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CPT: Camptothecin

DDR: DNA Damage Response

DSBs: Double Strand Breaks

DTC: Distal Tip Cells

HU: Hydroxyurea

IR: Ionizing Radiation

NEBD: Nuclear Envelope Breakdown

ssDNA: single stranded DNA

UV: Ultra Violet light

1 INTRODUCTION

1.1 Cell cycle regulation and checkpoints

Cell division is an essential process required for organism development and reproduction. Misregulation of cell proliferation leads both to disease and cellular dysfunction and therefore it has been extensively studied during the last decades. Indeed, lack of proper cell cycle regulation and accumulation of genomic instability are among the main characteristics of tumor cells and a hallmark in cancer disease. Therefore, a deep understanding of cell cycle and DNA maintenance mechanisms is still one of the major challenges from basic to applied biomedical science.

1.1.1 Cell cycle regulation

Cell cycle is tightly regulated in eukaryotes. Progression through diverse phases is controlled by the activity of cyclin-dependent kinases (CDKs). Cyclins are a group of proteins expressed in specific phases of the cell cycle, which regulate CDKs activity (Figure 1). Different cyclin-CDKs complexes phosphorylate effector proteins allowing cell cycle progression. CDKs are very well conserved from yeast to mammals, however the number of kinases differs between organisms. In yeast there is only one CDK forming diverse complexes with various cyclins, whereas at least four CDKs (CDK1, 2, 4 and 6) have been found in higher organisms.

Fine-tuned regulation of CDK activity is mediated by diverse mechanisms. First, the availability of cyclins, which rely on transcriptional control and protein degradation since they have a short half life (Morgan, 1995). Second, post-translational modifications, mainly phosphorylation, either activate or inactivate CDK depending on the modified residue. CDKs activation requires phosphorylation by CAK (CDK-activating kinase) (Kaldis, 1999), whereas inactivation of CDK1 and CDK2 is caused by phosphorylation of inhibitory residues (Thr14 and Tyr15). This modification depends on the balance between the activities of the WEE1 and MYT1 kinases, and the CDC25 family of phosphatases (Parker and Piwnica-Worms, 1992; Nilsson and Hoffmann, 2000). Inactivation of CDK4 and 6 are also linked to a Tyr residue phosphorylation, however the kinase involved is still unknown (Terada et al., 1995). Further, cyclin-CDK complexes are inactivated by interaction with inhibitor proteins which have been

grouped in two families: INK4 and CIP/KIP. INK4 members (p16, p15, p18 and p19) inhibit CDK4-6 complexes whereas CIP/KIP proteins (p21, p27 and p57) are able to inactivate several cyclin-CDK complexes (Figure 1) (Sherr and Roberts, 1999).

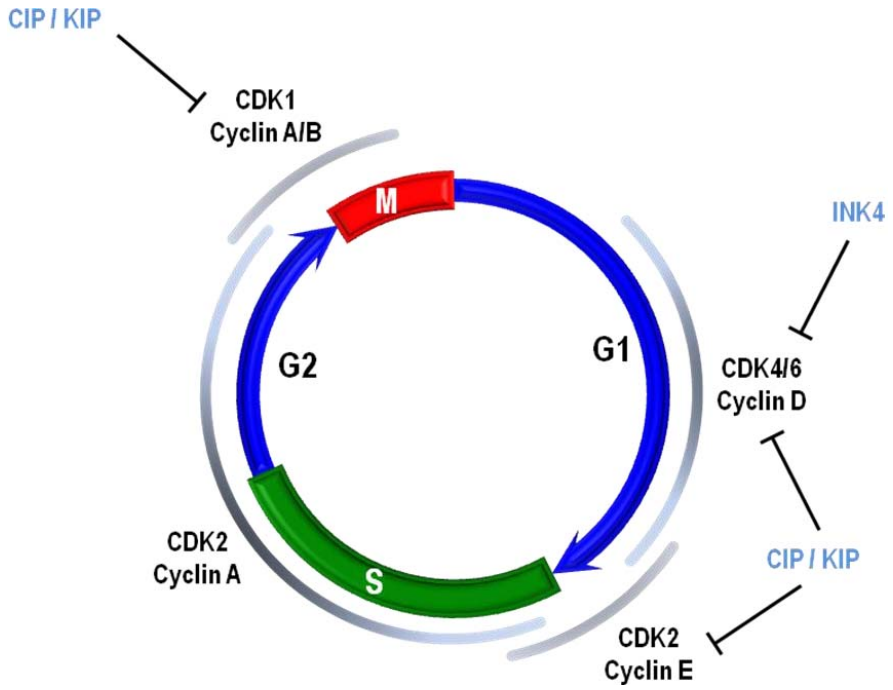


Figure 1. Cell cycle regulation in higher eukaryotes.
Activity of CDK/Cyclin complexes during different phases of the cell cycle. S: DNA replication phase, M: mitosis, G1 and G2: gap phases. CDK4/6-Cyclin D controls progression during G1. CDK2-Cylin E controls G1/S transition and beginning of S phase. CDK2-Cyclin A controls S phase progression and G2. Finally, CDK1-Cyclin A/B controls G2/M transition and Mitotic progression. Cyclins are indicated by their class name. Inhibition of different CDK complexes by CIP/KIP and INK4 proteins is also indicated (Modified from Boxem, 2006).

1.1.2 Checkpoints

After DNA damage, cells respond through surveillance mechanisms in order to maintain genomic stability by activating signal transduction cascades that constitute the DNA damage response (DDR). Such mechanisms promote mainly inhibition of cell cycle progression, DNA damage repair and induction of apoptotic cell death if DNA repair fails. As part of the DDR, status of DNA stability and elements required for cell division (such as histones or mitotic proteins) are verified at different points during the cell cycle, called checkpoints. G1/S transition is the first checkpoint where factors required for DNA replication are monitored

before allowing the entry in S phase. Once DNA replication has started, proper DNA synthesis and fidelity is evaluated by the intra-S phase checkpoint. Later, in G2, DNA integrity and correct completion of genome replication are evaluated as well as levels of components needed for the subsequent division. Finally, during mitosis, the spindle checkpoint (also called mitotic checkpoint) ensures accurate chromosome segregation.

