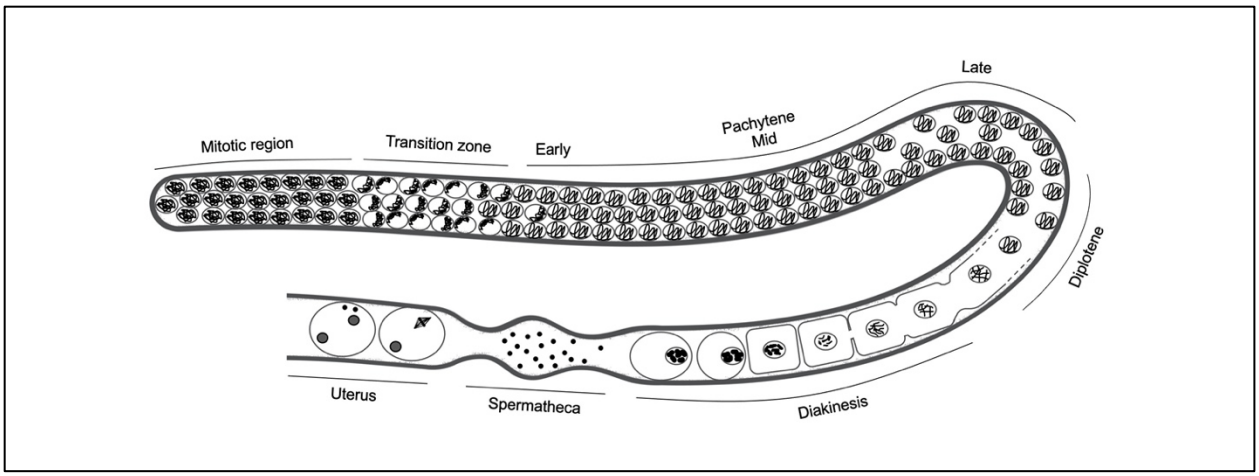


Figure 5: Schematic of an adult gonad. One arm of an adult hermaphrodite gonad. The different stages from mitosis to meiosis I are highlighted. Created with Biorender.com.

Having a model system that allows the study of different meiotic stages, became important to not only identify the mechanisms of resolution of DSBs generated during meiosis but also to identify those involved specifically in DSBs damage generated after a genotoxic insult (McClendon et al., 2016a). This study of factors that can affect genome stability is detrimental, taking into consideration that the genetic material will be carrying the genomic information across generations. Clear evidence of how this model organism is recognized as a useful tool to study DNA repair, is the adaptation of techniques that are standard components of the toolbox to study this process in cell lines (Nicolai et al., 2021)(Kocsisova et al., 2018)(Imanikia et al., 2016). This aided the increased number of publications with information regarding DNA repair pathways in this model organism, like HR (Barber et al., 2008; Lee et al., 2015; Silva and Jantsch, 2016), NHEJ (Vujin et al., 2020), BER (Asagoshi et al., 2012; Elsakrmy et al., 2020; Papaluca et al., 2018), NER (Arczewska et al., 2013; Boyd et al., 2010; Meyer et al., 2007; Oh et al., 2020; Thijssen et al., 2021), MMR (Meier et al., 2018) and ICL (MacKay et al., 2010; Wilson et al.,

2017), among others. Below, some cases where *C. elegans* germline studies on DNA damage gave important results are discussed.

1.2.3 DNA repair in *C. elegans*

1.2.3.1 DSBs as a double-edged sword

During meiosis, a key step and the aim of sexual reproduction is the formation of crossovers, which allows the exchange of genetic material between homologous chromosomes. This exchange requires as an initial step, the formation of DSBs by the topoisomerase VI-type enzyme SPO11 (Keeney et al. 1997; Dernburg et al. 1998) and the subsequent repair by homologous recombination (HR). Thus, while DNA damage can be deleterious, it is exploited in the germ line for this very important event that leads to genetic variation. Not surprisingly, the generation of external DSBs in the gonad uses in the repair of at least some of the proteins involved in the repair of endogenous DSBs. For example, one of the most important factors involved in the resolution of endogenous DSBs, is the conserved protein MRE-11 (MRE11 homolog) required for short-range resection (Nairz and Klein, 1997). This protein participates in the repair of IR-generated damage in the germ line and consequently is important for the maintenance of genomic stability (Chin and Villeneuve, 2001). In 2016, McClendon *et. al.* (McClendon et al., 2016a) showed that a protein involved in germline development and crossover formation on the X chromosome, known as XND-1 (no human homolog identified so far), is also involved in the repair of DSBs generated by genotoxic lesions. This newly identified role in genome stability could be evidenced, among other causes, as a reduction in fecundity that decreased over time and sensitivity to IR in *xnd-1* mutants. Although not directly involved in the regulation of the HR pathway, its

effect could be exerted by the alteration of chromatin marks involving the acetyltransferase MYS-1 (KAT5 homolog) (Wagner et al., 2010).

1.2.3.2 A protein is known by the pathway it belongs

The key step to understanding the underlying mechanism(s) involved in the maintenance of genome stability is the specific genetic pathways to which a gene belongs. In this section, I provide several examples of how this approach has been used to provide a framework for the studies in this thesis.

One example is the WRN-1 protein (homolog of Werner syndrome protein, WRN). Studies performed in the *C. elegans* model helped to unravel the mechanism employed by this protein in DNA repair and DNA damage signaling (Lee et al., 2010). By studying genetic interactions of this protein with different checkpoint pathways after treatment with different types of genotoxins, WRN-1 was found to be involved in the initial steps of checkpoint activation after either inhibition of DNA replication or ionizing radiation exposure. Another example is the case of the protein GEN-1 (GEN1 homolog), which is involved in Holliday junction resolution, a late step in DSB repair (Bailly et al., 2010). In this article, Bailly *et. al.* showed how the *C. elegans* homolog also has a role in the first steps of IR-induced DNA damage by participating in the induction of cell cycle arrest and apoptosis. It was also possible to elucidate how the monoubiquitinated form of FANCD1 is responsible for the recruitment of FAN-1 (FAN1 homolog) to sites of DNA damage and its consequent involvement in the resolution of ICLs (MacKay et al., 2010). Furthermore, initially discovered as one of the proteins recruited by the complex 9-1-1 to sites of DNA damage, the RHINO protein was shown to be necessary to promote CHK1 activation and achieve full

activation of ATR (Cotta-Ramusino et al., 2011). A few years later, using the *C. elegans* model, it was possible to discern that the *C. elegans* homolog, ZTF-8, is required to promote DNA repair at stalled replication forks in mitotic cells as well as in meiotic cells by DNA damage checkpoint activation (Kim and Colaiácovo, 2014). These studies not only shed more light on the mechanism involved in DNA repair, but also showed the involvement of this protein in the maintenance of germline genome stability.

1.2.3.3 Discovering the connection of different pathways with DNA repair

Studies in *C. elegans* also helped identify a novel role of a member of the linker and nucleoskeleton and cytoskeleton (LINC) complex, UNC-84 (SUN2 homolog), in DNA damage repair and meiotic recombination (Silva and Jantsch, 2016). This role pertains to the inhibition of NHEJ and promotion of HR at sites of DSBs generated as a consequence of inter-strand crosslinks, involving the recruitment of the FAN-1 (FAN1 homolog) nuclease. An additional example concerns the histone demethylase JMJD-1.1 (KDM7A homolog), which was shown to participate in later steps of homologous recombination (Lee et al., 2015), and this role was hypothesized to be influenced by relaxation of chromatin structure or regulation of genes that are involved in DNA repair. It also helped to prove that contrary to what was believed, the NER pathway has a more critical role in ICL repair than the FA pathway and that TLS polymerases POLZ-1 (POLZ homolog) and REV-1 (REV1 homolog) are essential to ICL repair (Oh et al., 2020).

1.2.3.4 New neighbors in the hood

In addition to identifying conserved roles for known components of the machinery that safeguards genome stability, the worm has also facilitated the discovery and characterization of new or conserved members of DNA damage response pathways. For example, the identification of the functional homolog of the yeast helicase SRS2 first came from work in *C. elegans* through the identification of RTEL-1 (RTEL1 homolog), which is involved in the elimination of unwanted recombination events (Barber et al., 2008). Moreover, genetic screens allowed the discovery of new factors involved in IR-induced DNA damage repair, identifying the phosphoinositide-3 kinase SMG-1 (SMG1 homolog) which may be involved in the phosphorylation of DNA repair proteins (González-Huici et al., 2017). Most importantly, SMG-1 doesn't show to be part of any of the three main pathways involved in DSBs repair, namely HR, NHEJ, and MMEJ.

1.2.3.5 Similar is not the same

One of the reasons previously mentioned that make *C. elegans* a good model, is the high homology of its genes with their human counterparts. However, the ability to study DNA repair pathways in detail allowed the identification of exceptions that refer to the mechanism involved. For example, CtIP is required for the loading of RAD51 at exogenously induced DSBs while the *C. elegans* homolog, COM-1, is not (Penkner et al., 2007). Another example is that the homolog of CHK2 does not play a significant role in DSBs repair (Higashitani et al., 2000) and that could be one of the explanations why HSR-9 (53BP1 homolog) does not participate in checkpoint activation like its human homolog (Ryu et al., 2013). Surprisingly, the NHEJ accessory factor, NHEJ-1, does not have a homolog in humans, showing that even the most conserved pathways can have exceptions.

1.2.4 Characterization of mutator genes in *C. elegans*

Phenotypic evaluation of double mutants of a gene under study, with a set of mutants implied in the maintenance of genome stability, aids in the characterization of a gene as a mutator. Below, we will discuss two examples of genes used to generate double mutants and their specific role in genome stability, as well as a long-established mutator assay that makes use of the mutant phenotype to simplify the detection of spontaneous mutations.

1.2.4.1 *dog-1*

DOG-1 is the ortholog of mammalian FANCJ helicase, that aids in DNA replication through G-quadruplex structures formed by G-rich DNA sequences (Youds et al., 2008). Thus, *dog-1* mutants have trouble replicating these regions of the genome and show an increased rate of mutagenesis (Tarailo-Graovac et al., 2015) seen most frequently as deletions specifically located in the G-rich sequences (Meier et al., 2021). These mutations are generated as a consequence of the stall or collapse of replication forks due to the DNA secondary structure (Boulton et al., 2004) which can be restarted or repaired by several mechanisms that include HR or TLS (Lehmann, 2002). The functional relevance of the DOG-1 protein (and its homolog) lies in the prevalence of G-rich DNA sequences in the human genome that are found in key locations of the genome such as telomeres, recombination hotspots, and gene promoters, among others (Simonsson, 2001). In *C. elegans*, mutations in genes involved in HR and replicative repair enhance the defects associated with loss of *dog-1*. This double mutant analysis is therefore commonly used to determine if a gene functions in these repair pathways (Youds et al., 2006)(Meier et al., 2021).

1.2.4.2 *helq-1*

HELQ-1 (HELQ in humans) is a conserved DNA helicase that contributes to the faithful replication of secondary DNA structures that are generated on the lagging strand (Meier et al., 2021). It was also shown to be involved together with RFS-1 (RAD51D in humans), in the resolution of HR repair intermediates during meiosis (Ward et al., 2010). Recent studies have shown that HELQ contributes to SSA and MMEJ, in addition to HR. Each of these pathways requires a DNA annealing step, suggesting that the function of HELQ in DNA repair relies on its ability to displace RPA from ssDNA (Anand et al., 2022)(Kamp et al., 2021). Thus, the involvement of HELQ in different ramifications of DNA repair, which include those pathways involving RAD51 nucleofilament (HR) or not (SSA, MMEJ); as well as its contribution to the correct replication of DNA secondary structures (loops), makes it a pleiotropic reporter of genome stability.

1.2.4.3 *unc-58*

Spontaneous mutations can arise as a consequence of defects in DNA replication, as well as in mechanisms involved in the elimination of mutagenic DNA lesions. For example, defects in MMR or NER pathways increase the spontaneous mutation rate (Schär, 2001). The increase in mutations observed in HR defective cells arises from the use of alternative repair pathways such as TLS and NHEJ (Schär, 2001). This highlights the importance of the appropriate pathway choice during the phase of the cell cycle when the damage is taking place.

In order to evaluate the rate of spontaneous mutations in *C. elegans*, a specific mutant of the ortholog of human KCNK9 (Potassium Channel subfamily K member 9), that has an easily

distinguishable phenotype has been employed. The dominant mutation *unc-58(e665)* results in almost paralyzed and small hermaphrodites (Brenner, 1974). This mutation can be suppressed by intra- or extragenic mutations, giving rise to worms with normal size and movement. Thus, by the generation of double mutants with the *unc-58* mutated gene, a gene can be assessed for its propensity to induce spontaneous mutations.

As mentioned at the beginning of this chapter, one of the main advantages of using *C. elegans* as a whole model organism, is the possibility to study the effects of harsh environmental conditions or genotoxic treatment on subsequent generations. This is possible due to germline immortality. Taking into account that the study on DNA damage responses is focused on the germline, we can assess the effect of genome instability present in one generation into the progeny for many generations later. But what does germline immortality involves and what can transgenerational consequences on genome instability represent? In the next chapter, we will discuss in detail examples of both, that will further our understanding of *C. elegans* as the ideal whole model organism.

1.3 TRANSGENERATIONAL INHERITANCE OF GENETIC INFORMATION: *C. elegans* AS THE LEADING MODEL

In order to optimize energetic requirements, cellular processes like transcription and protein expression taking place in oocytes and sperms are only limited to those genes that are vital for the first stages of embryonic development. One way of achieving it is by the compaction of gametes' genomes. As a consequence, their genotypes and chromatin modifications are different from those of somatic and germ cells. Another way to contribute to energy optimization during the first stages of development is by the presence of factors that are already provided by the mother and don't need to be transcribed by the zygote (Toralova et al., 2020; Wu and Vastenhouw, 2020). Those maternal factors involve RNA and proteins (Burkhart et al., 2020), called "maternal effector genes" (MEG), that will be gradually degraded as zygotic development takes place, and will be substituted by factors that will be transcribed and expressed by the zygote, a process that is known as "maternal to zygotic transition" (MZT) (Mitchell, 2022). In the specific case of *C. elegans*, there are two instances of maternal mRNA clearance; the first takes place during the transition from oocyte to one-cell embryo and is regulated by a consensus sequence in the 3'UTR, while the second takes place in the somatic blastomeres during embryonic development (Quarato et al., 2021).

Another way to transmit information across generations involves epigenetic modifications (Andersen and Horvitz, 2007; Greer et al., 2014; Katz et al., 2009). Those modifications acquired during the life of the mother may be replicated in the genome of the zygote. These epigenetic marks plus those acquired during the progeny's own lifetime will then be passed on to the next generations. Thus, a multigenerational memory of prior insults can have profound effects on

offspring generations in the future (Kishimoto et al., 2017). The transgenerational effect of epigenetic modifications has been shown to affect the expression of genes essential for fertility (Heestand et al., 2018), DNA damage repair (Xu et al., 2012), and telomere maintenance (Ahmed and Hodgkin, 2000; Yamamoto et al., 2021), all aspects detrimental to the survival of the progeny.

C. elegans, once again, turns out to be an excellent model for the study of genes involved in transgenerational memory. One of its advantages is its short development periods and conserved genetics. Genetic screens were performed to identify maternal effect genes, that when mutated produce zygotic lethality (mel- maternal-effect lethal), defects in fertility (mes- maternal-effect sterile), or behavioral and developmental abnormalities (maternal effect viable) (Figure 6). Among those genes are the maternal effect sterile *mes-2, -3, -4, -6* discovered by Capowski *et al* (Capowski et al., 1991; Xu et al., 2001), which are part of a complex whose absence causes necrotic death of germ cells during larval development. Included in the maternal effect lethal mutations are 41 genes discovered by Hekimi *et. al.* (Hekimi et al., 1995) that led to morphological or behavioral defects (Hekimi et al., 1995). In addition to these mutations which have defects in F1 or F2 offspring, another class of mutations confers a phenotype known as mortal germline (Mrt), which is defined by lineage sterility after numerous generations.

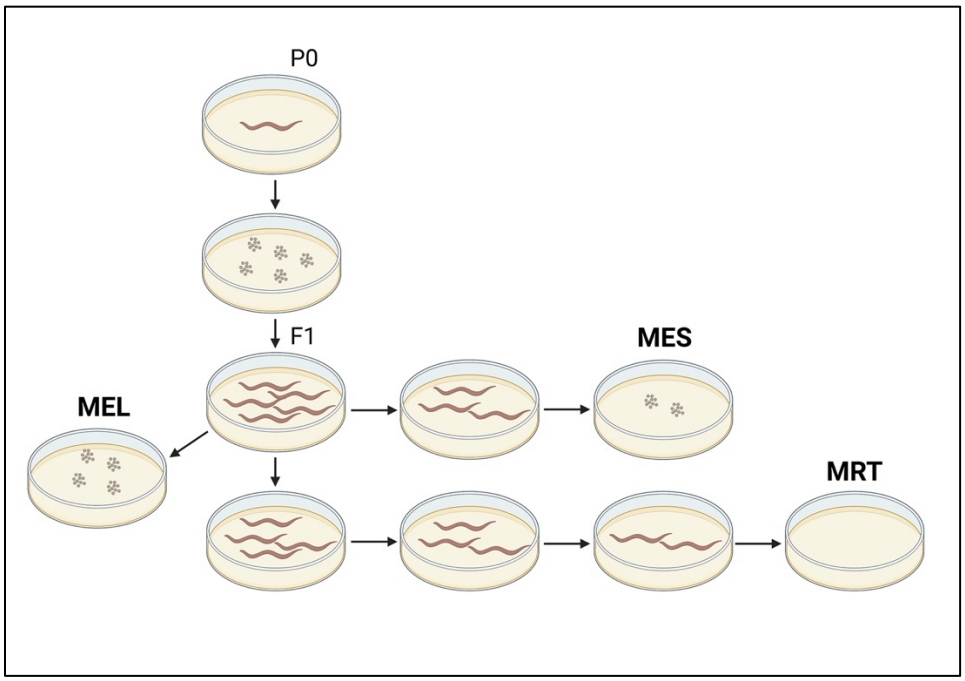


Figure 6: Representation of maternal effect genes. A homozygous mutant is plated (P0) and the eggs laid grow to be F1 progeny. If the gene is maternal-effect lethal (MEL) the eggs laid by F1 worms will not hatch because the gene is vital for zygotic development. If the gene is maternal-effect sterile (MES), the eggs laid by F1 reach adulthood and lay eggs but those eggs do not hatch because the gene is required for fertility. Finally, if the gene generates a mortal germline (MRT) phenotype, sterility is reached after several generations.

1.3.1 Forever young: Germline immortality

Germ cells have the burden of carrying genetic information over generations, and as a consequence, the pressure of species survival lays on them. Due to their paramount task, they are given certain tools that both differentiate them from somatic cells and make them more resistant to damage to prevent the accumulation of defects in genetic information.