Mutations fixed during the replication phase of the cell cycle are propagated through the cell progeny. Accumulation of mutations is involved in diverse processes that impair cellular function and regulation, like cancer and aging. Most carcinogens and genotoxic agents, and some oncogenes, generate either lesions or stress conditions that particularly affect DNA replication, promoting mutagenesis and carcinogenesis (Kaufmann, 2007; Halazonetis et al., 2008). S and G2/M checkpoints detect DNA damage occurring during DNA replication, ensuring proper completion of DNA synthesis. The mitotic checkpoint verifies that each pair of sister chromatids are properly bound to ensure equal chromosome segregation. However, DNA damage is not detected by this checkpoint unless it affects the centromere region (Rieder et al., 1995; O'Connell et al., 2008). Therefore, S and G2/M checkpoints defects, could lead to chromosome missegregation in mitosis, producing DNA instability that compromises cell viability. Consequently, these checkpoints have a predominant role to maintain DNA replication fidelity and DNA integrity.

1.1.2.1 S phase checkpoint

Activation of S phase checkpoint is triggered by different conditions that generate replication stress. This occurs, for instance, when DNA polymerase finds an obstacle that impairs its processivity (DNA adducts, breaks or certain DNA modifications produced by alkylating agents), or when there is a deficiency in substrates needed for DNA replication, like dNTPs (effect caused by drugs like Hydroxyurea -HU-). At this point, DNA replication needs to be stopped in order to prevent the collapse of the stacked replication forks that generate double strand brakes (DSBs), which in turn leads to deletions and chromosome abnormalities in mitosis (Gottifredi and Prives, 2005). To avoid these deleterious defects, S phase checkpoint activates mechanisms that promote firstly, the inhibition of late-origin firing and the slowing of fork progression and, secondly, the stabilization of stacked replication forks (Grallert and Boye, 2008). There are many proteins working together to regulate this process (Gottifredi

and Prives, 2005; Petermann and Caldecott, 2006; Harper and Elledge, 2007) (Figure 2). However, the most characterized pathway activated in response to replicative stress is the so-called ATR pathway (Ataxia-Telangiectasia and Rad-3 Related protein). DNA replication blockade leads to excessive accumulation of single stranded DNA (ssDNA). This structure is recognized and stabilized by the RPA protein (Replication protein A). Subsequently the 9-1-1 complex (formed by RAD9-HUS1-RAD1) is recruited to the ssDNA-RPA sites. Other complexes like the MRN (MRE11-RAD50-NBS1) also take part in the response, although this one is mainly recruited to DSBs sites. These complexes in combination with other mediators (ATRIP, CLASPN, TopBP1) activate the ATR kinase, which in turn phosphorylate effector proteins, such as CHK1 or p53, that further signal to promote the appropriated checkpoint response. Another kinase, ATM (Ataxia Telangiectasia Mutated protein), acts in parallel to ATR when the replication blockade is triggered by DSBs. In both cases, the outcome of the pathway could be either the cell cycle halt coupled to DNA repair or apoptosis, depending on dose and type of damage. The cell cycle arrest is predominantly achieved through inactivation of the CDC25A phosphatase, which normally activates CDK2 to allow DNA replication progression. DNA repair is regulated by activation of DNA-PK, RAD-51 or FANC2 proteins, whereas the apoptotic response is predominantly controlled by the P53 activity (Gottifredi and Prives, 2005).

1.1.2.2 G2/M checkpoint

Cells that suffer DNA damage during G2 or with lesions that were not detected or repaired by the S phase checkpoint, are able to prevent entry into mitosis. This is mediated by the G2/M checkpoint. As part of the DDR, S and G2/M checkpoints involve similar protein complexes, nonetheless, their contribution and the effector pathways could be different. This variation depends on the DNA damage triggering the response (e.g. DSBs or ssDNA accumulation) and the cell cycle specific proteins that must be targeted (Figure 2). DSBs in G2 can directly activate ATM, and indirectly, via ATM-dependent strand resection, can lead to ATR activation. In this case, the MRN complex plays an important role, first recruiting ATM to the DSBs and then generating ssDNA that would also recruit ATR to amplify the checkpoint signal (Jazayeri et al., 2006). Subsequently, CHK2 and CHK1 are activated by ATM and ATR, respectively. The principal aim of this pathway is to stop entry into mitosis, and thus both CHK kinases inactivate CDC25B and C to prevent CDK1 activation. This signal is

reinforced by inducing WEE1 kinase, another inhibitor of CDK1 activity (Raleigh and O'Connell, 2000). This response is very rapid because is transmitted through phosphorylation, however if the arrest is further maintained, p53 can also contribute by transcriptional regulation: 1) repressing CDK1 and cyclin B expression and 2) promoting p21 transcription which inhibits the activity of the CDK1/cyclin B complexes (Taylor and Stark, 2001). Apart from this canonical pathway, p38 is also able to induce G2 arrest by inhibiting CDC25B and C. This inactivation is mediated by the kinase activity of MK2 (MAPKAP kinase-2) and it seems to be specifically initiated in response to the bulky DNA lesions generated by ultra violet light (UV) (Manke et al., 2005). DNA repair and apoptotic effectors are common for the S and G2/M checkpoints.

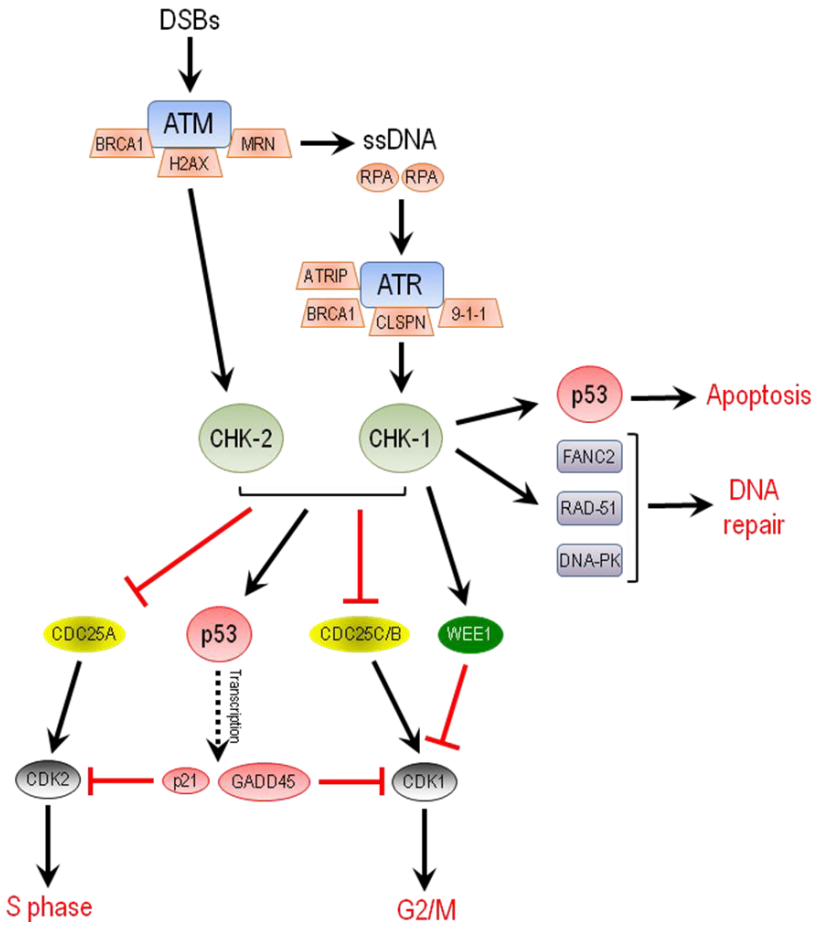


Figure 2: Checkpoint regulatory pathways in mammals.
The scheme shows the core of the checkpoint pathway that controls the intra S phase and G2/M checkpoints in response to either double strand breaks (DSBs) or single stranded DNA (ssDNA) (Modified from Dai and Grant, 2010).

1.1.3 14-3-3 proteins in cell cycle and checkpoint regulation

14-3-3 proteins are an evolutionarily conserved family implicated in multiple cellular processes. 14-3-3 proteins work as molecular dimmers that bind mainly to serine phosphorylated motifs and regulate the function of many proteins by controlling aspects like subcellular localization, stability or activity (van Heusden, 2005). In fact, PUBMED search for "14-3-3" results in ~3750 hits and proteomic approaches have identified hundreds of 14-3-3 putative interacting proteins (Jin et al., 2004; Meek et al., 2004; Pozuelo Rubio et al., 2004; Benzinger et al., 2005; Ge et al., 2010; Pozuelo-Rubio, 2010). As a consequence of their large number of functions, 14-3-3 proteins have been related with several human diseases although most of the studies are focused in their roles in cancer and neurodegeneration (Tzivion et al., 2006; Morrison, 2008; Steinacker et al., 2011). This emphasis comes from 14-3-3 roles in cell cycle, apoptosis and stress signaling regulation (Berdichevsky and Guarente, 2006; Porter et al., 2006; Gardino and Yaffe, 2011).

According to the subject of this thesis, description of known 14-3-3 functions will be focused on their roles in cell cycle and checkpoint. 14-3-3 proteins are necessary for proper cell cycle arrest following DNA damage in yeast, flies and mammals (Figure 3). Checkpoint-related function for 14-3-3 proteins was firstly discovered in fission yeast (*Schizosaccharomyces pombe*), where Rad24 and Rad25 were found to be required for delayed mitotic entry after UV and ionizing radiation (IR) (Ford et al., 1994). In *Drosophila melanogaster* there are two 14-3-3 proteins (ζ and ε) that function in cell cycle regulation during development by suppressing Cdk1 activity and therefore inhibiting the entry into mitosis (Su et al., 2001). Mammalian 14-3-3s have expanded to seven proteins corresponding to isoforms encoded by individual genes (β, γ, ε, η, σ, τ, ζ) and their function in cell cycle control is conserved. However, the contribution of the different isoforms and the mechanism underlying is still under exploration. 14-3-3 functions in checkpoint regulation are mediated by interactions with several cell cycle regulators that will be mentioned in the following sections. To facilitate the global understanding of 14-3-3 family functions, they will be referred as "14-3-3 proteins", no matter which organism the evidences came from.