One of the elements that promote immortality is the expression of telomerase, a ribonucleoprotein that uses its RNA component as a template for telomere replication (Harrington, 2003). Maintaining the length of telomeres across many replication events ensures they do not become critically short and behave as an uncapped DNA end, which leads to fusion or activation of apoptosis. In addition to ensuring replicative capacity, the genetic information must also be protected from DNA damage, preventing the accumulation of spontaneous mutations (Suter et al., 2004). When studying DNA damage repair is important to take into consideration the possible existence of differences as far as the identity and nature of the DNA repair pathways are concerned. The consequences of damaging genomic DNA from germ cells are more detrimental for the species than damaging somatic cell DNA.

Another mechanism collaborating to promote germline immortality involves epigenetic modifications, including histone acetylation and DNA methylation. The tight regulation of genes required to be expressed during specific developmental stages is key to ensuring the right propagation of information (Suter et al., 2004). This applies to both genes and also repetitive elements like transposons, since the maintenance of their epigenetic silencing has proven to be essential for genome stability (Bestor, 2003). Failure to accomplish transposon silencing can lead to the incorporation of mutations through transposition (Hajkova et al., 2002). Another level of control of germ cell genome quality involves a selection mechanism, which involved controlling that the genetic information is not altered previous to the formation of the zygote, at the time of haploid gametes selection (Smelick and Ahmed, 2005). Consequently, aneuploidy or deleterious mutations would prevent fertilization or viability of the zygote.

To date, there are four cellular functions that have been shown to be transduced into transgenerational sterility when their function is compromised. They involve components of the RNAi (RNA interference) pathways, epigenetic factors, proteins involved in telomere maintenance, and DNA repair. Below, we will discuss specific cases of mutants from each case that eventually develop transgenerational sterility.

1.3.2 Manifestations of transgenerational genome instability

1.3.2.1 RNA interference (RNAi)

RNAi was discovered by studies in *C. elegans* (Fire et al., 1998). RNAi uses dsRNA to target and degrade specific mRNAs. Endogenous small RNA pathways related to RNAi have been shown to be involved in many important processes that include development, metabolism, cell fate, and cell death (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Vastenhouw et al., 2003). Another function regulated by RNAi- related processes is the anti-viral response. The latter is taken care of by the exo-RNAi response that involves degradation of RNAs derived from exogenous sources. Processes affecting the regulation of the organism biology are controlled by the endo-RNAi response, involving endogenously derived small RNAs. This endo-RNAi response involves three distinct classes of small RNAs: microRNAs, endo-RNAs, and piRNAs (Piwi-interacting RNAs). They all bind to the Argonaute effector proteins that will lead them to the recognition of their target transcript and the eventual down-regulation of its expression. Specifically, the endo- and piwi-RNAs are the ones involved in the maintenance of germline immortality.

The *C. elegans* Argonaute protein CSR-1 is known to protect active genes from piRNA silencing in the germline, promote germline transcription, regulate the biogenesis of histone mRNAs, participate in chromosome segregation, histone mRNA maturation, germ-line gene expression, and fine-tune germline mRNAs loaded into oocytes (Quarato et al., 2021). Thus, CSR-1 activity is essential for fertility and chromosome segregation (Quarato et al., 2021). The effect of CSR-1 on maternal mRNAs might be a conserved mechanism required for the MZT and mRNA clearance across species.

Piwi proteins are paralogs of Argonaute proteins and are primarily expressed in the germ line. Piwi is known to be involved in the maintenance of germ cell immortality as its absence causes transgenerational replicative aging of the germ cells. Strikingly, *Simon et. al*. (Simon et al., 2014) showed that the reduction in IGF-1 signaling, known to extend somatic lifespan, is able to suppress the germ cell mortality of the *prg-1* (piwi in humans) mutants. These results highlight the intricate mechanisms involved in RNAi pathways and how they are able to interconnect in order to maintain such an important function as germ cell immortality and the propagation of specie attached to it.

SET-24 (no human homolog) is another *C. elegans* germline-specific protein that is required to maintain immortality. *set-24* mutant animals reach sterility in fewer than 20 generations when chronically exposed to restrictive (25^{o}C) temperatures. Preliminary results by *Frezal et. al*. indicate that SET-24 is required for the transgenerational maintenance of silencing via the nuclear RNAi pathway (Frézal et al., 2018). This is also true for mutations in the *rsd-2* and *rsd-6* (RNAi spreading defective/TDRD5 in humans) genes which regulate accumulation and target silencing

of the endo-RNAs 22G-RNAs (Zhang et al., 2012). *rsd-2* and *rsd-6* mutants show a highly penetrant Mrt phenotype at the stressful temperature of 25ºC, but are completely fertile at the ideal growth temperature 20ºC. These defects in transgenerational fertility at higher temperatures were accompanied by the de-silencing of repetitive loci, which authors suspect could be the driving cause of the infertility.

As with any complex regulatory mechanisms, RNAi pathways are also interconnected with epigenetic regulation for the maintenance of germline immortality. The protein MORC-1 (MORC1, MORC2 in humans), for example, is essential to maintain the heterochromatin mark H3K9me3 at certain endogenous nuclear RNAi targets (Weiser et al., 2017). Thus, MORC-1 acts as a protective factor in order to prevent the spread of the H3K9me3 marks provided by MET-1 (NSD1, NSD2 in humans). In this article, they discovered that the small RNA methyltransferase *henn-1* is required for piRNA stability during embryogenesis, but not in the adult germline. So, not only epigenetic marks in the chromatin are involved in the maintenance of transgenerational germline immortality but also the action of the RNAi pathways.

1.3.2.2 Epigenetics

Epigenetic changes involve DNA modifications that regulate if a gene is expressed or not. These types of changes are performed by epigenetic factors, which include DNA methylation, histone modifications, and microRNAs. However, not only do these factors determine which genes are expressed, but they are also a way of transmitting information that has roles in longevity and fertility. An important characteristic is that these modifications can be transmitted across generations. For example, two epigenetic marks known to be involved in the formation of

heterochromatin are the H3K9me2, enriched on DNA transposons and telomeres, and H3K9me3, enriched on retrotransposon families. On the other hand, euchromatic post-translational modifications involve, among others, H3K36me3, catalyzed by MET-1 (NSDE-1 and NSDE2 in humans), while MES-4 (no homolog in humans) catalyzes H3K36me2 and H3K36me3. These epigenetic factors regulate chromatin to influence gene expression, but they also play part in the maintenance of germline immortality.

Many studies have been performed in *C. elegans* regarding epigenetic factors and their roles in fertility. SET-2 (SETD1A/SETD1B in humans) is a methyltransferase with an important role in fertility that encompasses germline immortality by the incorporation of epigenetic marks of active transcription in H3K4 (Robert et al., 2020). Its absence causes transgenerational loss of germline identity that eventually leads to sterility. This effect is achieved by the misexpression of genes in early generations (priming), that will continue to be mis-expressed in subsequent progeny until sterility is reached. On the other hand, there are also demethylases involved in the maintenance of fertility. For example, SPR-5 (LSD1 in humans) is a demethylase whose absence causes transgenerational infertility. Lack of this demethylase also leads to the accumulation of DNA methylation of adenines (6meA) as well as the euchromatin mark H3K4me2 that goes along with a decline in the heterochromatic mark H3K9me3 (Katz et al., 2009). H3K4me2 should be rebooted to the initial state in developing gametes or in the zygote to prevent the inappropriate transmission of epigenetic memory from one generation to the next one. For that, the *spr-5* demethylase is the one involved in the erasure of those marks in the PGCs (primordial germ cells) (Katz et al., 2009).

When studying epigenetic marks and the balanced regulation of activities involving methylases-demethylases, it becomes important to take into consideration more than one epigenetic factor at the same time, since the activity of one factor may require the activity of another that can have the same or opposite activity. For example, mutation of the methyltransferase *met-2* (SETDB1/SETDB2 in humans) results in a germline mortality phenotype and acts in cooperation with SPR-5 to reestablish the epigenetic ground state in the germ line (Andersen and Horvitz, 2007; Kerr et al., 2014). Also, during germline transcription, the euchromatin mark H3K4me2 is deposited in activated genes by *set-17* (PRDM7 in humans) and *set-30* (SMYD1, -2, -3 in humans) (Katz et al., 2009). This epigenetic mark then is an important part of the maintenance of the immortal germ line.

1.3.2.3 Telomeres and DNA damage repair

The telomeres are repeats of non-coding repetitive sequences located at the end of the chromosomes. They protect the genome from nuclear degradation, unnecessary recombination, repair, and chromosomal fusions (Chen et al., 2009; O'Sullivan and Karlseder, 2010). They are coated with a protein complex named Shelterin to prevent the activation of the DSB response to the chromosome ends, which can be detected as DSB (Palm and de Lange, 2008). Two components of that complex, TRF1 and TRF2, bind the double strand region of the telomeres. The component TIN2 interacts with both proteins and recruits the TPP1 protein that interacts with the POT1 protein that binds to the single strand of telomeres. The last component of the complex, RAP1, interacts with TRF2. Altogether, they form a loop structure that protects telomeres from exonucleolytic degradation (van Steensel et al., 1998). In each round of replication, telomeres are shortened, eventually leading to cellular senescence or apoptosis if left unattended. This is known

as the end-replication problem, where the DNA replication machinery is unable to copy the last few nucleotides of the lagging strand, leaving a single-stranded overhang (Gilson and Géli, 2007). This consequence is avoided in germ cells by the presence of the reverse transcriptase telomerase, which extends the 3' end of chromosomes by retro-transcribing in an iterative fashion the template region of the associated telomerase RNA (Cong et al., 2002). In this way, the length of germ cells' telomeres remains constant, allowing for the immortal phenotype of this type of cell. One of the problems encountered during the replication of telomeres is the presence of heterochromatin-like structures, especially G-quadruplexes and t-loops, which represent a threat to the replication fork, causing its stalling (Cong et al., 2002; Lee and Paull, 2005). Double strand breaks at telomeres are recognized by the MRN complex, which in turn activates the ATM/ATR kinases, leading to the activation of the HR and alt-NHEJ DNA repair pathways (Lee and Paull, 2005; Sobinoff and Pickett, 2017). As a consequence, there is a strong link between DNA damage responses and telomeres to prevent errors generated during chromosome end replication.

The absence of any of the genes involved in telomerase activity, *trf1, trt2, smg7* in mice, and *cdc13 (in yeast)*, results in late-onset sterility and end-to-end chromosome fusions that arise as a consequence of the shortening of telomeres (Langston et al., 2020; Tong et al., 2011; Wang et al., 2018b; Zhang et al., 2001). Defects in germline stability that are a consequence of telomere maintenance, can be evidenced in two interconnected processes involving telomere length and activation of DNA damage responses. The absence of any component of the complexes protecting telomere structures leads to the activation of DSB repair pathways. Recently, two paralogous proteins that bind telomeric dsDNA have been identified in *C. elegans* by a protein-interaction screening (Yamamoto et al., 2021). These proteins, DTN-1 (TRF1 in humans) and DTN-2 have

redundant roles in the maintenance of germline immortality, and in the absence of both genes, worms become progressively sterile, show X chromosome non-disjunction and long telomeres. They propose that the most probable cause for the chromosomal defects and mortal germline is the activation of DNA-damage response pathways as a consequence of the deprotection of the telomeres.

One of the most studied – and the first-- genes in *C. elegans* that has a role in response to telomere DSBs response is *mrt-2* (RAD1 in humans). MRT-2 is part of a checkpoint pathway that is able to delay the cell cycle in response to a single DSB. Since telomeres can be considered as DSBs with short overhangs buried in the double-stranded telomeric DNA, when these telomeric loops unfold during S phase, the DSBs-like ends stalling of the replication fork at the sites of telomeres, could activate a checkpoint response. Although *mrt-2* mutants have normal brood sizes in the F2 generation, there is a gradual decrease in brood size until reaching sterility, indicating an accumulation of damage over the generations (Ahmed and Hodgkin, 2000). In late generations *mrt*-2 mutants, one can see the results of defects in telomere maintenance: chromosome fusions that ultimately cause aneuploidy. A similar phenotype with the same chromosomic consequences is seen in the *hus-1* (HUS1 in humans) mutant (Hofmann et al., 2002). In this case, after IR treatment, it was also shown that there is a significant increase in the mRNA levels of the pro-apoptotic gene *egl-1* mainly in the germline. Together with *cep-1 (p53 in humans), egl-1* (no humans homolog) induction by *hus-1* activates the apoptotic machinery.

Another protein that has a key role in regulating telomere length, as well as DNA damage response is CLK-2 (TELO2 in humans). Its role in reproduction can be inferred because of its requirement for the two- to four-cell stage embryonic development and consequently high levels of *clk-2* mRNA in the oocytes (Bénard et al., 2001). This maternally deposited gene is able to interact with the ATM and ATR kinases (Ahmed et al., 2001) in response to the presence of telomeric DSBs. In addition to this, mutants exhibit changes in the length of the telomeres that can be shorter or longer, depending on the specific cellular context (Guo et al., 2021). Recently, CLK-2 was also implicated as part of the nonsense-mediated mRNA decay (Guo et al., 2021). The DNA damage response regulator MRG-1 (MORF4L1 and MORF4L2 in humans), activates the *clk-2*-dependent checkpoint when DNA lesions fail to be repaired which in turn leads to the activation of *cep-1*-dependent germ cell apoptosis (Xu et al., 2012). The *mrg-1* mutants have a decreased brood size, which is partly a consequence of the increased embryonic lethality. These mutants also experience defects in crossover formation and defects in X chromosome non-disjunction, explaining the need for *mrg-1* for DNA damage repair on meiosis.

Proteins involved in transgenerational genome stability encompass different cellular functions, including chromatin modifications and DNA damage responses. Below, we will discuss a specific case that is involved in both functions and that will be the focus of this thesis, the chromatin remodeler SMARCAD1.

1.4 SMARCAD1: A VERSATILE PROTEIN

In eukaryotic cells, the double-stranded DNA is wrapped around an octamer of histones to form the nucleosomes. Each octamer has two copies of each of the H2A-H2B and H3-H4 dimers. They are further packed with linker histones and other proteins into higher-order chromatin structures. As a consequence, in order to access DNA for replication or transcription, for example, those chromatin structures have to be temporally modified in order to allow access to the genes. Defects in chromatin remodelers frequently result in defects in embryonic development and pluripotency (Hota and Bruneau, 2016). Those nucleosome remodeling mechanisms involve the disruption of histone-DNA contacts in an energy-dependent process (Gavin et al., 2001; Havas et al., 2000). The mechanisms involved in chromatin remodeling include sliding the histone octamer across DNA, changing the conformation of nucleosomal DNA, and changing the composition of the histone octamer. But histones are not only a protein complex acting as a scaffold for the DNA to be wrapped around, but they also play an active role in the remodeling process. There are specific histone modifications such as acetylation that act as recruitment marks and even specific histone tails, such as the H4 tail, that act as catalytic regulators (Clapier and Cairns, 2009).

ATP-dependent chromatin remodelers include among their domains a helicase motif, which is formed by two helicase sub-domains, one of which is responsible for ATP hydrolysis. As a consequence of this chemical process, these two subdomains experience a structural change with respect to the other, which allows translocation through the DNA. Proteins that contain helicase motifs have been subdivided into several superfamilies. Among those, is the helicase-like superfamily 2 (SF2), which includes the Snf2 family, consisting of proteins that have a helicase-like region with a similar primary sequence to *Saccharomyces cerevisiae* Snf2p. SMARCAD1 is

in the Swr1-like or INO80 subfamily of Snf2 remodeler together with the INO80, Swr1, and EP400 (Clapier and Cairns, 2009; Tyagi et al., 2016).

SMARCAD1 consists of two N-terminal CUE domains named for "coupling of ubiquitin to ER degradation". The catalytic domain is split into ATPase1 and ATPase2, connected by an extended loop (Figure 7). The N-terminal CUE domains interact with binding partners that help in the recruitment of SMARCAD1 to the genome (Ding et al., 2018; Lim et al., 2019), while the C-terminal catalytic domain is required for maintaining genome integrity (Sachs et al., 2019). Although SMARCAD1 has been shown to be able to bind to both naked DNA and nucleosomes, its catalytic activity is more potently activated through the binding to nucleosomes. It was also shown to interact with individual histones and is even able to interact with histones in the absence of DNA, which is related to its function of being able to promote *de novo* nucleosome assembly (the histones are not taken from other DNA fragments) (Markert et al., 2021). The catalytic domain makes direct contact with the H4 and H3 N-terminal tails. For the assembly of nucleosomes that start from free histone complexes, its ATPase activity is not required. However, most of its functions relies on ATPase activity, as can be seen when examine in detail the different processes in which SMARCAD1 is involved.

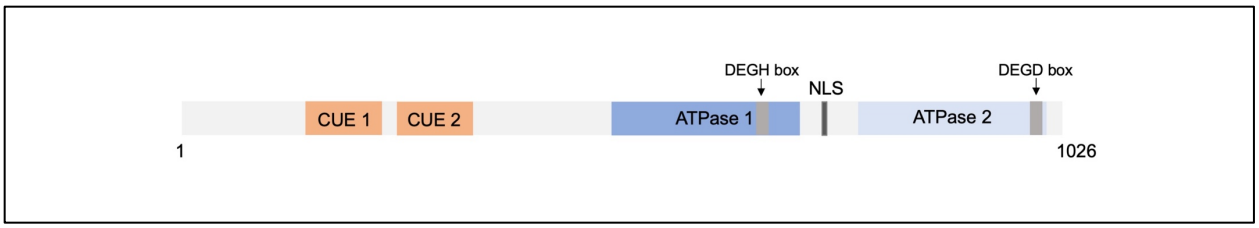


Figure 7: Cartoon representation of the domains of human SMARCAD1 protein. In the N-terminal region, the two ubiquitin binding domains, CUE domains (Coupling of Ubiquitin to ER degradation), CUE1 and CUE2. In the C-terminal region, two ATPase domains, ATPase 1 also known as Helicase ATP-binding domain, and ATPase 2 also known as Helicase C-terminal domain. The DEGH and DEGD boxes located in ATPase 1 and ATPase 2 domains, respectively, are represented in gray. The nuclear localization sequence (NLS) is represented in dark gray.

1.4.1 Beyond nucleosome eviction: chromatin post-translational modifications, replication, and transcription

1.4.1.1 Post-translational modification related to SMARCAD1 function

SMARCAD1 has been found to interact with the scaffold protein KAP1 (KRAB-Associated protein 1/ also known as Trim28), and this interaction is responsible for the maintenance of normal levels of H3 acetylation and H3K9 methylation (Rowbotham et al., 2011). As a consequence, in the absence of SMARCAD1, there is an upregulation of acetylation and downregulation of H3K9 methylation. Although, mutation of the ATP binding domain of SMARCAD1 is still able to localize normally, the ATPase function is required to exert its role in the modulation of histone modifications (Ding et al., 2018).