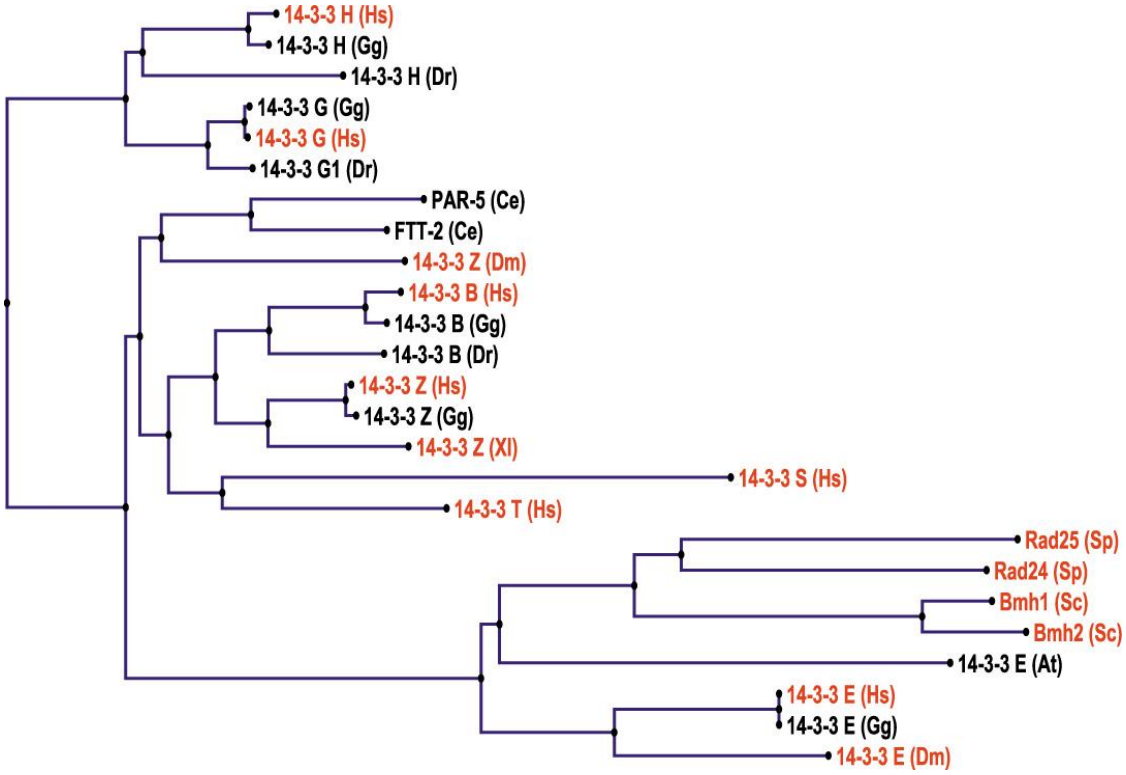


Figure 3. **Phylogenetic tree of 14-3-3 family proteins**.
14-3-3 ortholog sequences were aligned using ClustalW. CLC Sequence Viewer was used to generate the tree using the Neighbor Joining algorithm. Names in red correspond to 14-3-3 members, which have been either related to cell cycle control or shown to physically interact with checkpoint/cell cycle proteins (Hermeking and Benzinger, 2006). (At) *Arabidopsis thaliana*, (Ce) *Caenorhabditis elegans* (Dm) *Drosophila melanogaster*, (Dr) *Danio rerio*, (Gg) *Gallus gallus*, (Hs) *Homo sapiens* (Sc) *Saccharomyces cerevisiae*, (Sp) *Schizosaccharomyces pombe*, (Xl) *Xenopus laevis*.

1.1.3.1 G1/S transition

G1 progression is controlled by the CDK4-6/cyclin D complexes, which phosphorylate pRB family of proteins, allowing the dissociation and activation of E2F transcription factors. E2F family members induce the expression of genes involved in G1/S transition. One of these genes is cyclin E, which binds to CDK2 to maintain pRB phosphorylation, keeping a positive feedback loop over E2F-mediated transcriptional activation. Moreover, besides cyclin E levels, CDK2 activity is also controlled by phosphorylation. Specifically, CDK2/cyclin E complexes are activated by CDC25A which removes the inhibitory phosphorylation of CDK2, allowing G1/S transition. Upon DNA damage, the checkpoint response triggers two mechanisms to inhibit CDC25A, which depends on CHK1 mediated phosphorylations. In first place, CDC25A phosphorylation (Ser78) promotes its degradation through the β-TrCP

ubiquitin ligase and the proteasome (Jin et al., 2003). In addition, a second mechanism involves 14-3-3-dependent inhibition of CDC25A function. 14-3-3 proteins bind to CDC25A phosphorylated at Ser178 and Tyr507 residues (Chen et al., 2003). Although the mechanism is still under exploration, it has been proposed that 14-3-3 binding to CDC25A prevents the interaction between this phosphatase and the CDK2 (Gardino and Yaffe, 2011). By this mechanism, 14-3-3 proteins would prevent G1/S transition by reinforcing checkpoint mediated inhibition of CDC25A (Figure 4).

On the other hand, in unperturbed cells, 14-3-3 proteins play a role that favors G1/S transition. As mentioned above (section 1.1.1), CDK2/cyclin E activity is also controlled by the CIP/KIP family of proteins. Specifically, p27 and p21 bind and inactivate CDK2, preventing the G1/S transition. However, 14-3-3 mediates cytoplasmatic sequestration of p27 (Sekimoto et al., 2004) and also promotes proteasomal degradation of p21 (Wang et al., 2010). Therefore, 14-3-3 contributes to G1/S transition in unchallenged cells, whereas participates in the G1/S phase arrest upon DNA damage.

1.1.3.2 S phase progression

The first part of S phase is controlled by the CDK2/cyclin E complex, which promotes the recruitment of replication complexes to DNA in order to initiate DNA synthesis. Later, cyclin E is degraded and CDK2 associates with cyclin A to promote the progression through S phase. Since CDC25A controls CDK2 activity, 14-3-3 proteins are able to halt S phase progression by inhibiting CDC25A. Similarly to what happens in G1/S transition, this 14-3-3 inhibitory function is dependent on checkpoint activation. In addition to this mechanism, 14-3-3 is also able to bind directly to CDK2, preventing its nuclear localization that is necessary for cell cycle progression (Laronga et al., 2000). Therefore, 14-3-3 participates in the intra S phase checkpoint (Figure 4).

Apart from this function, there are several evidences implicating 14-3-3 proteins with the DNA replication complexes (Zannis-hadjopoulos et al., 2007). First, 14-3-3 proteins binds cruciform DNA, which is a DNA structure related with replication origins in eukaryotes (Alvarez et al., 2002; Callejo et al., 2002). Furthermore, 14-3-3 binding to cruciform DNA seems to affect DNA replication initiation in budding yeast (Yahyaoui et al., 2007).

Supporting such a function, 14-3-3 proteins are also able to interact with the topoisomerase II (Kurz et al., 2000) and several proteins of the MCM2-7 complex which are components of pre-replicative complexes (Zannis-hadjopoulos et al., 2007). However, since 14-3-3 proteins are mainly cytoplasmatic in eukaryotic cells, its direct function in DNA replication is still under investigation.

1.1.3.3 G2/M transition

The control over S and G2/M phases converge in the regulation of the CDK1/cyclin B complex. This is because DNA damage in these phases promotes checkpoint mediated inactivation of this complex, which is the major mediator of the entry into mitosis. The activity of CDK1 is regulated by the phosphorylation of two residues (Thr 14 and Tyr15). Because these residues are within the ATP-binding domain, they inactivate the kinase activity of CDK1 if phosphorylated. Phosphorylation of these residues relies on the balance between the WEE1/MYT1 kinases, and the CDC25B/CDC25C phosphatases, which add and remove inhibitory phosphorylations, respectively. Inactivation of the CDC25 phosphatases is mainly dependent on 14-3-3 binding to their phosphorylated motifs, mediated by CHK1, CHK2 and MK2 checkpoint kinases. 14-3-3 binding decrease the phosphatase activity of CDC25C (Kumagai et al., 1998) and also prevent the binding of CDC25B to the CDK1/cyclin B complex (Giles et al., 2003). An additional mechanism of CDC25 inhibition mediated by 14-3-3 would be its cytoplasmatic sequestration, preventing its interaction with CDK1 in the nucleus (Lopez-Girona et al., 1999; Davezac et al., 2000; Graves et al., 2001).
The role of 14-3-3 in G2/M arrest is further mediated by its association with the WEE1 kinase, which is also phosphorylated by the checkpoint kinases. In this case, 14-3-3 binding favors the nuclear localization and increase the kinase activity of WEE1 (Lee et al., 2001; Rothblum-Oviatt et al., 2001). Therefore, 14-3-3 proteins prevent G2/M transition by two synergistic mechanisms, the inhibition of CDC25 family members and the activation of WEE1 upon checkpoint activation. This function is further reinforced by the 14-3-3 binding to CDK1, which prevents its nuclear localization (Chan et al., 1999; Laronga et al., 2000) (Figure 4).

1.1.3.4 Mitosis

After nuclear envelope breakdown (NEBD), once the cell is committed to mitosis in a process mediated by the CDK1/cyclin B complex, microtubule polymerization starts the spindle assembly from the two separating centrosomes. Each pair of sister chromatids generated by replication of one chromosome must attach to opposite poles and just one pole at a time. This is ensured by an error-correcting machinery mediated by the Aurora B kinase complex (Cimini et al., 2004). In addition, in order to ensure proper chromosomal segregation, a checkpoint mechanism detects the presence of chromatids that are not bound to the microtubules. In this case, to delay chromosome separation (anaphase), the checkpoint promotes an inhibitory complex involving MAD1, MAD2, BUBR1, and other proteins (Kops, 2008). This complex prevents the degradation of securin (necessary for chromosome disjunction) and cyclin B, which is essential for mitotic exit (telophase and cytokinesis). Even though 14-3-3 proteins are not major players in this checkpoint, they contribute to anaphase delay, probably by interacting with MAD and BUB checkpoint components (Grandin and Charbonneau, 2008) (Figure 4).

However, 14-3-3 proteins promote cytokinesis in unperturbed cells by at least two mechanisms. One of them involves 14-3-3 interaction with PKCε in telophase, which is required to allow complete cell abscission in cytokinesis (Saurin et al., 2008). The other is achieved by 14-3-3 mediated regulation of the mitotic switch from cap-dependent to cap-independent translation. This switch allows the production of the CDK11 cap-independent form (p58-PITSLRE), a requisite to complete the final stages of mitosis (Wilker et al., 2007).

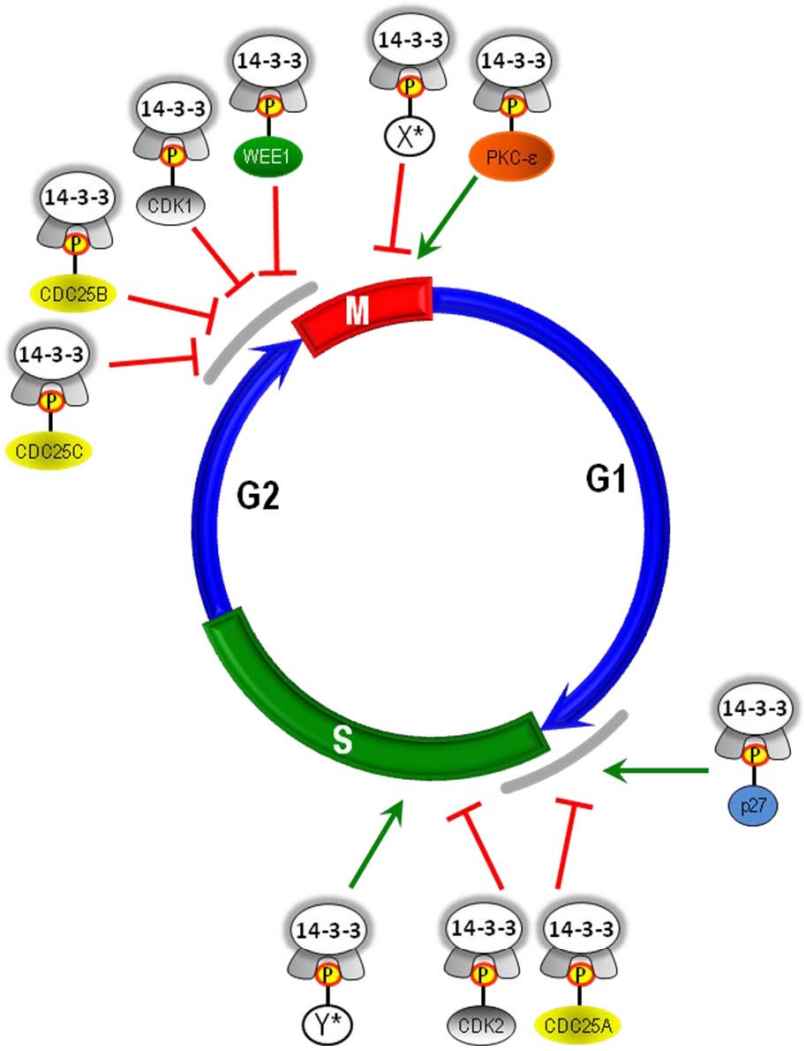


Figure 4. 14-3-3 interaction with different cell cycle regulators.
14-3-3 proteins regulate G1/S and G2/M transition, and also progression throughout S and M phases, by interacting with diverse cell cycle regulators. Most of these interactions are mediated by phosphorylation of the 14-3-3 partners. In unperturbed cells 14-3-3 contributes to cell cycle progression at different points (green arrows), whereas upon DNA damage 14-3-3 proteins are required to halt the cell cycle (red inhibitory symbols). X*: represents several partners involved in DNA replication such as MCM-3, 5 proteins or topoisomerase II. Y*: represents unidentified partners involved in spindle checkpoint, likely MAD or BUB proteins (Based on Gardino and Yaffe, 2011).