KAP1 is also involved in normal development and differentiation, and just like SMARCAD1, is involved in chromatin replication and DNA repair (Cheng et al., 2014a, 2014b). The SMARCAD1-KAP1 association has been found in multiple cell lines, developmental stages, and pluripotent and differentiated cells. SMARCAD1 interacts with KAP1 through its CUE1 domain and that KAP1 is required to retain SMARCAD1 in the nucleus of mouse ESCs (embryonic stem cells). Although the CUE domains bind ubiquitin, these are non-canonical ubiquitin-binding domains, since the KAP1 binding is not mediated by ubiquitin (Ding et al., 2018).

SMARCAD1 has also been shown to be involved in histone deacetylation, and consequently, gene silencing. This function of the protein is proposed to be mediated by an interaction with HDAC1 and HDAC2 (Histone Deacetylase 1 and 2) (Rowbotham et al., 2011). These interactions are thought to promote histone modifications at the pericentric heterochromatin. Thus, it is not only the regulation of the general genome chromatin architecture, but also of specific and important regions of chromosomes that will ensure mitotic fidelity.

1.4.1.2 Replication

SMARCAD1 has been shown to interact *in vivo* with the PCNA (Proliferating Cell Nuclear Antigen) complex at replication sites (Rowbotham et al., 2011). This interaction is mediated by the SMARCAD1 N-terminal region, which is responsible for its localization in the replication fork. This localization in turn help to maintain PCNA levels at replication forks, thus ensuring accurate and timely DNA replication. This function of SMARCAD1 at replication forks is so important that its absence leads to the activation of the ATR-mediated checkpoint pathway (Lo et al., 2021).

The role of the SMARCAD1-PCNA pair during replication was reported to be only under unperturbed conditions (Lo et al., 2021). When a fork is stalled as a consequence of genotoxin treatment, like hydroxyurea, both SMARCAD1 and PCNA dissociate from the stalled fork. Although not required for the resolution of stalled forks, SMARCAD1 is involved in the efficient restart and further progression of replication forks, which explains why cells lacking this gene are sensitive to replication stress-inducing agents. This goes hand in hand with the observation that there is a poor recovery of PCNA levels at restarting stalled forks, which subsequently causes inefficient fork restart and severe defects in fork progression. Unsurprisingly, this role in the maintenance of PCNA levels involves SMARCAD1 chromatin remodeling activity, which antagonizes 53BP1 accumulation at the active replication forks through the eviction of 53BP1-associated nucleosomes and consequently, the displacement of the 53BP1-ATAD5 (ATPase Family AAA Domain Containing 5) complex involved in preventing PCNA recovery at restarted forks (Lo et al., 2021).

SMARCAD1 is also required for HR-mediated repair of DSBs since it regulates long-range resection (discussed later, section 1.4.3). The ATPase activity of SMARCAD1 is essential for this function, but the deletion of its N-terminal domain, NΔ-SMARCAD1, is still proficient in HR (Lo et al., 2021). Nevertheless, NΔ-SMARCAD1 is sensitive to drugs that cause replication stress, indicating a separation of functions between these two domains. Thus, SMARCAD1 is critical to limit fork stalling under unperturbed conditions and promote fork restart and fork progression upon replication stress. In the absence of the N-terminal region, SMARCAD1 still associates with chromatin but is not enriched at sites of replication (Lo et al., 2021).

The role of SMARCAD1 in replication is further supported by the recent finding on the *Saccharomyces pombe* homolog, Fft3 (Fission yeast Fun Thirty 3). Fft3 interacts with DNA replication polymerases δ and ε (Pol δ and ε) (Ait-Saada et al., 2019). In the presence of replication fork stalling, after treatment with genotoxins, Fft3 was found to promote SSA and HR-mediated replication fork restart.

1.4.1.3 Role for SMARCAD1 in transcription

Histone acetylation is an important regulator of transcription. This modification is made by histone acetyltransferases (HATs) including CREB-binding protein (CBP). Acetylation of conserved lysines in histones the relaxation of DNA-histones interaction, allowing access to those genes to be transcribed. Additionally, ATP-dependent chromatin remodelers can act in combination with the HATs. That is the case with *Drosophila* melanogaster SMARCAD1 homolog, which is recruited to the promoter region by CBP (Doiguchi et al., 2016) where it stimulates CBP acetylation of H2A on K5A and K8A. Consequently, when SMARCAD1 is knocked down, some genes are downregulated while others are upregulated, which can be explained by the interaction of SMARCAD1 with KAP1 to maintain heterochromatin marks and with HDAC to remove silencing marks like acetylation.

The involvement of SMARCAD1 in transcription does not end in the regulation of HAT activity. After the transcription has started, RNAPII (RNA polymerase II) has to deal with nucleosomes located throughout the gene coding sequence; those have to be temporally disassembled as the sequence is transcribed and immediately reassembled after RNAPII has

finished. One of the chromatin modifiers during RNAPII elongation is the histone chaperone FACT complex (Facilitates Chromatin Transcription), which disassembles the histones from the nucleosomes ahead of RNAPII and immediately recycle them (Mason and Struhl, 2003). In this way, the chromatin state of the transcribed region undergoes a temporal modification to allow its transcription.

Recently, the *S. cerevisiae* homolog of SMARCAD1, Fun30, has been shown to help RNAPII overcome the nucleosome barrier to transcription. This role explains the enrichment of this protein over transcribed regions, as well as the promoter and terminators (Lee et al., 2017). Unlike the FACT complex that is involved in the disassembly and reassembly of nucleosomes, SMARCAD1 appears to only be involved in nucleosome disassembly. Importantly for this function, Fun30 directly interacts with RNAPII in a CTD-phosphorylation-independent manner and with other factors involved in transcription elongation like Spt6 (SuPpressor of Ty) (Venkatesh and Workman, 2015).

1.4.2 Role of SMARCAD1 in development and reproduction

In mice, the *smarcad1* homolog, previously known as *etl-1*, was shown to be expressed in many cell types throughout development, suggesting its involvement in multiple processes during embryogenesis. This is further supported by the finding of core pluripotency transcription factors NANOG, OCT4 and SOX2 bound to the human *smarcad1* gene (Ding et al., 2018).

ETL1 protein expression in adult tissues is highly variable and even in the same tissue its levels were found to change significantly during development (Soininen et al., 1992)(Schoor et al., 1993). In somatic cells, ETL1 localizes in the nucleoplasm, showing regions of increased concentration. During metaphase, as a consequence of nuclear membrane breakdown, it is evenly distributed throughout the cytoplasm (Schoor et al., 1999). ETL1 is not readily detected in the mature oocyte and zygote, it can however be detected again by immunohistochemistry at the two-cell stage. This increase at the mid-late two-cell stage suggested that *etl-1* synthesis during early cleavage depends predominantly on maternal mRNA. The fact that is absent in the pronuclei indicates that is not required for DNA replication before the first cell division. Considering that SMARCAD1 was previously shown to be important for the stability of the replication fork, these results could mean there are other proteins involved in this stage to supplement SMARCAD1 function. The high expression of ETL1 at the two-cell stage raises the possibility that it is involved in the transcription of zygotic genes. There is another increase in the expression between the morula and blastocyst stages that itself depends on zygotic gene expression.

Drosophila melanogaster SMARCAD1 follows a similar expression pattern to the mouse. The protein is located in the nucleus and constitutively expressed in early-stage embryos, suggesting its contribution to embryogenesis and is also expressed in adult cells (Doiguchi et al., 2016).

Studies in mice showed that SMARCAD1 is not essential for ES cell viability, proliferation, and embryonic development. However, homozygous mutants had a retarded growth and a decreased postnatal viability and fertility (Schoor et al., 1999). The authors hypothesized

that the reduced postnatal viability could be associated with physical defects like skeletal dysplasia, which explains the reduced thoracic volume that could be leading to respiratory failure. Most homozygous litters were smaller than the heterozygous and *wild-type* counterparts, showing a reduction in total body weight of around 25%. By analyzing these phenotypes in two different genetic backgrounds they showed that ETL1 mutations are strongly modified by the genetic background.

A study in yeast about the role of SMARCAD1 in meiosis was the first to focus on a mechanistic consequence of this chromatin remodeler in reproduction (Storey et al., 2018). Due to its post-translational modifications of chromatin activities, Fun30 protein was identified as a meiotic hotspot factor, that is a protein that helps to create regions of the genome that are active for meiotic DSBs and crossovers.

In humans, the only evidence of the role of SMARCAD1 in fertility involves the work of Bansal *et. al.*, who analyzed the population of RNA transcripts in human spermatozoa. Surprisingly, the SMARCAD1 transcript was specifically down-regulated in the asthenozoospermic patient samples (Bansal et al., 2015).

1.4.3 DNA damage repair in the chromatin: SMARCAD1/Fun30 function

ATP-dependent chromatin remodelers belonging to the four families (SWI/SNF, INO80, CHD and ISWI) have been shown to be involved in the repair of DSBs. However, the *S. cerevisiae* homolog of SMARCAD1, Fun30, has been the only member of these families shown to be directly

involved in long-range resection (Chen et al., 2012). There is a considerable amount of evidence supporting this conclusion, including delayed long-range resection and decreased loading of RPA and RAD51 at 5kb from the break site in the absence of Fun30. In addition, Fun30 and double mutants with the exonucleases responsible for long-range resection, Exo1 or Sgs1 have a more severe impairment to respond to genotoxic agents, although the effect with Sgs1 is stronger (Costelloe et al., 2012). Lastly, recruitment of Fun30 to DSBs does not takes place in resection defective mutants *sgs1* and *exo1*. While this could mean that Fun30 has a later role HR, further studies indicate its function is limited to the first steps of HR, since it did not show to be involved in strand invasion or later steps (Eapen et al., 2012).

One hypothesis for the role of Fun30 in long-range resection involves its ATP-dependent chromatin remodeling activity. Specifically, through its histone dimer exchange activity, it would remove those γH2A-H2B dimers that are bound to RAD9. In this way, Fun30 would be favoring HR over NHEJ, similar to BRCA1 does (Tong et al., 2020). This is supported by the fact that after FUN30 deletion, there is an increase in the rate of NHEJ (Eapen et al., 2012). Another piece of evidence is the fact that many of the binding partners of Fun30 have been found to be shared with RAD9 and they even share the same binding site of Dpb11 in yeast (Bantele et al., 2017). The activity of Fun30 at damage sites appears to be regulated by checkpoint proteins CDK1 which phosphorylates Fun30 on Ser20 and Ser28 in order for long-range resection to take place (Chen et al., 2016).

There is still a lack of knowledge regarding the mechanism of action of SMARCAD1 under normal and damage conditions that would allow identifying its differential role in replication and

DNA repair. On one hand, as previously mentioned, studies in human cell lines and yeast showed that SMARCAD1 associates with PCNA during replication; while on the other hand, it was shown to associate with the 9-1-1 complex during DNA damage response in yeast (Bantele and Pfander, 2019). The first evidence that helps differentiate the SMARCAD1/Fun30 role in replication fork vs DSB repair is the involvement of its CUE domains. Those domains were shown to be involved in the interaction with PCNA, but are not required for resistance to genotoxic agents (Bi et al., 2015). By the generation of an ATPase-only mutant, cells showed to be proficient in DSB repair. These evidence leaves an important concept when treating SMARCAD1/Fun30 null cells with genotoxins, that involves the question of whether the sensitivity is a consequence of replication fork instability and/or defects in DSB repair consequence of defects in long-range resection. To make things more complex, replication fork stalling is also known to generate DSBs in response to HU, CPT, and MMS. Then, an important point to consider when studying Fun30 would be to use cell cycle coordinated cells.

Based on its pleiotropic roles in resection and replication, SMARCAD1 has been tested for its ability to protect cells against genotoxic agents. Both in cell lines or yeast, SMARCAD1/Fun30 is required for resistance to CPT. This function appears to involve its role in long-range resection since overexpression of Exo1 in Fun30 deficient cells suppresses sensitivity to CPT (Bi et al., 2015). In contrast to CPT, the sensitivity of *Fun30* mutants to HU required high concentrations of genotoxin. Similarly, Fun30 alone does not appear to be highly sensitive to MMS and was not sensitive to treatment with UV light. However, it does appear to be sensitive to treatment with PARP inhibitors (Costelloe et al., 2012). Considering the type of damage induced by these genotoxins and their preferred mechanisms of repair (CPT and HU: HR (Kjeldsen et al., 2018;

Petermann et al., 2010); UV:NER (Bergink et al., 2007) and MMS: mostly BER (Alseth et al., 2005)), these sensitivities suggest that the role of SMARCAD1 in genotoxic resistance is through its role in HR.

Another DNA repair pathway in which SMARCAD1 is involved is the mismatch repair pathway (MMR). This pathway ensures that those mismatches generated during DNA replication that could not be corrected by the proofreading activity of polymerase, are repaired. Just like for replication, this repair process will have to take place embedded in nucleosomes assembled behind the replication fork. In this specific situation, the mechanism of nucleosome exclusion is mediated by an Msh2-mediated reaction (Terui et al., 2018)(Goellner, 2020). In this context, Msh2 is required for the recruitment of SMARCAD1 to the mismatch site. Contrary to what could be expected, the presence of this remodeler is not related to nucleosome remodeling directly, but it appears to assist with an Msh2-dependent nucleosome exclusion to counteract chromatin assembly.

The pleiotropic functions of SMARCAD1 in key cellular processes became clear after a plethora of studies performed in yeast and human cell lines. Specifically, its role in HR and replication links its chromatin remodeler activity with specific processes that go beyond simply the regulation of gene expression by the modification of chromatin compaction. In fact, the discovery of its involvement in HR through the favoring of long-range resection, provides compelling evidence for a role in DNA damage responses. However, the study of the causes that can explain its role in fertility observed in mice has lagged behind.

Thus, in this work, we will start addressing the question of the mechanism behind the decreased fertility of null organisms. Specifically, we will focus on the role its DNA repair function has in fertility. In order to do that, we will take advantage of the whole model organism *C. elegans*, not only because of the previously mentioned advantages mentioned in sections 1.2 and 1.3, but also because it will facilitate the studies of DNA damage at the level of the germline and the transgenerational role genotoxic agents have on fertility. This will allow us to connect for the first time the studies made at the cellular level with their effect on fertility.

1.5 Overarching hypothesis

The putative *C. elegans*' SMARCAD1 ortholog has been predicted based on sequence homology and its functional characterization has not been addressed so far. Thus, based on previous results on null mice we hypothesize that the worm ortholog is involved in fertility and that this role is mainly influenced by the function of SMARCAD1 in germline genome stability.

Taking into account the functional characterization of human and yeast SMARCAD1/Fun30, we hypothesize that the role in germline genome stability involves the function of *C. elegans'* SMARCAD1 ortholog in long-range resection as part of the homologous recombination DNA repair pathway.

Finally, based on observations during this study, we hypothesize that worm SMARCAD1 is not a mutator gene.

2.0 GERMLINE GENOME STABILITY IS REGULATED BY THE CHROMATIN REMODELER SMARCAD1 IN *C. elegans*

2.1 MATERIALS AND METHODS

2.1.1 Culture and strains

Worms were cultured on MYOB plates seeded with OP50 and grown at 20^{o}C unless otherwise noted (Brenner, 1974).

m03C11.8 encodes the worm ortholog of SMARCAD1. For the remainder of this thesis, we will refer to this locus as *smrd-1*. To study the role of SMRD-1 in mitotic and meiotic HR in the germline, the following mutant strains were used: *mre-11(iow1), unc-58(e665), pot-2(tm1400), exo-1(tm1842), helq-1(tm2134), dog-1(gk10), sws-1(ea12) and smrd-1(ea92)*. Double and triple mutants generated for this work were done using standard genetic techniques (Table 1).

Table 1: Generated strains

Strain	Genotype
QP2126	*smrd-1(ea92)/hT2 III*
QP2164	*smrd-1(ea92) III, helq-1(tm2134) III*
QP2213	*pot-2(tm1400) II; smrd-1(ea92) III*
QP2186	*smrd-1(ea92) III; mre-11(iow1)/nT1 V*
QP2163	*smrd-1(ea92) III, exo-1(tm1842) III*
QP2166	*smrd-1(ea92)/hT2 III; sws-1(ea12) V*
QP2185	*smrd-1(ea92) III; unc-58(e665) X*
-	*dog-1(gk10) I;smrd-1(ea92) III*

2.1.2 Generation of *smrd-1(ea92)*

Unique CRISPR guides near the start codon of *smrd-1* were selected using the CRISPR design tool at http://crispr. mit.edu (see Table 2 for sequences of the primers used in single guide RNA design). Generation of the null mutant was obtained by incorporating a universal STOP-IN cassette as described by Wang *et. al* .(Wang et al., 2018a). *dpy-10* was used as an injection control. An injection mix consisting of *dpy-10(cn64)* and *smrd-1* repair oligos, each genomic RNA (gRNA) (one for *dpy-10*, two for *smrd-1*) diluted in TE buffer was prepared and injected into *N2(wt)* day 1 adult hermaphrodites. Roller progeny [*dpy-10(cn64)*/+] of injected hermaphrodites were isolated and allowed to have Roller progeny that was lysed in worm DNA isolation buffer (0.1 M Tris, pH 8.5, 0.1 M NaCl, 0.05 M EDTA, 1% SDS, 0.1 mg/ml proteinase K). The worms that incorporated the STOP-IN cassette were identified by a primer that bound to the cassette and another primer that bound to the *smrd-1* gene at ~400 bp from the cassette. The positive strain was homozygous and a PCR product of the region containing the cassette was purified (NucleoSpin PCR Clean-up Kit, Macherey-Nagel) and sequenced to corroborate the presence of the expected sequence. Non-

rolling offspring of the F2 animals were used to establish the stocks analyzed in this thesis. Animals were outcrossed and backcrossed to the GFP containing *qC1* or *hT2* balancers to get stable heterozygous stocks. These stocks were maintained by picking green offspring at each generation. Experiments at different temperatures equilibrated the balanced stock at the different temperatures prior to use and then F1, non-green homozygous animals were picked.

2.1.3 Generation of *smrd-1::3xHA*

CRISPR guides near the stop codon were selected using the CRISPR design tool at http://crispr. mit.edu (see Table 2 for sequences of the primers used in single guide RNA design). The repair template was amplified from the vector 3xHA pUCIDT- Kan Golden Gate which contains the 3xHA tag sequence. The injection control and content of the injection mix were as discussed for the generation of the *smrd-1* null. The worms that incorporated the tag were genotyped using a primer that bound to the 3xHA sequence and another that bound to the *smrd-1* sequence. The positive strain was homozygous and a PCR product of the region containing the cassette was purified (NucleoSpin PCR Clean-up Kit, Macherey-Nagel) and sequenced to corroborate the presence of the expected sequence.

2.1.4 snoRNA detection

To determine whether the insertion of the STOP-IN cassette into the *smrd-1* gene did not affect the transcription of the intronic snoRNA H04D03.5, a PCR-based method to detect small

RNAs was performed as described by Ro *et. al*. with minor alterations (Ro et al., 2006). Briefly, 1 mg of total RNA was polyadenylated with E. coli poly(A) polymerase (NEB,M0276S) and the product purified by acid-phenol:chloroform. 4 mg of polyadenylated RNA was retrotranscribed with SuperScript II using the RTQ primer followed by treatment with RNAse H (NEB, M0297S). The retrotranscribed product was purified (NucleoSpin PCR Clean-up Kit, Macherey-Nagel) and used for snoRNA detection using the reverse universal primer RTQ-UNIr and a forward primer specific for the snoRNAs H04D03.5 and K07C5.11 (used as positive control). See Table 2 for primers.