1.2 *C. elegans* as a model to study checkpoint responses and cell cycle regulation

The *Caenorhabditis elegans* germline has become a powerful model for the study of gene function. There are several important advantages that make *C. elegans* an excellent model to test fundamental questions before scaling up to mammalian systems. First of all, as multicellular organism, it represents a model where interactions between different cell types and tissues can be studied, providing significantly more information about cellular processes when compared to unicellular organisms like yeast or *in vitro* systems like cultured cell lines. In this sense, this model allows to study gene function not only at the cellular level but also at the systemic level. Furthermore, *C. elegans* is perhaps the eukaryotic organism where gene knockdown can be more feasible thanks to RNA interference approaches. RNAi in *C. elegans* produces a systemic and inheritable silencing that simplifies gene function studies. In addition, RNAi can be administrated by convenient ways such as feeding or soaking, and the existence of two RNAi libraries covering ~85% of worm genes allows high-throughput screening. Phenotype characterization in worms is also affordable due to their transparent body and the large collection of markers and reporters. Moreover, cellular lineage is stereotypical and well characterized allowing accurate developmental studies.

Since cell cycle and DDR genes are very well conserved from yeast to mammals, *C. elegans* is becoming widely used to study genes involved in these processes. The next section contains a description of some of the main aspects of *C. elegans* biology to understand its utility as a model in the study of gene function, giving special attention to checkpoint and cell cycle related genes.

1.2.1 *C. elegans* biology

C. elegans is a small nematode of about 1mm in adulthood that can be found as free-living organism in soil, where it survives by eating microorganisms, predominantly bacteria. In the laboratory, *C. elegans* are maintained between 15ºC and 25ºC, having a life cycle of about 3 days at 20ºC. *C. elegans* populations are mainly composed of hermaphrodite animals which produce approximately 250-300 embryos each, by auto-fecundation. However, the existence of males allows cross mating, which is useful for genetic studies. Mutant worms are readily

obtained by chemical mutagenesis or exposure to IR. Worm strains can be frozen (by using an appropriate freezing solution) for long periods of time. Also they can tolerate harsh environmental conditions, like starvation, by switching to a facultative diapause stage which allows the animals to survive for prolonged periods.

The life cycle of *C. elegans* comprises the embryonic stage, four larval stages (L1 to L4) and adulthood (Figure 5). The end of each larval stage is marked by a cuticle molt, a layer of collagen that surrounds the worm, secreted by the underlying epithelium. At adulthood stage, the worm body is composed of 959 somatic cells arranged in different organs and tissues: epithelial, nervous, muscle, excretory, coelomocyte (putative immune) and alimentary systems. Additionally, the hermaphrodite adult worm has nearly 2000 germ cells organized in the gonad, which is the only proliferating lineage in the adult (Altun and Hall, 2009).

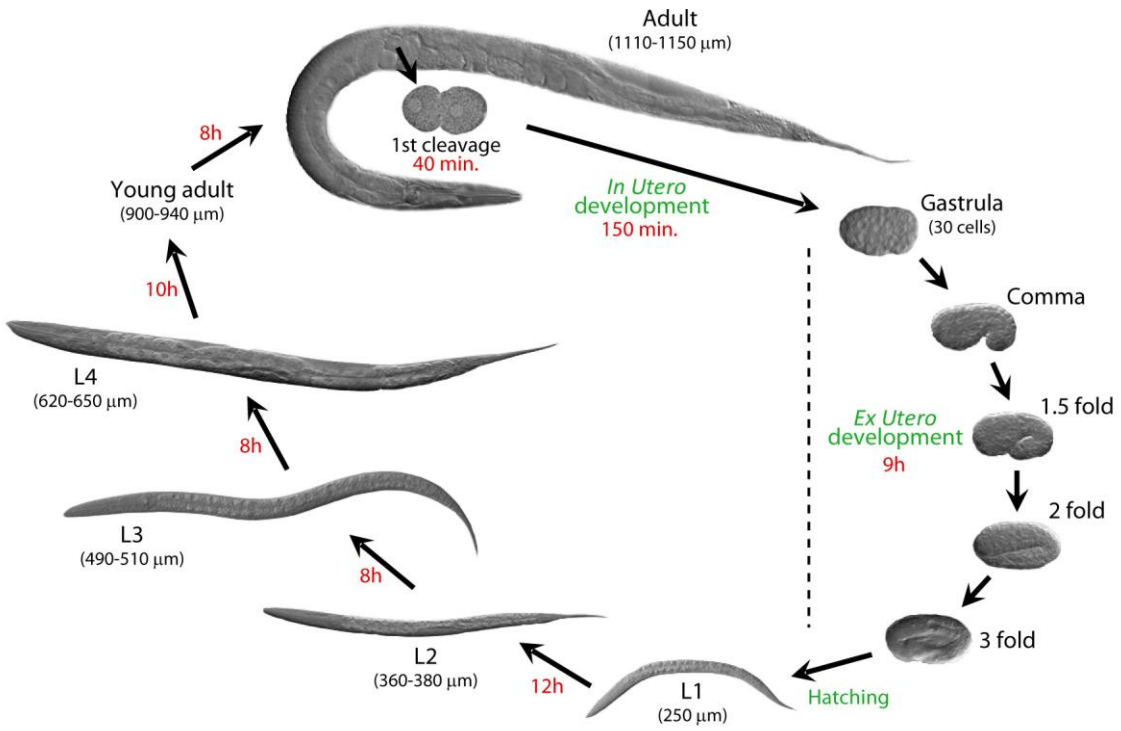


Figure 5: *C. elegans* life cycle and development.
Unperturbed worm life cycle at 22º. Numbers in red indicate the length of time the animal spends at a certain stage. Stages of dauer (at L1) and adult reproductive diapause provoked by starvation or stress conditions are omitted (Modified from Altun and Hall, 2009).

1.2.1.1 Germline organization

The *C. elegans* germline represents an excellent system to study cell cycle because germline precursors are continuously proliferating to generate gametes. The hermaphrodite adult has two symmetric gonads. Each gonad is shaped in a tubular, bent arm structure. The majority of the germ cells are arranged in a monolayer at the tube periphery. The nuclei are partially separated from each other by plasmatic membranes, but all of them have access to a common core cytoplasm known as rachis (except for the mature oocytes). Therefore, the germline is a sort of syncytium where the cytoplasmatic components can migrate. Furthermore, germ cells are organized into a spatial/temporal gradient along the distal-proximal axis (Figure 6). In the distal germline (also called proliferative region), cells proliferate to produce new germ cell precursors by mitotic divisions, which are followed by nuclei in pre-meiotic S phase and other subsequent stages of meiotic prophase (transition zone). Further proximally, germ nuclei enter in pachytene (which are the most abundant meiotic cells), where the chromosomes are fully aligned and synapsed forming “spaghetti like” structures. Later, cells progress to diplotene at the bend of the gonad and after to diakinesis, where DNA condensation makes six bivalent chromosomes that can be observed in the oocytes located in the proximal region. Mature oocytes are followed by sperm cells arranged in a structure called spermatheca. When the oocytes pass through this structure they get fecundated to produce embryos.

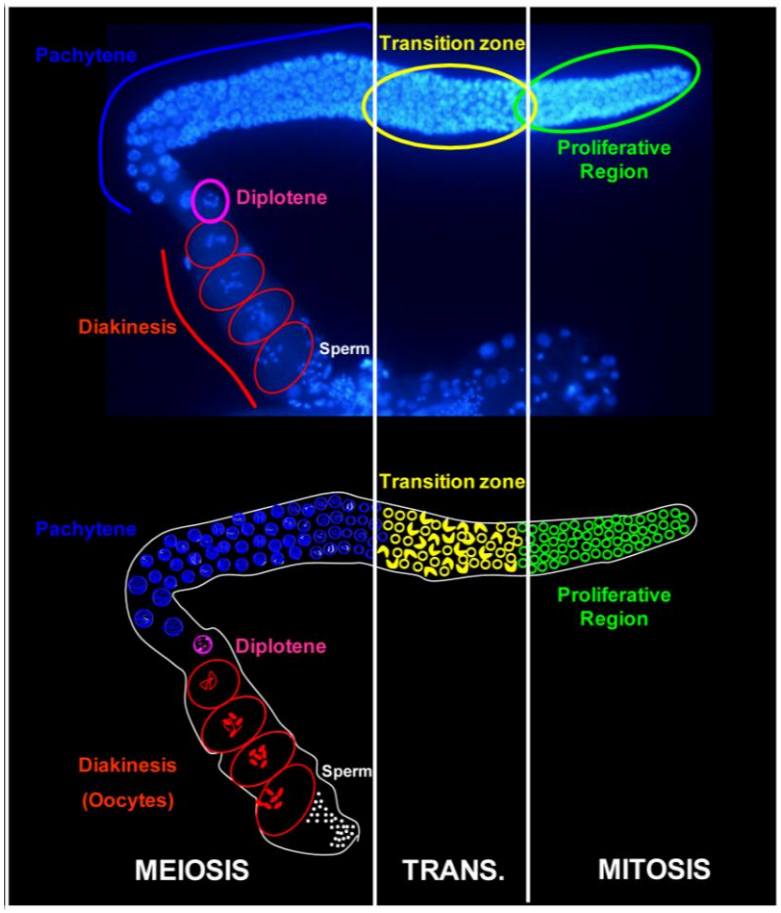


Figure 6: Germline spatial organization of hermaphrodite adult worms.
In the distal germline, cells proliferate to produce new germ cell precursors (green zone). Next, cells abandon the proliferative region to pass into the transition zone (in yellow) before starting the meiotic phases (in blue, pink and red) to finally give rise to the oocytes in the most proximal region (diakinesis stage).

Besides germ cells, the gonad also consists of somatic components such as DTCs (Distal Tip Cells), gonad sheath, spermatheca, spermatheca-uterine valve, and the uterus. DTCs are large somatic cells located at the tip of each gonad arm, forming a cap with cytoplasmic extensions. The gonad sheath consists of five pairs of cells that form a single layer covering the germline. The spermatheca, where fecundation takes place, is an accordion-like structure that contains sperm, followed by the spermatheca-uterine valve that allows the passage of fertilized embryos to the uterus, an epithelial chamber that links the two ganadal arms. Embryos develop inside the uterus approximately until the 30 cell stage, when they are expelled outside via the vulva.

1.2.1.2 Germline development

Germline development is divided into two phases: the proliferative phase from L1 to young adult, and the maintenance phase during the adult stages. After hatching, the gonad comprises two primordial germ cells flanked by the somatic gonad precursors. During L1 and L2, germ cells proliferate expanding the germline population up to 60 cells. Then in L3, the most proximal cells enter into meiosis, establishing a boundary of mitosis/meiosis between distal and proximal regions. Following gonad elongation in L4, the first meiotic cells commit to spermatogenesis producing about 150 sperm precursor cells per gonad during this larval stage. Then, by mid-L4 the newly generated meiotic cells switch to oocyte commitment, although the first oocytes are not produced until the beginning of the adult stage. At the same time germ cell precursors keep dividing at the distal gonad which results in about 400 germ cells by mid-L4 (Kimble and Crittenden, 2005). After L4, young adults produce only oocytes, while germline proliferation proceeds to generate approximately 2000 cells (1000 per gonad) (Figure 7). In later adult stages, this number is maintained steady in a balance between continuous germ cell proliferation, occurring in the distal part of gonad, physiological apoptosis, and embryo generation. In this balance, it is estimated that half of the germ cells are eliminated by programmed cell death during progression through the prophase of meiosis I, which mostly correspond to cells in pachytene stage at the gonad bend. It is thought that these apoptotic cells supply cytoplasmic components that flow through the rachis, becoming essential for the growth of the developing oocytes (Gumienny et al., 1999). The average length of a germ cell cycle is 4 hours during larval proliferation (Kipreos et al., 1996) and 16–24 hours in the adult maintenance phase (Crittenden et al., 2006). Although germ cells precursors share a common cytoplasm, they do not divide synchronously at any time of germline development, therefore they behave autonomously.