2.1.5 Brood analyses

L4 hermaphrodites of a given genotype were individually plated and transferred to a new plate every 24 hr until egg-laying ceased. Three to four days later, each plate was scored for the number of hermaphrodites and males. The procedure was followed for multiple generations. Data from each individual parent was combined to give the total adult brood and total males for each generation. To calculate male frequency, the total number of males in each generation was divided by the total number of adults. Percentage of fecundity was calculated only by considering the fertile worms and dividing the average brood size of the mutant over the average brood size of *wt*, multiplied by 100. The percentage of sterile worms in each generation was calculated by dividing the total number of sterile worms over the total number of analyzed worms, multiplied by 100.

2.1.6 Characterization of sterile worms

Morphology of the gonad and the number of chromosome bivalents (DAPI bodies) were analyzed under a Leica DM6 B microscope. *smrd-1* mutants were analyzed in the generation before becoming 100% sterile (F7).

2.1.7 Embryonic viability

F2 L4 hermaphrodites of a given genotype were individually plated and transferred to a new plate every 12 hr until egg-laying ceased. After transfer, the number of eggs and L1's on the plate was counted and recorded. Three to four days later, each plate was scored for the number of adult hermaphrodites. The percentage of viability was calculated by dividing total adults by total eggs and multiplying by 100. For the study of the rescue of embryonic viability by the male contribution of SMRD-1, F2 L4 *smrd-1(ea92)* hermaphrodites were crossed with *N2(wt)* males. Their progeny was individually plated and 12 h later the eggs laid were counted. . Three to four days later, each plate was scored for the number of adult hermaphrodites. The percentage of viability was calculated as previously mentioned.

2.1.8 Genotoxin sensitivity assays

Camptothecin (CPT) (C9911, Sigma-Aldrich) exposure was performed as described with minor alterations (Kessler and Yanowitz, 2014). Briefly, young adult hermaphrodites were

incubated in 0, 250, 500, and 1000 nM CPT dissolved in 1x M9, pH 6.0, buffer and 0.2% DMSO for 18 hr at room temperature with mild agitation. Following exposure, worms were washed, transferred to plates, and allowed to recover for 3 hr. Post recovery, worms were plated (five worms per 3-cm dish) and allowed to lay for 4 hr before removal and egg counts. Viable offspring were counts 3-4 days later. Percentage survival was calculated as the number of adult progeny divided by the number of eggs/L1's multiplied by 100. For each condition, 80 adults were used over two trials.

Hydroxyurea (HU) (H8627, Sigma-Aldrich) plates were prepared as follows. 6-cm dishes with 10 mL NGM were previously seeded with live OP50 and left at RT for 3 days. 250µL of the HU dilutions were added to have a final concentration of 0, 8, 12, and 25 mM, and let soak ON at RT. Young adult hermaphrodites were plated and incubated for 20 hr at room temperature. Following exposure, worms were transferred to drug-free NGM and live OP50 plates and allowed to recover for 3 hr. Post recovery, worms were plated (five worms per 3-cm dish) and allowed to lay for 4 hr before removal and egg count. Viable offspring were counted 3-4 days later. Percentage survival was calculated for CPT treatment.

For IR treatment, day one adults were plated on each of four 6-cm plates with 100 worms per plate and exposed to 0, 10, 50, or 100 Gy of IR from a 137Cs source (Gammacell1000 Elite, Nordion International Inc.). Twelve hours post-irradiation, worms were plated (three worms per 3-cm dish) and allowed to lay for 12 hr before removal and egg counts. Adult offspring and calculations are described as above previously mentioned.

Treatment of L1 hermaphrodites with CPT, HU and IR was conducted using the maximum dose for the treatment of young adults. L1s were treated overnight with CPT and HU at 25ºC or irradiated and processed immediately for IR. After treatment, for these two drugs, worms were put on drug-free NGM with live OP50 plates. Plates were incubated at 25ºC until adulthood. Adults were DAPI stained to assess germline morphology.

For the studies of SMRD-1::3xHA localization, day one adults were treated with the maximum doses used for HU and CPT treatments and incubated for 20 and 18 h, respectively. After treatment, worms were dissected and immunostained as described below with primary antibody rabbit α-HA (Sigma-Aldrich,H6008).

2.1.9 Immunofluorescence

Day 1 adult hermaphrodites were dissected in M9/levamisole and fixed in 0.5% triton/1% paraformaldehyde for 5 min in a humid chamber. Slides were freeze-cracked and immersed in methanol for 2 min followed by a brief immersion in acetone. Following fixation, slides were washed with PBST (phosphate-buffered saline with Tween20) and incubated in primary antibody (α-RAD-51, kindly provided by Verena Jantsch, 1:2,000; α-XND-1 (Wagner et al., 2010), 1:2,000) overnight at 4ºC. Next day, slides were washed in PBST and incubated in secondary antibody (α-rabbit Alexa 568, 1:2,000; α-guinea pig Alexa 633, 1:2,000, Molecular Probes) for 2 hr at room temperature in the dark. After the final wash with PBST, slides were mounted in Prolong Gold with DAPI (Life Technologies) and imaged on a Nikon A1r or Leica Stellaris confocal microscope. 2D images were taken using Volocity 3D imaging software (PerkinElmer). RAD-51

foci were quantified by individually scoring foci in each nucleus. RAD-51 counts were confirmed by examining 3D renderings of individual nuclei. Graphs represent the averages of three germ lines for each genotype. For HA-tagged SMRD-1, the procedure was the same as for RAD-51, with the exception of immersion in ethanol for 2 min and using as primary antibody rabbit α-HA (Sigma-Aldrich,H6008), 1:1,000.

2.1.10 Mutation frequency

Mutation frequency of *smrd-1(ea92)* was assessed as described previously (10.1534/genetics.106.058701). Briefly, *smrd-1(ea92);unc-58(e665)* and *unc-58(e665)* homozygotes were grown on 40 6-cm plates until starvation and then transferred to 40 10-cm plates containing a streak of OP50 opposite to the agar. Plates were incubated for 4 days and scored for the presence of unc revertants that could reach the OP50. Mutation frequency was calculated as described (Harris et al., 2006). The mutation frequency of *smrd-1(ea92)* in the *dog-1* background was assessed as previously described (Youds et al., 2006). Briefly, *dog-1(gk10)* hermaphrodites were crossed with *smrd-1(ea92)* males and 10 worms from the progeny were individually plated and genotyped for *dog-1(gk10)* and *smrd-1(ea92)* to confirm the heterozygosity for both genes. From one plate started from heterozygous hermaphrodites that were let to self-cross, 60 worms were individually plated and genotyped for both genes. Since none of the plates had homozygous *dog-1*(gk10), 16 worms from those plates that were started from hermaphrodites *dog-1(gk10)/+;smrd-1(ea92)* and *dog-1(gk10)/+;+* were individually plated and confirm homozygosity for both genes. Double mutants were created and used immediately to prevent the accumulation of mutations in the stocks. Freshly outcrossed *dog-1(gk10)* was used as control.

Briefly, 100 F3 *dog-1(gk10), smrd-1(ea92), wt* and *dog-1(gk10);smrd-1(ea92)* day 1 adults grown at 25ºC were individually lysed in buffer for DNA isolation. The poly G/C tract of *vab-1* was amplified by PCR (primers and conditions described in *Youds et. al.* (Youds et al., 2006)) and resolved on a 2% agarose gel. The presence of one or more bands below the expected product size signified a deletion event.

2.1.11 Cell cycle analysis by EdU labeling

F3 *N2* and *smrd-1(ea92)* day one adults grown at 25ºC were washed in buffer M9 and added to 25 µL M9 in one well of a 96 well plate. 25 µL of EdU were added and mixed by pipetting to be then incubated for 15 min at RT covering the plate with aluminum foil. Worms were washed with M9 and the gonads were dissected. Then, the gonads were put into a 5 mL pyrex tube by diluting in 2 mL 3% paraformaldehyde and incubated for 15 min at RT. After washing 3x with PBST, they were incubated overnight in 2 mL 100% MeOH at -20ºC. Gonads were washed 3x with PBST and 100 µL of EdU processing solution were added to proceed with the incubation for 1h at RT covered with aluminum foil. Finally, samples were washed 3x PBST and ~25 µL DAPI were. The mix was pipette into a 2.5% agarose pad and put on a coverslip. The slides were left to settle overnight at RT.

Table 2: Primers, gRNAs and repair templates sequences used in this thesis

Primer/gRNA/Repair template	Sequence	Description
wm-gRNA1-M03	TGCAACACGCACCCGTTCTC CGG	*gRNA1 for STOP-IN cassette in M03C11.8*
wm-gRNA2-M03	AGCCGATATGGACGCCAAAA AGG	*gRNA2 for STOP-IN cassette in M03C11.8*
wm-RT-M03	atcccaaattctaggatcaATGTCAACGACGAGCGATTTTCAAACCG GAGGGGAAGTTTGTCCAGAGCAGAGGTGACTAAGTGATAAG CTAGCAAAAGGCGCTTTTGTCTCAAAATCTGgtaaattttccgaatttta tagcct	*Repair template STOP-IN cassette in M03C11.8*
Wm-14	GTGGCGGCATAGGATTTATTCG	*STOP-IN cassette in M03C11.8 genotyping (fwd)*
Wm-15	GTCAAGTGGCCGTTATTCTGC	*STOP-IN cassette in M03C11.8 genotyping (rev)*
VV-189	CTTCTTCATCTTCACTAGGCATCG	*Primer to use with VV-190 (fwd)*
VV-190	gaggtgactaagtgataagctagc	*STOP-IN cassette internal primer (rev)*
VV-195	GACGGAGCTTTACGCTTTC	*Primer for M03C11.8 null sequencing (fwd)*
VV-196	cttgcggtgtttgcgtac	*Primer for M03C11.8 null sequencing (rev)*
VV-249	cgattttgtccccgcttttc	*Primer repair template for m03c11.8::3xHA (rev)*
VV-250	CCTGTCGAAAAAGAGCAGC	*Primer repair template for m03c11.8::3xHA (fwd)*
VV-233	GCCCCCATTATCTTTCCATC	*Primer snoRNA H04D03.5 (fwd)*
VV-247	CCTCGCTTATAAGCCGG	*Primer snoRNA K07C5.11 (fwd)*
gRNA1-M03_Sclt_C-term	cTatttttgggtgaaaatgaacg	*gRNA1 for 3xHA tag in M03C11.8*
gRNA2-M03_Sclt_C-term	agtgaTTAtGCgGAaGTgGA	*gRNA2 for 3xHA tag in M03C11.8*
Repair template for *M03C11.8::3xHA*	CGATTTTGTCCCCGCTTTTCCGTGAATTTATGAGGATTTTGACTATTTTTGGGT GAAAATGAACGGGAAAAAAGTGATTAAGCGTAATCTGGAACATCGTATGGG TATCCCGCATAGTCTGGGACATCGTATGGATATCCTGCGTAGTCAGGCACAT CATAAGGATATCCGGCTCCTCCGGCTCCTGCGGAAGTGGAAGGTTGAGATT CTTCCTCTTTTTGCTGCTCTTTTTCGACAGG	*Repair template of M03C11.8::3xHA (amplified with VV-249 and VV-250 from plasmid 3xHA pUCIDT-Kan Golden Gate)*
VV-249	cgattttgtccccgctttttc	*M03C11.8::3xHA tag amplification (rev)*
VV-250	CCTGTCGAAAAAGAGCAGC	*M03C11.8::3xHA tag amplification (fwd)*
VV-251	GCGTAATCTGGAACATCGTATG	*M03C11.8::3xHA tag genotyping internal primer*
TBM-332	TGTATTCTTTCCCTGCCT	*helq-1(tm2134) wt/mutant*
TBM-333	TCTTCCACCATCTCCAATC	*helq-1(tm2134) wt*
TBM-334	ACGTATTTAGCAGACCCA	*helq-1(tm2134) mutant*
BES-6	GTTCGAAGTCGACTGGAACTGTTGTG	*pot-2(tm1400)*
JLY-964	CTCGTTCGACCGTCGTATTGAAGC	*pot-2(tm1400)*
ZK-242	CAATTTGAAGCACTTCTTCAAAAGTG	*mre-11(iow1)*
ZK-251	CTATCATCAAAATTCTCGTTGCCGT	*mre-11(iow1)*
ZK-252	CCTAGACGCGTACTAGTCGACTGACATCATCAAAATTCTCGT TGCTAC	*mre-11(iow1)*
ZK-216	GGTCGCAATCGATGTGAGTTGC	*exo-1(tm1842)*
ZK-217	GCAGCTGTGCTCAACCCGAC	*exo-1(tm1842)*
ZK-99	CACAGAGTACCCGATGCGAGGACTATA	*dog-1(gk10) wt/mutant*
LR-199	GAAGAGCCACCGGAAGAAGAGG	*dog-1(gk10) mutant*
LR-202	GACGCGTTCAAGCCAAGCTC	*dog-1(gk10) wt*
Sws-1(NM-082)	AGCGGGAATTTGAAGATG	*sws-1(ea12)*
Sws-1(NM-083)	AGCTGGAAACTCTGAAAC	*sws-1(ea12)*

2.2 RESULTS

The first observations regarding SMARCAD1 function in mice came from the generation of knock-out animals whose main reported phenotype involved an impaired fertility and the generation of litters that exhibited a retarded growth and decreased post-natal viability (Schoor et al., 1999). Years later, the human ortholog was again related to reproductive functions by the studies performed by *Bansal et. al*. (Bansal et al., 2015), in which they show that the group of males with asthenozoospermia exhibited a down-regulation of SMARCAD1 transcripts in sperm samples. Based on these observations, we hypothesize that the *C. elegans'* ortholog of SMARCAD1 is involved in fertility.

2.2.1 *smrd-1* mutant worms have a transgenerational decrease in fertility

The *C. elegans* ortholog of *smarcad1* is predicted by sequence homology to be the gene *m03c11.8* located in chromosome 3 (Figure 8A), that from now on will be referred to as *smrd-1*. Its sequence is on the complementary strand and consists of 9 exons and 8 introns. Between exons 6 and 7, on the leading strand, is the sequence corresponding to the snoRNA H04D03.5. The only information regarding the snoRNA functions comes from RNAseq studies that claim its expression is influenced by the P granule components encoded by *pgl-1 (no human homolog)* and *glh-1 (Ddx4)* and the checkpoint protein encoded by *cep-1 (p53)*, according to Wormbase (www.wormbase.org). The presence of this snoRNA, thus, prevented the construction of an *smrd-1* null strain by deletion of the whole gene. Thus, the strategy to knock out this gene consisted of the incorporation of a 43 bp STOP-IN cassette by CRISPR-Cas9 at the beginning of exon 1 (Figure

8B). This cassette contains three STOP codons in three different frames, that aims to prevent the transcription of the gene. As a consequence, most of the sequence of the gene remains unaltered.

The homozygous null worms were viable at 20ºC and the newly generated allele was named *ea92*. However, from the first generations, few worms in the population presented a white-clear phenotype, that appears as a clear region around the vulva and is related to the absence of a gonad, and consequently sterility. Before proceeding with the experiments, *smrd-1(ea92)* worms were balanced with the genetic balancer *hT2* which contains a GFP marker so the balanced heterozygotes and ea92 homozygous mutant animals could easily be distinguished. In this way, the newly generated null strain will be maintained as heterozygous preventing deleterious effects of homozygosity to accumulate in the strain.

In order to confirm that the incorporation of the STOP-IN cassette did not alter the transcription of the snoRNA located between exons 6 and 7, we determined the presence of the snoRNA by PCR in homozygous null worm lysates. As a control, randomly selected snoRNA of similar size (127 bp), K07C5.11, located in chromosome V. Again, like our snoRNA of interest, there is the same information available regarding its function. As shown in Figure 8C, both snoRNAs are shown to be present in the null worms.

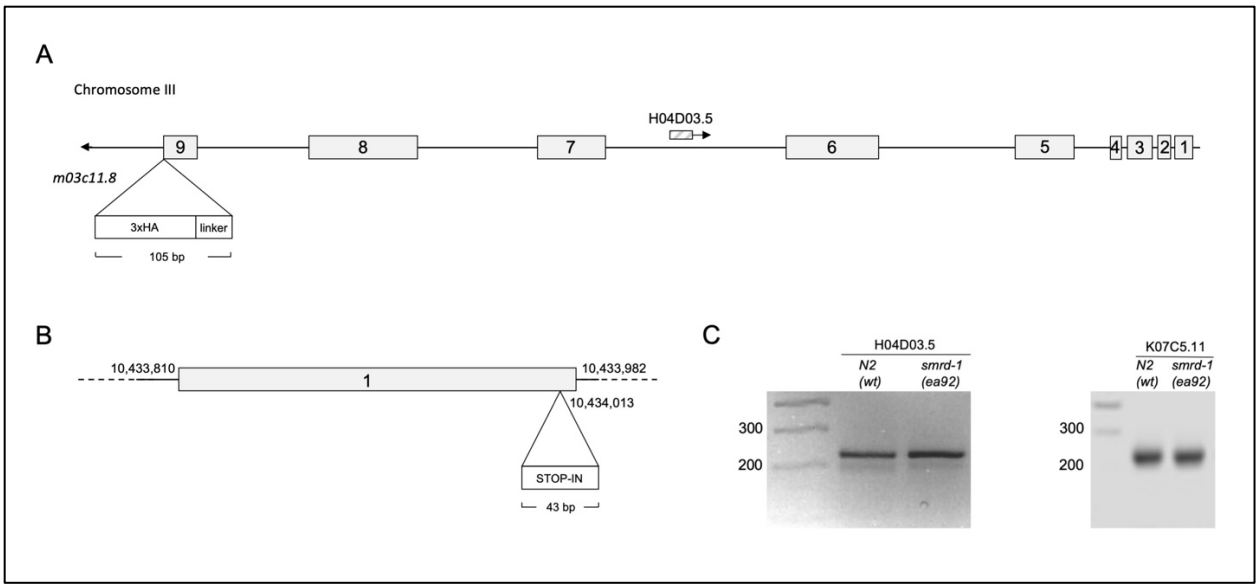


Figure 8: *smrd-1* null worms have an early stop codon incorporated in the form of a STOP-IN cassette. A- Diagram of *smrd-1* coding sequence. Shaded and numbered boxes represent the exons and straight lines the introns. The coding sequence of the snoRNA H04D03.5 that is contained in the intron between exons 6 and 7, is depicted as a striped box. The C-terminal 3xHA tag and linker added to smrd-1 for localization studies, are represented as a box on the 3' end of the coding region. This tag was used only in the full length *smrd-1*. B- Representation of the intron 1 to show the location of the insertion of the STOP-IN cassette, which corresponds to the 10,434,013bp of chromosome III. C- Results of the PCR-based method to detect the presence of the snoRNA H04D03.5 in the *ea92* mutant. Detection of the snoRNA K07C5.11 was used as a positive control.

Detection of the white clear phenotype associated with worm sterility in homozygous mutants, prompted us to start addressing the role of *smrd-1* in fertility across generations (Figure 9A). We started by assessing the brood size for multiple generations at 20ºC in order to determine if we were in the presence of a maternal-effect sterile gene. However, although we observed changes in the brood sizes, the mutant could be grown past F40 with no cases of sterility. For example, the first three generations at 20ºC (Figure 9B), showed a significant reduction in all generations compared to the control (F1, F2 and F3 $p<0.0001$, χ^2). We then asked whether in order

to increase the penetrance of the phenotype associated with this mutant we had to grow it under stress conditions. The difference in brood sizes became even more significant when growing them at 25ºC (Figure 9C) (F1, F2 and F3 $p<0.0001$, χ^2), a temperature that represents a stress condition. Under this condition, we notice two main differences. First, *smrd-1(ea92)* brood sizes significantly decrease from F1 to F3. Second, there is a transgenerational increase in the number of sterile worms, indicating this gene has a mortal germline (Mrt) phenotype when grown at 25ºC. Thus, we then proceeded to address the causes of the transgenerational decrease in brood sizes and increase in sterility.

In order to determine whether the decreased brood size is related to a decrease in fertility, we determined the fertility only of those worms that were fertile (Figure 9A and 9D). Again, we see a transgenerational decrease in the percentage of the fecundity of fertile worms.

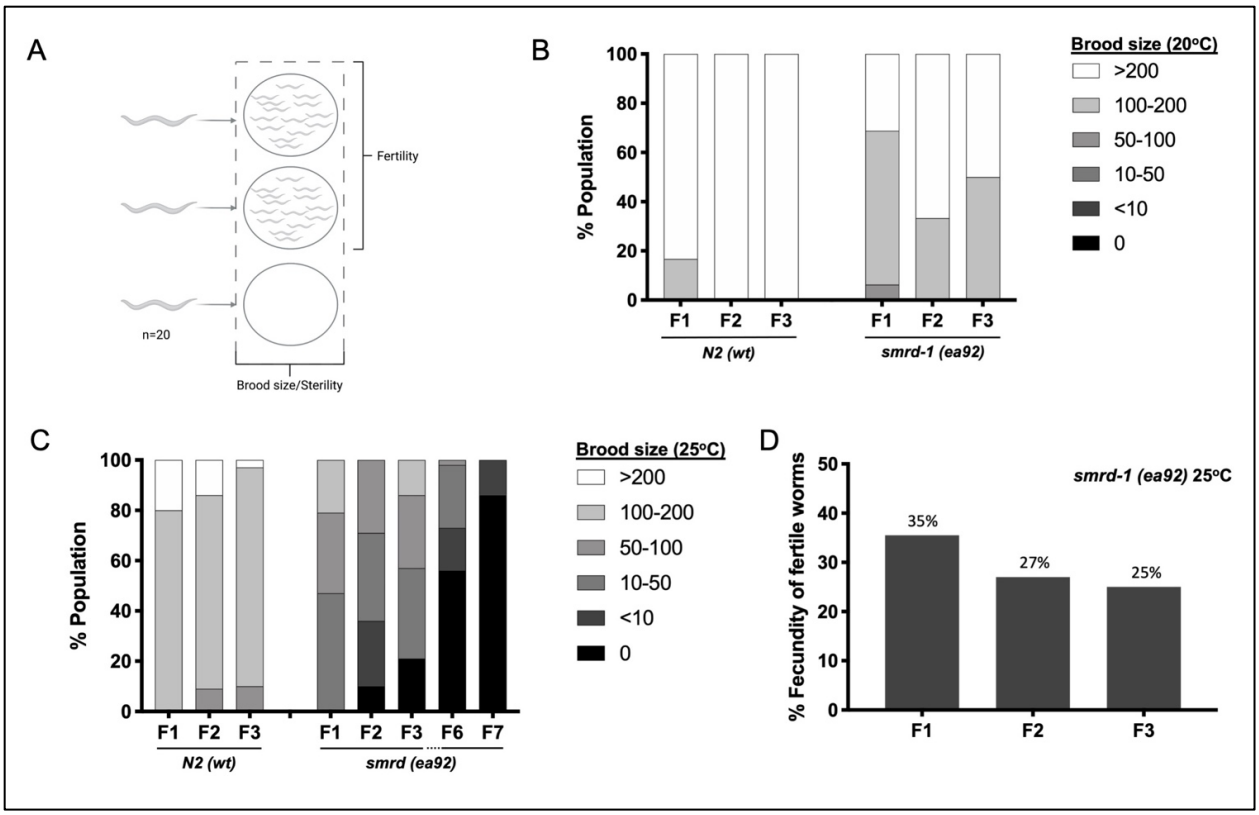


Figure 9: *smrd-1* null worms have decreased fertility that is exacerbated by stress conditions. A- Diagram showing which plates of the progeny are considered for the calculation of brood size (all plates), sterility (all plates) and fertility (only plates with progeny). B- Graph of the brood sizes of F1 to F3 of *wt* and mutant worms grown at 20ºC. The number of population in each case is divided in ranges that are represented in different shades of black. For *N2(wt)* n=6 each (F1,F2 and F3). For *smrd-1* n=16(F1), n=15 each (F2 and F3). Brood size distributions of *smrd-1(ea92)* were significantly different compared to their *N2(wt)* counterpart (****p<0.0001, χ^2) C- Analog to B but growing the worms at 25ºC. *smrd-1* worms were grown beyond F3 until the onset of sterility. For *N2(wt)* n=40(F1), n=44(F2), n=39(F3). For *smrd-1* n=34(F1), n=22(F2), n=20, n=6(F6), n=6(F7). Brood size distributions of *smrd-1(ea92)* F1 to F3 were significantly different compared to their *N2(wt)* counterpart (****p<0.0001, χ^2) D- Percentage of fecundity calculated by considering only the plates with progeny for each generation of worms described in C. n=34(F1), n=22(F2), n=20.

2.2.2 *smrd-1* is required for germline development

The total number of viable progeny can also be influenced by effects on development. If this were the case we would observe that not all of the eggs hatch or some of the animals arrest as young larvae. Thus the final, viable brood size would be decreased. In order to evaluate the embryonic viability of the mutant, we started with the heterozygous, balanced worms, plated homozygous progeny (F1) until we had F2 adults (Figure 10A). Those adults were then plated individually to lay eggs, and the percentage of embryonic and larval viability was determined by counting the number of adult F3 worms (see methods). As shown in Figure 10B, F3 mutant eggs have a significantly decreased embryonic viability ($p<0.0001$, t-test unpaired). Not surprisingly, we also noticed that fertile F2 mutant worms also laid fewer eggs compared to *wild-type* (*wt)* worms ($p<0.001$, t-test unpaired), contributing to the decreased brood size (Figure 10C).

There are two explanations for reduced embryonic viability. One possibility is that the oocytes produced by the *smrd-1* mutant are defective and cannot support development of the embryos; the other is that *smrd-1* is required for the embryonic development. In order to distinguish which one of these is contributing to the reduced embryonic viability, we reasoned that we could outcross the mutant *smrd-1* mothers with wild-type males. If the eggs are defective, embryonic lethality would still occur. If *smrd-1* is required zygotically for development, then the paternal contribution of *wt smrd-1* will provide this function and should rescue the lethality. Therefore, we crossed F2 mutant hermaphrodites with *wt* males and determined the embryonic viability of the progeny (Figure 10D). As shown in Figure 10E, the paternal contribution significantly improved embryonic viability ($p<0.0001$, t-test unpaired), implying that the main contributor to the reduced embryonic viability in the mutant is the absence of *smrd-1* for zygotic development.

In addition to the effects on embryogenesis, there was a striking number of worms with few to no eggs and these could not be rescued by outcrossing suggesting that the sterility is not due to defects in sperm production or sperm signaling in the *smrd-1* mutants. Therefore, we next quantified if the percentage of sterility increased in each subsequent generation. As shown in Figures 9C and 10F, we see an increase in sterility in the mutant populations. This increase continued until the whole population became sterile in F7.

To begin to address the underlying causes of the sterility, we began with an examination of the germ lines using whole-mount fixation and staining with the DNA intercalating agent, DAPI. One potential cause of sterility is defects in crossover formation during meiosis which can be observed by looking at the diakinesis oocytes. In wild type, 6 DAPI bodies, corresponding to the 6 bivalents are seen (Figure 10G). By contrast, when performing DAPI staining of the sterile F7 worms, we noticed several types of defects in the 6 bivalents at diakinesis (DAPI bodies) (Figure 10G), which when quantified show to be significantly increased in F7. In this case, an increase in the number of DAPI bodies is associated with chromosome cohesion defects, but there is also the presence of chromosome fisions (arrow, Figure 10G). Oocytes with less than 6 DAPI bodies indicate the presence of chromosome fusions, while the absence of DAPI bodies is indicative of the absence of mature oocytes even though the gonad is present (Figure 10H) ($p<0.0001$, χ^2). But the defects were not only present at the level of the chromosome bivalents but also at the level of the gonad morphology, like in the example shown in Figure 8I, where there is an absence of a whole gonad and the other gonad does not produce mature gametes and has an altered morphology. When quantifying these morphological defects by comparing F3 with F7, we

see a significant increase in these defects that were already starting to show at F3 (Figure 10J) ($p<0.0001$, χ^2). Overall, these results indicate that the increase in sterility in *smrd-1* mutants is related to defects in gonad morphology and oocyte maturation that could be a consequence of defects in gonad development during zygotic development and/or defects in primordial germ cells' development into meiosis, as well as truncated meiosis.

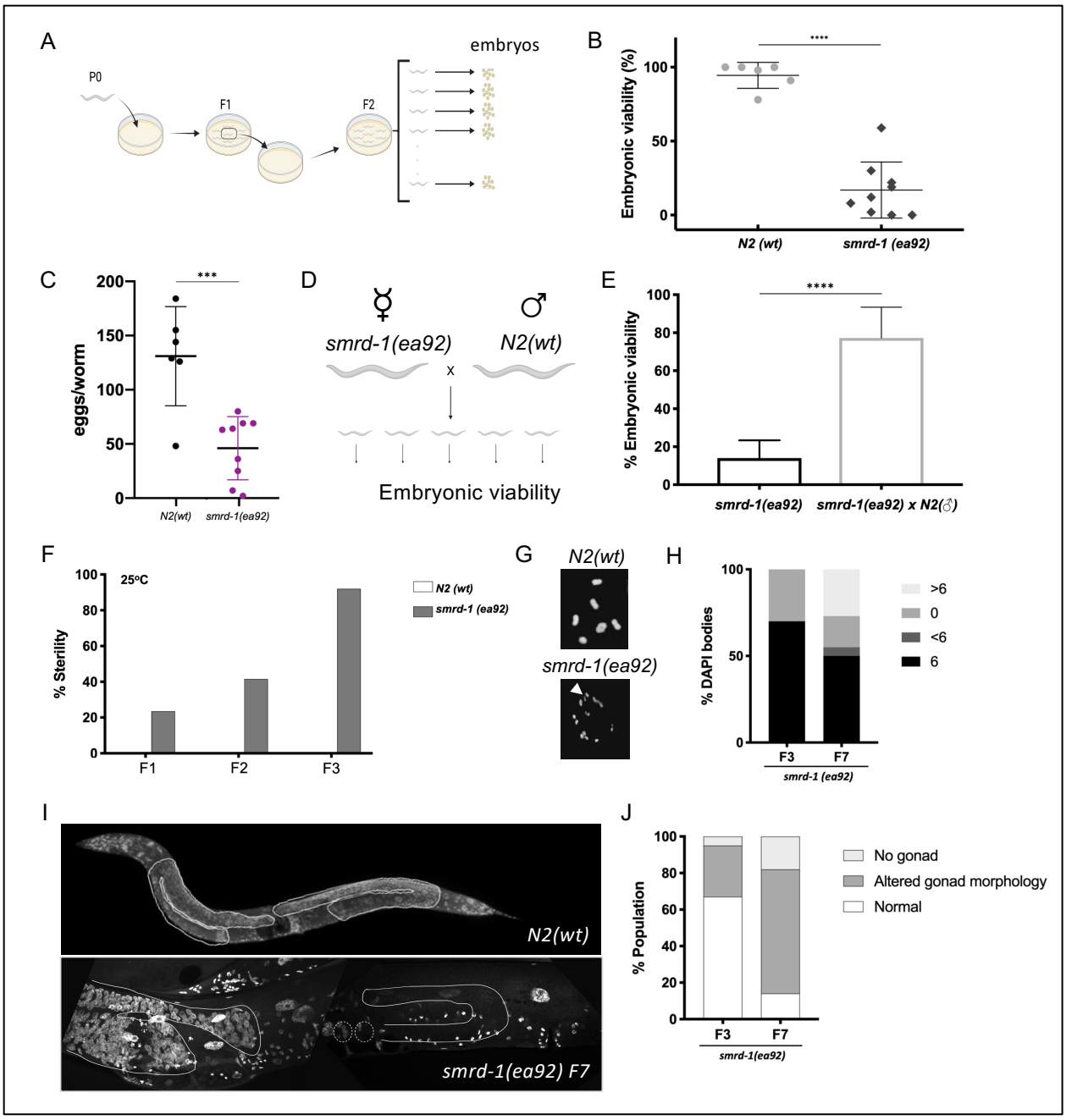


Figure 10: Reduced fertility in *smrd-1* mutant worms is consequence of a reduction in embryonic viability and increase in sterility. A- Diagram showing experimental procedure to quantify embryonic viability. One L4 worm was plated and let to have progeny (F1). From that progeny, one L4 was put on a new plate until having progeny (F2) from which 10 day one adult worms were individually plated and their eggs counted to determine 3-4 days later the percentage of those eggs that got to hatch and develop to adult worms. B- Graph of the percentage of embryonic viability of the embryos laid by F2 *wt* and mutant worms grown at 25ºC. For *N2(wt)* n=6. For *smrd-1* n=9.

****p<0.0001, t-test unpaired. C-Number of eggs laid per F2 *N2(wt)* and *smrd-1* worm grown at 25ºC and used on B for the calculation of embryonic viability. ***p<0.001, t-test unpaired. D- Diagram showing the experimental procedure to evaluate paternal rescue of embryonic viability. The progeny from the cross of F1 *smrd-1* hermaphrodites grown at 25ºC with *N2(wt)* males is individually plated and used for the calculation of embryonic viability as previously discussed. E- Graph of the percentage of embryonic viability of the embryos laid by F1 *wt* and mutant worms grown at 25ºC. For *smrd-1* n=9. For *smrd-1*x*N2(wt)* n=5. ****p<0.0001, t-test unpaired. F- Graph of the percentage of sterility of *wt* and mutant worms grown for three generations at 25ºC. n=174(F1), n=107(F2), n=48(F3). G- Representative image of the bivalents at diakinesis in F3 *N2(wt)* and *smrd-1* grown at 25ºC. Arrow indicates chromosome fisions. H- Quantification of the number of DAPI bodies (bivalent chromosomes in diakinesis) in F3 and F7 *smrd-1* worms grown at 25ºC showing a statistically significant increase in the number of defects. n=39(F3) and n=22(F7). ****p<0.0001, χ^2. I- Representative image of a *N2(wt)* gonad and one example of an F7 *smrd-1* gonad showing morphological defects. J- Quantification of the morphological defects observed in the gonads of F3 and F7 *smrd-1* worms grown at 25ºC, which show a significantly different pattern of defects. n=39(F3) and n=22(F7). ****p<0.0001, χ^2.

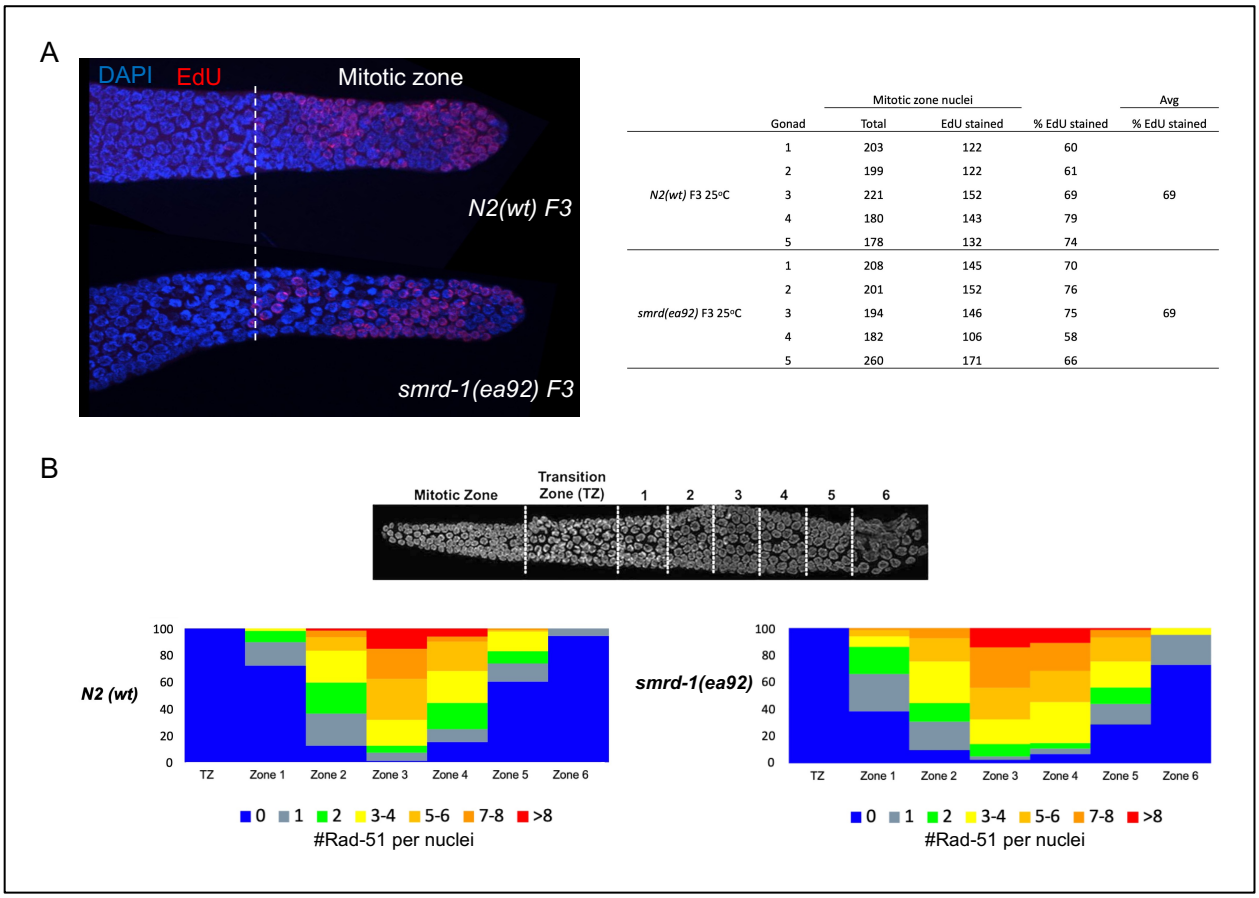
2.2.3 *smrd-1* is involved in the maintenance of genomic stability in germline's mitotic and meiotic regions

The decreased fertility of *smrd-1* mutant worms that showed to be related to the germline function and development, led us to hypothesize that the main cause behind the decreased fertility is due to the function of SMRD-1 in the maintenance of germline genome stability. The range of defects that we observed upon DAPI staining could arise from accumulation of DNA damage in the developing mitotic and meiotic germ cells or from epigenetic changes. In begin to address the nature of these defects in the *smrd-1* mutants, we focused on analyzing cell cycle progression and hallmarks of DNA damage. Taking into account that human and yeast orthologs of SMARCAD1

have been shown to be involved in replication, we studied address whether *smrd-1* loss affects the cell cycle by quantifying the S phase nuclei by EdU staining of mitotic zone nuclei. As shown in Figure 11A, the percentage of nuclei in S phase is the same in *smrd-1* as in *wt* worms, implying that the absence of *smrd-1* during reproductive growth does not induce changes in S phase progression. Therefore, we infer that the defective gonads do not arise from replicative catastrophe in mitotic cells.

Another way to study defects at the DNA level in the gonad is by analyzing the dynamics of RAD-51 foci. This is because unlike in human cell lines and yeast that detect DSBs by the immunostaining of phospho-H2AX (γ-H2AX), *C. elegans* does not have an ortholog for this histone. Thus, the way to detect the presence of DSBs is by the immunostaining of the nucleofilament protein RAD-51 (see section 1.1.1.1) (Lemmens and Tijsterman, 2011). In *wt*, we see the expected reduced number of RAD-51 foci in the mitotic region, while in the pachytene region the number of RAD-51 foci increases through early pachytene, and decreased at mid-late pachytene (Figure 11B). These RAD-51 dynamics are generated as a consequence of the activity of the protein SPO-11 which is responsible for the generation of DSBs that are required for the formation of crossovers between homolog chromosomes. By contrast, when we analyzed RAD-51 foci dynamics on *smrd-1* F3 mutants adults (day 1), we noticed a significant increase in the number of foci both in Zone 1 of the germ line ($p<0.0001$, χ^2), corresponding to leptotene/zygotene, and in Zones 4 - 6 ($p<0.0001$, χ^2), corresponding to mid-late pachytene. The excess RAD-51 foci in Zone 1 may be explained by damage that arose in the mitotic zone or during meiosis S phase that could not be repaired and was “carried through” to meiosis. Since, we already showed that S phase was unperturbed in the mitotic region, either damage arises during phases of

the cell cycle or *smrd*-1 mutants are defective in signaling to the checkpoint machinery to arrest cell cycle progression. We also observed increased RAD-51 foci in the mid-late pachytene region which is indicative of defects in DNA repair in the meiotic region. Thus, these results support the hypothesis that *smrd-1* is involved in the maintenance of genome stability in both, mitotic and meiotic regions of the germ line.



	Gonad	Mitotic zone nuclei: Total	Mitotic zone nuclei: EdU stained	% EdU stained	Avg % EdU stained
N2(wt) F3 25ºC	1	203	122	60	
	2	199	122	61	
	3	221	152	69	69
	4	180	143	79	
	5	178	132	74	
smrd(ea92) F3 25ºC	1	208	145	70	
	2	201	152	76	
	3	194	146	75	69
	4	182	106	58	
	5	260	171	66	

Figure 11: Maintenance of germline genome stability throughout the gonad relies on *smrd-1*. A- Representative image of EdU staining in the mitotic region of the gonad (left). Table of the number of mitotic nuclei and percent of EdU stained nuclei in each trial (right). n=5. B- RAD-51 foci dynamics for F3 *N2(wt)* and *smrd-1* day one adults worms grown at 25ºC. On the top of the image, a DAPI stained *wt* gonad used to indicate the localization of each pre-defined zone. Below each graph, the number of RAD-51 foci counted per-nuclei associated with each color. The RAD-51 foci distribution was significantly different in Zone 1 (****$p<0.0001$, χ^2) and Zone 4 to 6 (****$p<0.0001$, χ^2) .

2.2.4 *smrd-1* is required for the repair of DSBs induced by genotoxic agents in the gonad

Since we observed an increase in RAD-51 foci in the *smrd-1* germ line, we wanted to explore whether SMRD-1 is required for DNA repair in *C. elegans*. To do this, we turned to the treatment with genotoxic agents that induce different types of damage. As discussed in section 1.2, these agents help define whether a protein has a role in the repair of different DNA lesions. In this specific case, we want to examine the role of *smrd-1* in DNA damage repair in both the mitotic and meiotic regions (Figure 12A). Specifically, for meiotic region we will refer to the mid-pachytene stage, where the crossover between homolog chromosomes is formed and the synaptonemal complex (SC) aids in chromosome synapse (Figure 12A).

To determine whether *srmd-1* has an effect on the mitotic, germline stem cells, we treated L1 larvae with the different genotoxins. At this developmental stage, the germ line is comprised of somatic cells, Z1 and Z4 and two primordial germ cells, Z2 and Z3 (refer to section 1.2.2). Z2 and Z3 begin dividing in L1 in response to food. If there is unrepaired DNA damage in the developing germ cells, the cells arrest and the resultant gonad is devoid of germ cells. There can also be damage in the somatic blast cells at this stage leading to developmental arrest of the organism. As shown in Figure 12B (left) and in the summary table below, when considering the percentage of normally developed gonads after treatment, *smrd-1* mutants appear to be more sensitive to HU treatment, although there is also a slight sensitivity to CPT. However, the primordial germ cells show no sensitivity to IR treatment. Since the percentage values presented here correspond to one experiment, statistical analysis cannot be performed. However, they can give us an idea of the tendency to the different degrees of sensitivity to the different genotoxins, which is supported by the results shown in Figure 12C which will be discussed later.

The percentage of normally developed gonads after treatment, as shown in Figure 12B (bottom left), indicates that *smrd-1* mutant elicits unique responses to different genotoxins based on the different developmental outcomes of the germ line. The absence of gonad was more prevalent in HU treated L1s, and the absence of mature gametes was more prevalent in CPT-treated worms. In IR-treated worms, the L1 appeared slightly more resistant that *wild-type* and has more animals that develop with a germ line than controls. But, many of these animals had meiotic defects (#6: DAPI bodies equal less or more than 6). One possibility for the difference compared with *wild-type* would be that *smrd-1* loss allows for repair through mutagenic pathways that are otherwise unavailable in wild type. Taking into account that the HU drug affects the replication fork, the presence of more defects seen in the mitotic region when treating with this drug is expected, considering most of the cell cycle of the germline stem cells is spent in the S phase (Figure 12A). Sensitivities to CPT and IR will be addressed in more depth in section 2.3.

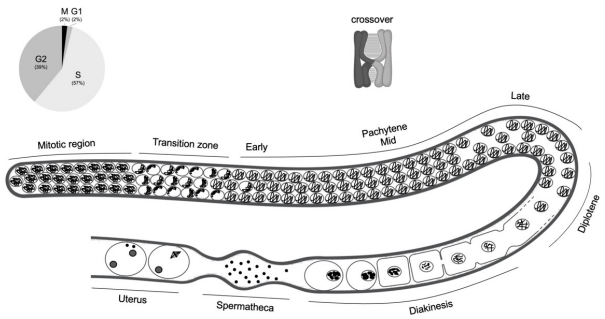
In order to determine if there is a role for *smrd-1* in meiotic repair, we focused on the response of pachytene nuclei to genotoxic treatment. This can be performed by treating adult worms and evaluating the embryonic viability ~24 h later. Based on our knowledge of *C. elegans* germ cell development and progression, we infer that the oocytes laid in this time window were going through zygotene and pachytene during the genotoxin exposure. Thus, if the damage cannot be repaired, and if the nuclei are not eliminated through apoptosis, then defective oocytes will be formed, fertilized, and produce non-viable embryos. As shown in Figure 12B (right) and table below, there is a marked sensitivity of *smrd-1* mutants to CPT (500 nM $p<0.01$ and 1000 nM $p<0.0001$, t-test unpaired) and to a less extent to HU (25 mM $p<0.01$, t-test unpaired). However,

there is no sensitivity of *smrd-1* to IR. These results are not unexpected if we take into account that in *the late* pachytene region of *C. elegans*, IR damage can be repaired by NHEJ and MMEJ (Macaisne et al., 2018). Overall, these results indicate that *smrd-1* is involved in the repair of CPT- and HU-induced DNA damage, in agreement with previous studies in other species. Moreover, it is then tempting to speculate that the DNA repair role involves the repair of DSBs induced by CPT and HU by HR, taking into account the involvement of *smrd-1* in this DNA repair pathway.

We then reasoned that if SMRD-1 is involved in the DNA damage response to HU and CPT in the mitotic and pachytene regions, then we should be able to visualize changes in its localization in response to damage. In particular, we anticipated that SMRD-1 may co-localized with the DNA, potentially in repair foci, such as observed with RPA and RAD-51 (Harrell et al., 2018). Our first attempt to make an N- or C-terminal tagged version of SMRD-1 using the fluorescent protein mScarlet was unsuccessful. Although in both cases the tag was successfully cloned by CRISPR, the protein could not be detected using live or fixation methods. However, its growth at 25ºC showed the same transgenerational sterility as our mutant obtained by the incorporation of the STOP-IN cassette, corroborating the phenotypes associated with the mutant are not influenced by the incorporation of the cassette sequence. Thus, we decided to incorporate a C-terminal 3xHA tag in the endogenous *wt smrd-1* gene using CRISPR-Cas9 (Figure 8A), confirmed that the tag does not confer mutant phenotypes, and examined those worms both before and after treatment with CPT and HU. We detected localization by immunostaining with anti-HA antibodies. As shown in Figure 10C, both in the mitotic or meiotic region of untreated animals, SMRD-1 has a nucleoplasmic localization. However, in the presence of genotoxins, although no foci are visualized, there is a clear migration of the protein to the periphery of the nuclear wall

where the chromatin is located. This increased concentration of SMRD-1 next to the chromatin is associated with an almost complete absence of protein in the center of the nuclei. We also noticed changes in the localization of the DNA in the mitotic region after treatment, showing a displacement towards the nuclear wall. This goes in hand with the fact that in the presence of DSBs, repair by HR requires the localization of the DNA in the inner surface of the nucleus membrane (Oza et al., 2009). This SMRD-1 location suggests an interaction with the DNA loops of the chromatin. Interestingly, the intensity of the immunostaining appears to be more marked in the periphery corresponding to the gonad location where their sensitivity was more prevalent, HU in the mitotic region, and CPT in pachytene.

A



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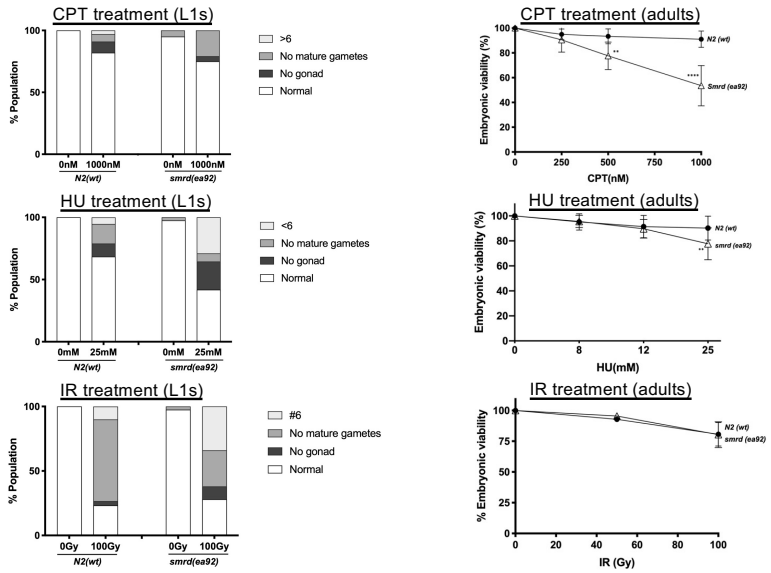


Normal gonad development (%)			
L1	*N2(wt)*	*smrd-1(ea92)*	Δ
CPT	82	79	-3
HU	68	43	-25
IR	23	29	+6

Embryonic viability (%)			
Adult	*N2(wt)*	*smrd-1(ea92)*	Δ
CPT	91	54	-37
HU	90	78	-12
IR	81	80	-1

C

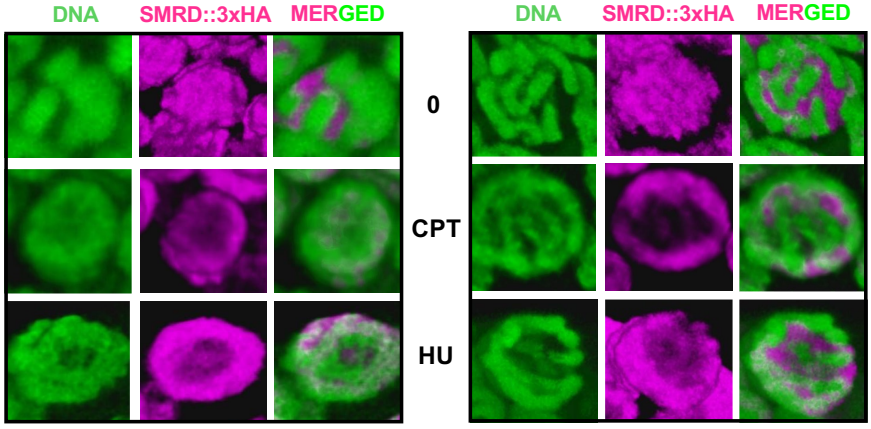


Figure 12: *smrd-1* null worms are differentially sensitive to genotoxic agents. A- Representation of an hermaphrodite gonad indicating the different nuclei stages of meiosis I. On top of the mitotic region, a pie chart indicates the average percentage of time mitotic cells spend on each cellular phase. On top of the mid-pachytene region, homolog chromosomes undergoing synapsis are shown, as an indicative of the main cellular event that is happening to the nuclei when they will be treated with the drugs. B- Graphs showing the percentage of population that has normal or defective gonads after treatment of L1 stage worms with CPT, HU or IR (left) (CPT treatment: n=33 for *N2(wt),* n=40-48 for *smrd-1.* HU treatment: n=19 for *N2(wt),* n=31-40 for *smrd-1*. IR treatment: n=30 for *N2(wt),* n=29 for *smrd-1*). For each treatment, the distribution of the type of damage generated in the mutant versus wt is significantly different (CPT treatment: *p<0.1, χ^2, HU and IR treatments: ****p<0.0001, χ^2). Since this experiment has an N=1, no statistical calculation can be performed as far as the percentage of population with normal gonads. On the right, graphs showing the percentage of embryonic viability after treatment of adult stage worms with CPT, HU or IR. (CPT treatment: n=12-15 for *N2(wt),* n=15-23 for *smrd-1*, **p<0.01, ****p<0.0001, t-test, unpaired. HU treatment: n=19-21 for *N2(wt),* n=12-18 for *smrd-1,* **p<0.01, t-test, unpaired. IR treatment: n=7-13 for *N2(wt),* n=15 for *smrd-1*). N=3. Below the graphs of L1 and adult treatment, the tables indicate for each genotoxin the percentage of normal gonads (for L1) or embryonic viability at maximal dose (for adults). C- Immunostaining of SMRD-1:3xHA in the mitotic and pachytene region of gonads before and after treatment with the maximal doses of CPT or HU.

2.2.5 *smrd-1* is part of the HR repair pathway in *C. elegans* germline

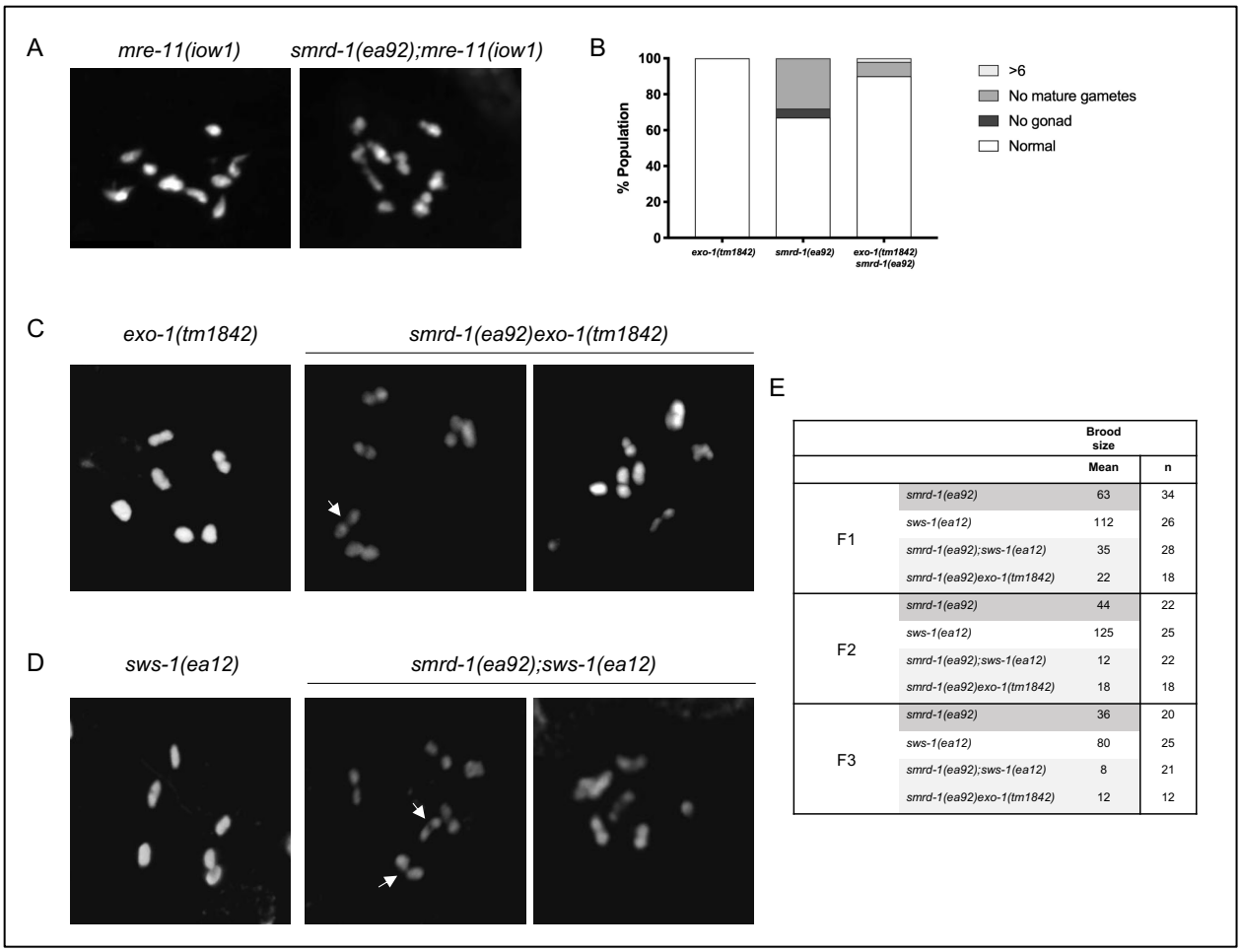
There is compelling evidence indicating that human and yeast SMARCAD1 is involved in HR by contributing to long-range resection, and based on the response of the mutant worms to the treatment of the genotoxins discussed before, we hypothesize that the role this protein has in germline genome stability involved its role in HR. Taking into account that this is the first study on the *C. elegans* SMARCAD1 ortholog, we wanted to determine if this function is conserved in this model organism. We, therefore, selected three genes that participate in different steps of the

HR pathway (refer to section 1.1.1.1, Figure 1). The first gene is the component of the MRN complex, MRE-11, which is the key protein responsible for short-range resection and formation of DSBs in meiosis. Thus, we used a separation-of-function allele that is specifically defective in short-range resection (Yin and Smolikove, 2013). Mutants carrying this allele, *mre-11(iow1)* present with chromosome fragmentation and aggregation at diakinesis, generated by the repair of the meiotic DSBs by end-joining instead of HR. The same phenotype is seen in the *smrd-1(ea92);mre-11(iow1)* double mutants (Figure 13A). These results are consistent with a function for *mre-11* upstream of *smrd-1,* and that in the absence of short-range resection, the repair is channeled to NHEJ.

We next analyzed *exo-1*, whose action in long-range resection is directly favored by the ortholog SMARCAD1 (Costelloe et al., 2012). In worms, single mutants of *exo-1* exhibit normal meiosis, have 6 bivalents at diakinesis (Lemmens et al., 2013) and are fully viable. In some mutant backgrounds, however, *exo-1* phenotypes become essential. In the *smrd-1exo-1* double mutant, the number of bivalents remains the same, but we do notice a slight spatial separation between the homologs which appear to be connected by chromosome bridges (Figure 13C, arrows). This phenotype has also been seen in mutants with defects in crossover resolution or cohesion (Ferrandiz et al., 2018; O'Neil et al., 2013). We also noticed worms that lack mature gametes and even have chromosome fragmentation (>6 DAPI bodies) (Figure 13B). However, these defects are less pronounced than what is observed in *smrd-1* single mutant raising the possibility that *exo-1* activity is required for the fragmentation seen in *smrd-1* mutants. However, the decreased brood size of the *smrd-1(ea92)exo-1(tm1842)* double mutant (Figure 13E) raises the alternative

possibility that the double mutant has a decreased survival rate and as a consequence, we are only seeing those double mutants that have higher fitness.

A similar result is obtained with the double mutant with *sws-1(ea12),* a component of the Shu complex that is involved in the stabilization of the nucleofilament of RAD-51 (Godin et al., 2016). Thus, this protein acts downstream to EXO-1, but still has a similar behavior as the *exo-1* double mutant. This means that two different proteins involved in different steps of the HR pathway have similar phenotypes when in *smrd-1(ea92)* doubles.



		Brood size	
		Mean	n
F1	*smrd-1(ea92)*	63	34
	sws-1(ea12)	112	26
	smrd-1(ea92);sws-1(ea12)	35	28
	smrd-1(ea92)exo-1(tm1842)	22	18
F2	*smrd-1(ea92)*	44	22
	sws-1(ea12)	125	25
	smrd-1(ea92);sws-1(ea12)	12	22
	smrd-1(ea92)exo-1(tm1842)	18	18
F3	*smrd-1(ea92)*	36	20
	sws-1(ea12)	80	25
	smrd-1(ea92);sws-1(ea12)	8	21
	smrd-1(ea92)exo-1(tm1842)	12	12

Figure 13: Genetic interaction of *smrd-1* with *mre-11, exo-1 and sws-1*, supports its involvement in HR pathway. A- Representative image of the bivalents at diakinesis in *mre-11* and *smrd-1;mre-11* mutants grown at 25ºC. B- Percentage of F3 *exo-1*, *smrd-1* and *exo-1smrd-1* worms grown at 25ºC with defects in gonad development, showing the double mutant a statistically significant distribution of defects (****$p<0.0001$, χ^2). n=39 for each genotype. C- Representative image of the bivalents at diakinesis in F3 *exo-1* and *smrd-1exo-1* mutants grown at 25ºC. Arrows indicate chromosome bridges. D- Analog to C, but for *sws-1* and *smrd-1;sws-1* mutants. E- Mean brood size of *smrd-1* double mutants with *exo-1* and *sws-1*.

2.2.6 *smrd-1* mutation is not a mutator

The sensitivity of *smrd-1* mutant worms to DNA damage-inducing agents and the transgenerational decrease in fertility can have different origins. Those can involve defects in replication through secondary structures, DNA repair mechanisms, telomere attrition, and defects in epigenetic marks, among others. In order to start characterizing the origin of the *smrd-1* mutant transgenerational phenotypes, we focused on replication and telomere attrition, since our previous results implicated *smrd-1* has sensitivity to genotoxins that affect replication and has altered phenotypes in double mutants of HR proteins (Figure 13).

We first started by analyzing the average brood size of *helq-1(tm2134) smrd-1(ea92),* double mutants for three generations at 25^{o}C (Table 3). *helq-1* worms have significantly increased brood size in all generations compared to the *smrd-1* single mutant, while the double mutant shows a decreased brood size across generations, with sizes that are similar to the *srmd-1* single mutant. The fact that *smrd-1* mutation does not show an additive effect with *helq-1* suggests that *smrd-1* is epistatic to *helq-1* and that these genes share a common pathway. These results are also supported by the lack of differences in the number of DAPI bodies from F1 to F3 (data not shown). Also, as mentioned in section 1.2.4.2, HELQ1 favors the exchange of RPA for RAD51 in ssDNA during HR. If *smrd-1* acts upstream of *helq-1* during HR or replicative repair, one possibility is that it may direct lesions into alternative (mutagenic) repair pathways that prevent the formation of the substrate upon which HELQ-1 acts.

Table 3: Mean brood size of *smrd-1* double mutants with genes involved in genomic stability. Single and double mutants were grown at 25ºC for three consecutive generations.

		Brood size	
		Mean	**n**
F1	*N2(wt)*	179	40
	smrd-1(ea92)	63	34
	helq-1(tm2134)	133	27
	helq-1(tm2134) smrd-1(ea92)	58	30
	pot-2(tm1400)	79	8
	pot-2(tm1400);smrd-1(ea92)	68	10
F2	*N2(wt)*	165	44
	smrd-1(ea92)	44	22
	helq-1(tm2134)	104	28
	helq-1(tm2134) smrd-1(ea92)	33	30
	pot-2(tm1400)	90	8
	pot-2(tm1400);smrd-1(ea92)	46	9
F3	*N2(wt)*	142	39
	smrd-1(ea92)	36	20
	helq-1(tm2134)	125	27
	helq-1(tm2134) smrd-1(ea92)	29	25
	pot-2(tm1400)	71	8
	pot-2(tm1400);smrd-1(ea92)	42	8

Similar results are obtained with the gene *pot-2*, which encodes a single-stranded telomere binding protein involved in the repression of telomerase (Cheng et al., 2012; Shtessel et al., 2013) (Table 3). This would indicate that *smrd-1* is involved in telomere maintenance. However, we did not see chromosome end fusions in diakinesis nuclei in *smrd*-1 mutants, which is a phenotype characteristic of telomere attrition. Thus, *smrd-1* may be sharing a pathway with *pot-2* alternative to telomere maintenance consistent with a role for *pot-2* in telomerase-mediated lengthening and

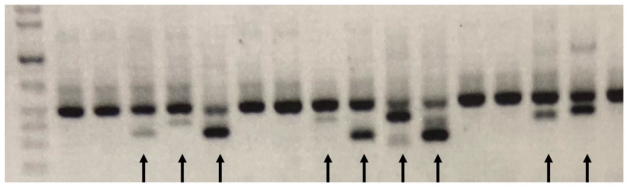
the ALT (alternative lengthening of telomeres) pathways in worms (Shtessel et al., 2013)(Cheng et al., 2012).

Since *smrd-1* is involved in genome stability and has a Mrt germline phenotype, we wanted to determine if it can be classified as a mutator gene. However, during its growth we never observed any phenotypically abnormal animals (i.e. no protruding vulvas (Pvl), uncoordinated (Unc), dumpy (Dpy) or roller (Rol) phenotypes) that are characteristic of mutation accumulation strains. Thus, we set out to determine if the mutation rate in *smrd-1* mutants is similar to *wild-type* controls using two assays to examine mutation frequencies: an Unc reversion assay that looks for intra- and extra-genic suppressors of *unc-58(e665)* point mutation (Section 1.2.4.3) as in previous studies in this laboratory (McClendon et al., 2016b); and the "*dog-1*" assay which looks at microsatellite repeat expansions and contractions (introduced in Section 1.2.4.4) previously mentioned in section 1.2.4. The Unc reversion assay showed no difference in mutation frequency in the *smrd-1(ea92); unc-58(e665)* double mutant compared to the *unc-58* mutant background alone (Table 4). This supports our finding that *smrd-1* loss does not lead to induction of spontaneous mutations throughout the genome. Consistent with these results, *smrd-1* also did not appear to be required for the maintenance of G/C tract stability in the absence of *dog-1* (Figure 14).

Table 4: Spontaneous revertant frequencies of *unc-58(e665)*. Reversion assay was carried out as described in section 2.1.10.

unc-58(e665) background	Trial	Plates per revertants/total plates
Wild type	1	1/40
	2	0/40
	3	0/40
smrd-1(ea92)	1	0/40
	2	0/40
	3	0/40

A



B

	n	# animals with deletions	%
dog-1	14	8	57
dog-1;smrd-1	82	47	57

Figure 14: *smrd-1* does not maintain G/C tract stability in the absence of *dog-1*. A-Amplification of the *vab-1* G/C tract in *dog-1;smrd-1*, showing deletions in the amplified region as fast-migrating bands on 2% agarose gel (arrows). B-Quantification of deletion frequency in *dog-1* and *dog-1;smrd-1* mutants.

2.3 DISCUSSION

The results presented in this thesis provide the first functional characterization of the *C. elegans* gene *m03c11.8*, confirming that this gene encodes the ortholog of the human *smarcad1* chromatin remodeler. Therefore, we have named this gene *smrd-1*.

smrd-1 null worms show a transgenerational decrease in *C. elegans* fertility, which is exacerbated by its growth under restrictive conditions (25ºC). The more penetrant phenotype is seen as a decrease in fertility and increased sterility that culminates with complete sterility of the line by generation 7 (F7). Thus, we can conclude that the absence of *smrd-1* produces a mortal germline (Mrt) phenotype. The decreased fertility is explained in part by reduced viability of the homozygous mutant embryos and in part by a decreased number of eggs laid. The former may be explained by a zygotic requirement for *smrd-1* function but might also be explained by epigenetic effects that are passed along from mother to embryo. Alternatively, it may reflect a defect in the hermaphrodite sperm. It remains to be addressed if *smrd-1* directly affects sperm quality or whether sperm defects contribute to the Mrt phenotype (see Appendix A.1 or further discussion on mutant sperm role).

Within the gonads of *smrd-1* deficient worms at the F3 generation, we see aberrations in gonad development, including defects in gonad morphology or even a complete absence of a gonad arm. The lack of a gonad arm suggests that the defects are arising in the differentiation and/or proliferation of the primordial germ cells (PGCs) Z2 and Z3. There may also be defects in the development or survival of the distal tip cell, which is a somatic cell that regulates germ cell proliferation (Kimble and White 1981). The impact of *smrd-1* does not appear to be directly on

the cell cycle, since we observed not difference in EdU staining in the mitotic region of the germ line and in agreement with results from human cell lines (Chakraborty et al. 2018), One possibility for the loss of germ cells, indicated by our results with DNA damaging agents, is that the initial proliferative germ cells committed to one germ line arm undergo replicative catastrophe and fail to divide, leading to a loss of this tissue. Further studies to analyze the early development of the PGCs and gonad in the *smrd-1* mutant will likely provide insights into these questions.

We also provide evidence that a subset of *smrd-1* oocytes are defective, with the appearance of chromosome fusions and fragments in some nuclei, defects in chromosome condensation in others, and separating bivalents in others. Each of these phenotypes may result from upstream defects in DNA replication and/or meiotic repair. We reported differences in the dynamics of RAD-51 in the gonad, indicating the presence of both carry-through damage from the mitotic region and persistent DNA damage from the mid-pachytene region into late pachytene/diplotene. Together, these results provide the first concrete evidence that *smrd-1* has an active role to promote germline function and, consequently, fertility.

While we see evidence of extra damage in the early meiotic germline (refer to Figure 11B), we cannot determine whether this damage arises from meiotic S phase or from the mitotic divisions themselves. Since we see no difference in cell cycle rate, either pre-meiotic damage may not activate cell cycle arrest in the *smrd-1* mutant (as recently observed for RNase H mutants in the worm (Smolikove, in press)). Strikingly, we observed that the number of RAD-51 foci at mid-pachytene does not differ between *smrd-1* and control. This implied that the extra RAD-51 seen in early meiotic nuclei either promote repair or are removed to allow repair through non-HR

pathways. However, *smrd-1* may also contribute directly to meiotic DSB formation and repair. If this were the case, the concomitant defect in meiotic break formation or processing would prevents excess accumulation of RAD-51. Distinguishing between these possibilities will likely shed important insights into SMRD-1 function in meiosis.

The increased damage that we observed in mid- to late- pachytene may explain the aberrant phenotypes of the diakinesis oocytes described above (and Figure 10G and H) and/or may lead to an increase in apoptosis. It is also possible that some unrepaired damage might be transferred into the zygote, as is known to happen on damaged sperm chromatin with maternal factors (Khokhlova et al. 2020).

Since our cytological studies of RAD-51 dynamics suggested that *smrd-1* has roles in both the mitotic and meiotic regions of the gonad, we focused our studies on genotoxin sensitivity on each region independently. These results support a role for SMRD-1 in HR. The sensitivity of *smrd-1* germ lines to HU in the L1 assay, when only mitotic germ cells are present, points to a role for SMRD-1 in either repair of ssDNA gaps or DSB breaks resulting from stalled and collapsed replication forks. We note that the sensitivity of *smrd-1* mutants to HU only occurs at higher doses, which is similar to what the authors saw in yeast (Bi et al. 2015), which suggests that the resultant damage may still be channeled to repair by the TLS pathway. The sensitivity to CPT in the L1 assay also indicates a role for *smrc-1* during replication since DNA-protein crosslinks serve as an impediment to DNA polymerase. Whether SMRD-1 is required to remove these Top1 linkages together with Spartan/TDP1 or whether it function in the repair of stalled/collapsed forks is an important area for future study. Top1 crosslinks are also a source of RNA:DNA hybrids formed

during the course of transcription (Promonet et al. 2020). Increased sensitivity to CPT may be suggest that there is increase in RNA:DNA hybrids (see below).

To analyze in more detail the causes of the *smrd-1* mutant sensitivity to CPT treatment at the mitotic region, I propose the following experiments: 1) To determine if the DSBs generated as consequence of the collision of the replication fork with TOP1ccs are being repaired by NHEJ instead of HR due to the lack of SMRD-1, we could determine if there is an increased level of the 53BP1 ortholog associated with DNA by immunofluorescence using a tagged version of this protein. The use of NHEJ over HR is always somewhat mutagenic since it is an error prone repair pathway. 2) Detection of increased levels of RNA:DNA hybrids can be done by immunohistochemistry using a structure-specific antibody (Smolka et al. 2021). 3) Finally, to determine if *smrd-1* contributes to DPC removal, the generation of double mutants and further analysis of CPT sensitivity will help us to answer this question. In particular, these studies should focus on double mutants of *smrd-1(ea92)* with *dvc-1* (encoding SPRT in humans), *tdpt-1* (encoding TDP1 in humans) and *ercc-1* (encoding the human repair protein ERCC1).

The lack of sensitivity of *smrd-1* mitotic germ cells to IR is particularly interesting since the main lesions induced by IR are DSBs (Borrego-Soto et al. 2015). This suggests either that another pathway, like NHEJ, may be taking the lead in repair of this damage in the mutant or that there is not a general requirement for *smrd-1* in HR-mediated repair.

The increased sensitivity of the meiotic region to CPT is more challenging to explain since DNA replication is not occurring at this time. Nevertheless, HR-mediated crossover repair is

thought to create a replication fork-like structure that might be sensitive to CPT. RNA:DNA hybrids could also be a problem in this region if the crossover repair intersects with the transcriptional unit. Therefore, I favor a model with which SMRD-1 works in DPC repair with proteases and endonucleases, like SPRT, TDP1, ERCC1. In fact, a recent publication on the yeast homolog Fun30, has shown that its role in long-range resection is important for the repair of CPT induced damage by TDP1 and Rad1-Rad10 (XPR-ERCC1 in humans) (Al-Natour et al. 2021). Thus, the generation of these double mutants and further evaluation of CPT sensitivity will be a highly informative experiment. However, it is also important to consider that Top1 is localized along the loops of the chromosomes (Pommier et al. 2022) and failure to repair these damage could led to defects in chromosome segregation (Pankratz and Forsburg 2005). This could be studied through the analysis of the chromatin in early embryos.

One of the most exciting results observations we made was the change in localization of SMRD-1::HA after HU and CPT treatment. These results support the involvement of this protein in DNA damage repair, because we see the loss of SMRD-1 from the nucleoplasm and its enrichment in the inner nuclear membrane region where the DNA is located. The lack of overlap with the DAPI signal suggests that SMRD-1 may be associated with the chromatin loops or that it is regulating structures at the nuclear envelope that are needed for repair. These results support prior data that showed the migration of the DNA to repair the damage to the inner nuclear membrane after DSBs (Lemaître et al. 2014). The fact that we did not see co-localization of SMRD-1::HA with DNA under un-treated conditions does not mean they do not interact, but could be a reflection of the sensitivity of the method used or that the bulk localization is preventing us from seeing a small fraction on the DNA itself.

The involvement of SMRD-1 in the repair of DSBs through HR was also supported by our results obtained with the double mutants of *mre-11*, *exo-1* and *sws-1*. We showed no interaction with *mre-11*, consistent with *Chen et. al.* who showed in yeast that in the absence of *mre-11*, SMRD-1 is not recruited to damage (Chen et al. 2012). However, our double mutants with *exo-1* and *sws-1* revealed defects, that can be seen as the more severe effects on transgenerational brood size. We also observed a new class of defects in diakinesis-stage chromosomes that could be an indicator of defects in cohesins or crossover resolution.

It would be tempting to favor defects in cohesions for the following reasons: First, it was previously shown that *smrd-1* does not act downstream *exo-1* (Eapen et al. 2012), so it may not be involved directly in HR resolution. Second, the double mutants have synergistic effects (defects in DAPI bodies and reduced brood size), which could imply that the role of *smrd-1* is not only involved in long-range resection. Similar observations were done in yeast, where they noticed the synergistic effect of *exo1*,*fun-30* double mutants, and they hypothesize that could involve the activation of a checkpoint pathway (Eapen et al. 2012), something that has not been proven. Interestingly, the observed phenotypes are similar for both *exo-1smrd-1* and *smrd-1;sws-1* doubles and is not seen in the *smrd-1* single mutant. Another explanation could be that *SMRD-1* is contributing to the stabilization, function, or location of EXO-1 and SWS-1, which in the absence would result in the destabilization of the chromatin loop structures.

smrd-1 null worms do not appear to result in the formation of spontaneous mutations, a phenotype known as a “mutator”. A major class of mutator genes are associated with transposition,

ruling out this as a cause of the *smrd-1* transgenerational sterility. However, considering that one of the causes of spontaneous mutations is the malfunctioning of the MMR pathway (Schär 2001) and that *smrd-1* in yeast is involved in MMR (Terui et al. 2018), this was an unexpected result. While we have not definitively proved that *C. elegans smrd-1* is not involved in MMR, the lack of a mutator phenotypes strongly favors this conclusion.

Thus, we have determined that the Mrt germline phenotype of *smrd-1* is not related to the accumulation of spontaneous mutations, transposition, or telomere attrition. Rather, we suggest that another mechanism involving *smrd-1*, possibility changes in epigenetic marks may play an important role. Mutations in the *lsd-1* histone demethylase also lead to a Mrt germ line seen by increased spermatogenic gene expression. The *mes-2*, *mes-3*, *mes-4,* and *mes-6* mutants of *C. elegans* lead to grandmaternal effect sterility caused by changes in H3K27 and H3K36 methylation that lead to inappropriate activation of the silent X chromosomes (Strome et al. 2014). This leads to empty gonads similar to what we see in a subset of our mutant *smrd-1* animals. Thus, a detailed analysis of both transcriptomic and epigenetic changes in the *smrd-1* animals will be an exciting area for future study.

Overall, the results presented here provide a new species for the study of SMARCAD1 function. This will allow the connection of the cellular functions of SMARCAD1 with the consequences on a whole organism. Additionally, this study supports the role of SMARCAD1 in fertility, which should lead to the incorporation of this gene into the panel of analyzed genes at the moment of diagnosis of infertility in the fertility clinics. Another consequence of the SMARCAD1 role in fertility is the further consideration of it as a potential late-life cancer promoter. This is

based on the recent connection made between the shared coexistence of factors between cancer and infertility that go beyond the strategies used during reproductive assistance (Cetin et al. 2008) and that affects women and men as well (Swerdlow et al. 2022).

2.4 FUTURE PERSPECTIVES

One of the key questions from this study is whether the quality of the sperm of *smrd-1* mutant males is leading to the decreased fertility and/or reduced zygotic viability. Preliminary results discussed in Appendix A.1, hint that the sperm is contributing to the mutant phenotypes described in this work. However, more studies to address this are needed. In particular, we need to definitely address whether mutant males are competent to fertilize, whether they transfer sperm, and whether this sperm is competent for support development. Since none of our crosses with mutant sperm produced offspring at 25°C, any of these possibilities are feasible.

In order to support our results on the *smrd-1* role in maintaining genome stability in the mitotic and meiotic regions of the germ line, we will study the RAD-51 dynamics of *smrd-1* mutants in a *spo-11* mutant background where no meiotic breaks are made. In this case, we would expect to visualize foci that are exclusively the result of processes impacted by the loss of *smrd-1* function. Related to this, I would suggest immunostaining the mitotic region of the *smrd-1* null with anti-phospho-Chk1 antibodies to corroborate that those cells are able to respond normally to DNA damage.

The genotoxin studies reported here open a wide range of studies that could be performed in order to further characterize the link between DNA damage response and *smrd-1*. For example, to increase the sensitivity to HU of the adult worms, we could perform the assay with *smrd-1* in a *rad-5* mutant background. As far as CPT is concerned, treating mitotic and meiotic regions of the mutant on a genetic background of the *C. elegans* homologs of *sprt, xpc, ercc1* and *ape1*, will help unravel the role the DPC and NER pathways may be having in the sensitivity to CPT. Finally,

regarding the lack of sensitivity to IR, we could evaluate it again but on a p53 mutant background, looking for the presence or increase in apoptotic cells.

It will be interesting to confirm the direct interaction of SMRD-1::HA with the DNA under normal growth conditions to validate its important role in the absence of exogenous DNA damage. Techniques such as ChIP could be performed to determine if there are particular regions enriched for SMRD-1 binding. Moreover, performing a co-immunoprecipitation of this tagged protein with the germline nuclei will contribute key information on its roles during meiosis. The immunostaining of *smrd-1* double mutants with *exo-1* and *sws-1,* with the antibody for the cohesion REC8 will be the first experiment to open a new line of research on the relationship between *smrd-1* and chromatin structure.

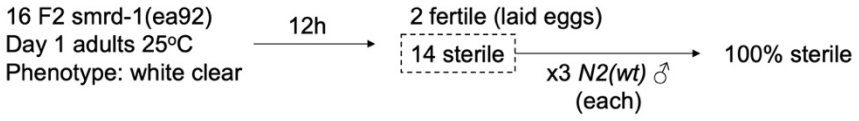
Lastly, the study of the effect of *smrd-1* on chromatin post-translational modifications that could be also involved in its role in reproduction could be explored in detail. Specifically, we would look at the changes on the H3K9me3 mark in late generations just prior to the onset of sterility. In these studies, we can also address whether the rescue of embryonic sterility after crossing mutant hermaphrodites with *wild-type* males involved epigenetic reprogramming in the progeny. In summary, this thesis has provided a novel genetic framework in which to ascertain conserved roles of SMARCAD1 in fertility.

Appendix A

Appendix A.1 Preliminary observations on the role of *smrd-1* in sperm viability

During this study, a *smrd-1(ea92)* male stock was maintained at 20ºC throughout many generations by the successive cross with homozygous mutant hermaphrodites. Although they would never throw as many males as the N2 male stock, they were clearly able to generate progeny. Interestingly, when we tried to grow the male stock at 25ºC, the number of males thrown were significantly reduced in each generation, indicating an effect of this growth conditions on mutant males as well as on mutant hermaphrodites that will be an interesting area for further investigation.

During the study of *smrd-1(ea92)* hermaphrodites' fertility, one possibility was that the mutation affects the function/production of the sperm produced by the hermaphrodite during its larval stage and that could be contributing to the reduced fertility. In order to address that possibility, we individually plated 16 F2 *smrd-1(ea92)* day 1 adults worms grown at 25ºC and incubate at that temperature for 12h to see if they laid eggs (see graph below).



Only 2 out of the 16 worms were able to lay eggs. Of the remaining 14 sterile worms, we crossed each one with 3 *N2(wt)* males and controlled the cross 2 days later. None of the crosses

produced progeny, indicating that the sterility in these cases was not related to the lack of sperm production.

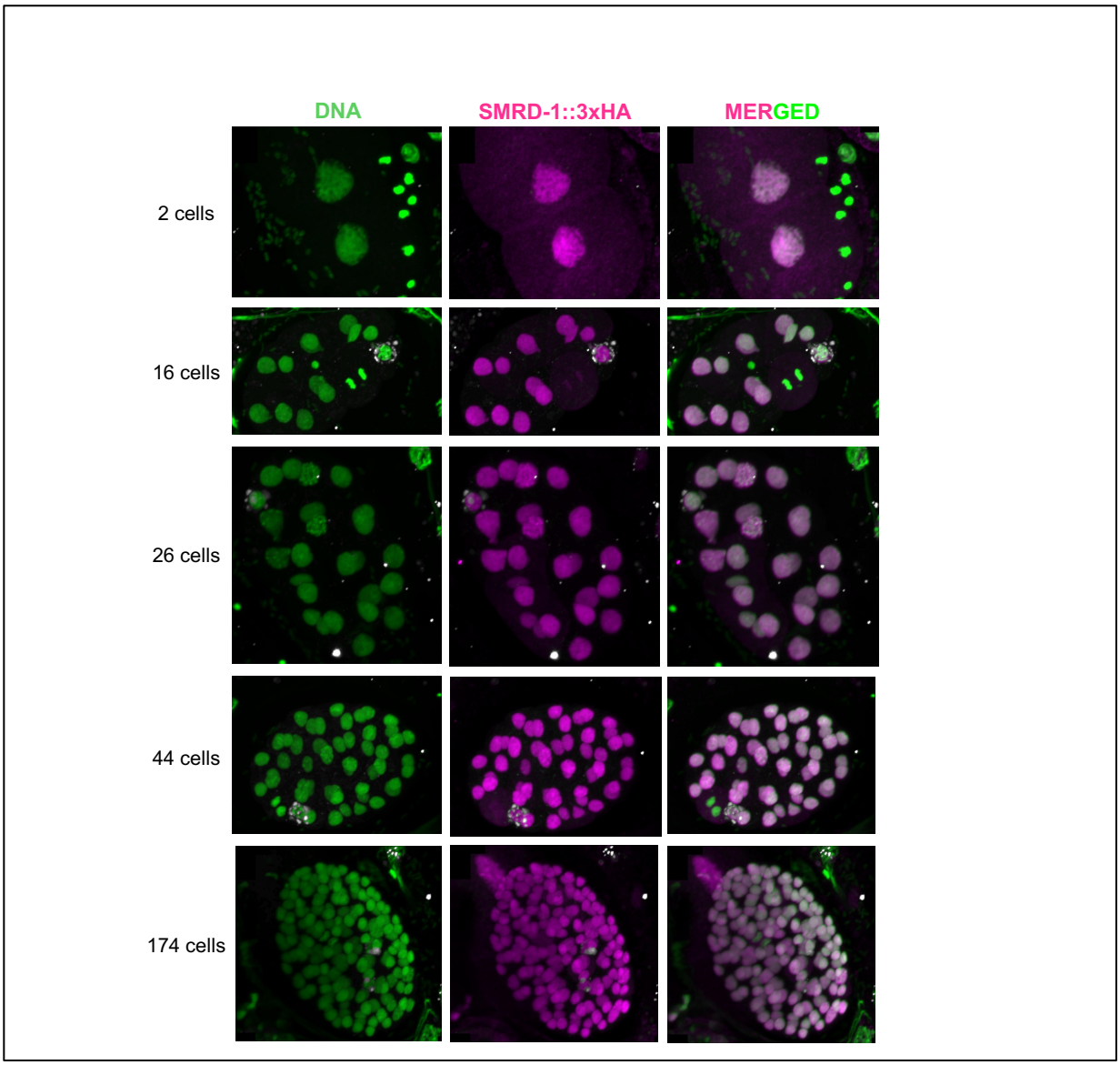
In order to start addressing the role of mutants males fertility, we maintained the male stock at 25°C for three generations and crossed them with *fog-2* females grown at 20°C. *fog-2* worms do not produce sperm and in order to be maintained have to be crossed with males and as a consequence is a good reporter of male fertility. Each *fog-2* female was crossed with 3 mutant or *wt* males.

Appendix Table 1: Male fertility of *smrd-1(ea92)* at 25°C. Female worms, *fog-2*, grown at 20°C were crossed with *smrd-1(ea92)* F3 males grown at 25°C with a 1:3 relation.

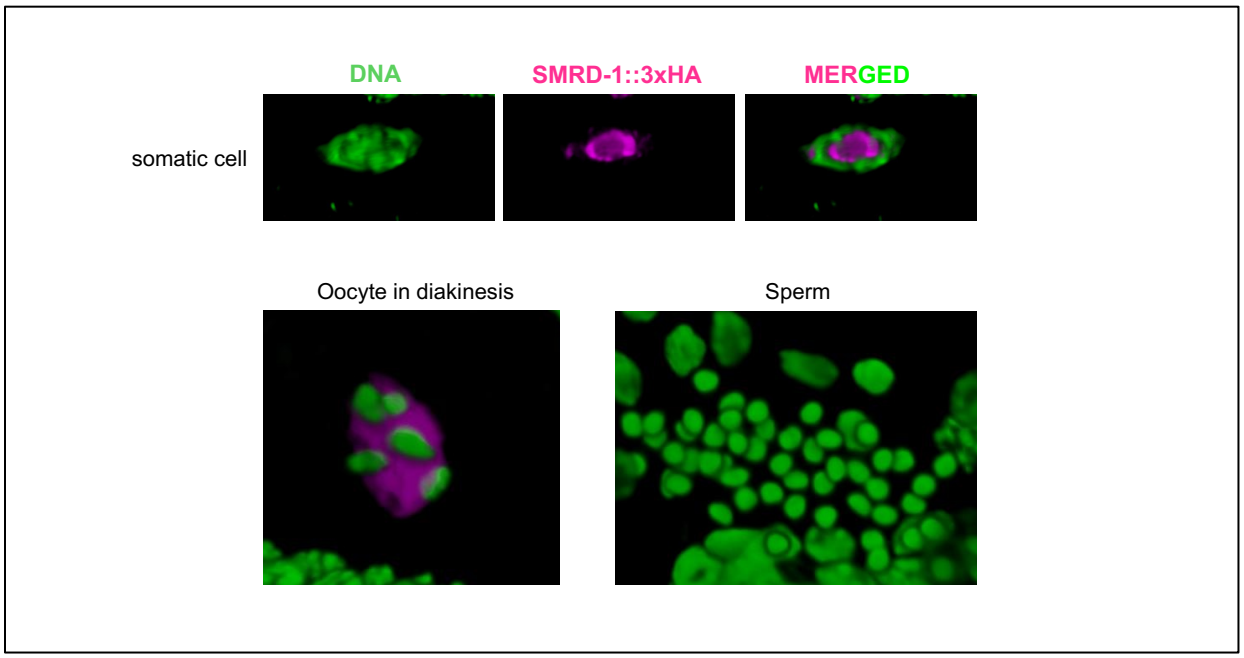
fog-2	Male (1:3)	Progeny	No progeny	Dead	Left plate	Fertility
14 worms	*smrd-1(ea92)*	0	7	3	4	0%
10 worms	*N2(wt)*	5	3	1	1	62.5%

Of the fog-2 females individually plated for the cross with *smrd-1(ea92)* (14 worms) and *N2(wt)* (10 worms) males, 7 and 8 were alive after two days of the cross with mutant and *wt* males, respectively (Appendix Table 1). These preliminary results indicate 100% infertility of the mutant males. However, since the number of repeats is low and we saw no cross progeny with some of the *wild-type* worms, we can only hypothesize that mutant males have reduced fertility. An in-depth study to address the contribution of SMRD-1 to sperm quality and to the hermaphrodite fertility is required.

Appendix A.2 Expression of SMRD-1::3xHA in somatic and reproductive cells



Appendix Figure 1: Expression of SMRD-1::3xHA during embryonic development. SMRD-1 has a nuclear localization during embryonic development. At the time of nuclear division, as can be seen in the 16-cell stage embryo, SMRD-1 acquires a cytoplasmic localization due to the absence of nuclear membrane. The antibody for PGL-1 was used to stain the P granules (shown in white) that are localized specifically to the primordial germ cells.

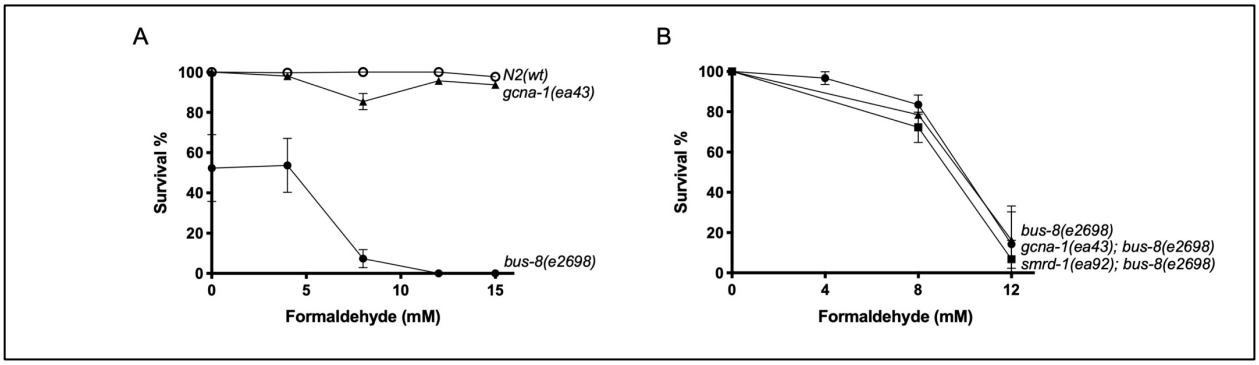


Appendix Figure 2: Expression of SMRD-1::3xHA in somatic and reproductive cell lineages. SMRD-1 has a nuclear localization in somatic cells that is decreased in the region of the nucleolus. During oocytes in diakinesis, SMRD-1 protein does not seem to co-localize with DAPI bodies but instead is located around them. No SMRD-1 could be detected in mature sperm.

Appendix A.3 Incorporation of *bus-8* mutation in formaldehyde sensitivity assays

Previous treatment with formaldehyde (FA) to study L1 sensitivity showed that our *wild type* strain as well as the mutant were resistant. In order to determine whether this resistance was associated with the permeability of the worm cuticle, we used a mutant of a membrane component that is among the strains used to evaluate chemicals toxicity using *C. elegans* as a model (Xiong et al. 2017), the mutant for the gene *bus-8*. When we treated *bus-8* and *N2* worms we see an increased sensitivity to FA. We used as a control the *gcna-1* gene mutant that was previously shown to be sensitive to FA treatment by other groups (Borgermann et al. 2019). As shown in

Appendix Figure 3A, this positive control is resistant to FA treatment, which could also involve the permeability of our strain to this chemical.



Appendix Figure 3: Percentage of survival of L1 larvae after formaldehyde treatment. A-Percentage of survival of *N2(wt)*, *gcna-1(ea43)* and *bus-8(e2698)* L1 treatment. The *bus-8* strain is in a different genetic background as the other two strains. B-Percentage of survival of L1 treatment of *bus-8* mutation in the same genetic background as the doubles with *gcna-1(ea43)* and s*mrd-1(ea92).*

Taking into account the increased sensitivity of *bus-8* worms, we then generated the strain containing this mutation but in the genetic background of this lab, as well as the generation of the *bus-8* double mutant with *smrd-1(ea92)* and *gcna-1(ea43)*. When analyzing the sensitivity of L1 worms to FA, we see there is an increased sensitivity for *bus-8* and for both double mutants (Appendix Figure 3B). This implies that the resistance to FA we are seeing on our genetic background could involve the presence of a suppressor or enhancer that can be making our strains more resistance to FA treatment. It will remain to be identified the nature of this resistance with a whole genome sequencing of these strains.

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