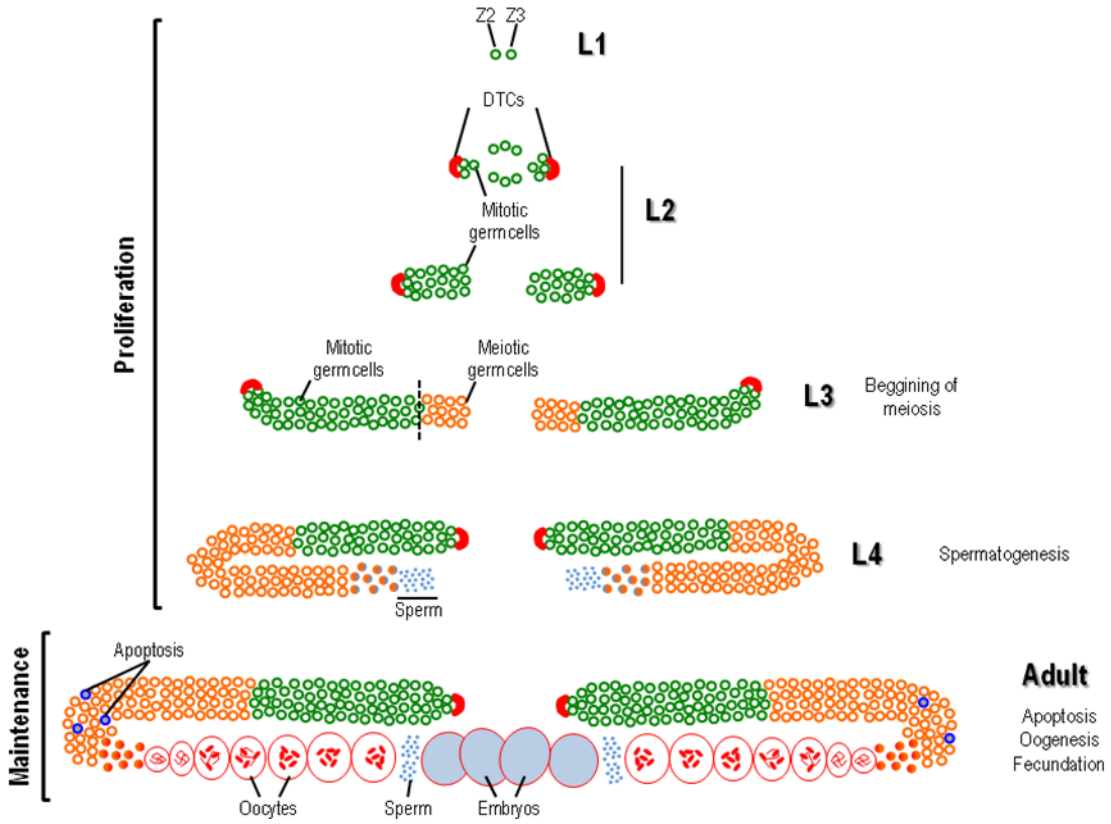


Figure 7. Germline development.

1.2.1.3 Germline proliferation

Germline proliferation depends on several factors. DTCs are necessary for germline proliferation during larval development and also in the adult. DTCs express the LAG-2 ligand that binds to the GLP-1 receptor present in the distal germ cells to stimulate their mitotic division (by activating the notch signaling pathway). Since DTCs are the only cells expressing LAG-2, a distally-enriched gradient of ligand is thought to regulate the mitotic division of the germline precursors. Additionally, GLP-1 expression is confined to distal germ cells, in part by action of the GLD-1 translational receptor, restricting the localization of proliferating cells at the distal end of the gonad (Figure 8) (Kimble and Crittenden, 2005). Even though DTCs are the only LAG-2 source in the adult, two AC/VU (anchor cell/ventral uterine) precursor cells express LAG-2 during L2, contributing to robust germline proliferation (Pepper et al., 2003).

In addition, germline proliferation is supported by sheath cells that promote germline amplification in parallel to the GLP-1 signaling. This proliferation is also involved in gonad elongation, and thus influences the developmental stage at which meiosis first occurs (Killian and Hubbard, 2005). However, the mechanism of interaction between sheath and germ cells is still unknown.

Control of germline proliferation is coupled to the regulation of the mitosis/meiosis transition. The core of this system involves GLD-1, GLD-2, FBF-1/2 and NOS-3, all of which are involved in post-transcriptional gene regulation (Figure 8). FBF-2 is a direct target of LAG-2/GLP-1 signaling, promoted by the DTC. FBF-1/2 are RNA binding proteins that are required for the maintenance of mitotic division by repressing numerous mRNA targets that favor the entry into meiosis, such as *gld-1* and *gld-3.* This signaling maintains the mitotic division at the distal end of the gonad. The mechanism is more complex since *fbf-1/2* are repressed by FBF itself and GLD-1/3 proteins. Further from the DTC, LAG-2 levels decrease, reducing the activation of FBF and therefore allowing the increase of GLD expression. GLD proteins and NOS-3 are key regulators of the entry into meiosis. There are two branches of regulation. On one hand, GLD-1 represses mRNAs required for germline mitosis (*glp-1* among them) and NOS-3 further activates GLD-1. On the other hand, GLD-2 is part of a translation activator complex that promotes the expression of the meiosis-promoting RNAs, while GLD-3 enhances GLD-2 activity (Kimble and Crittenden, 2007).

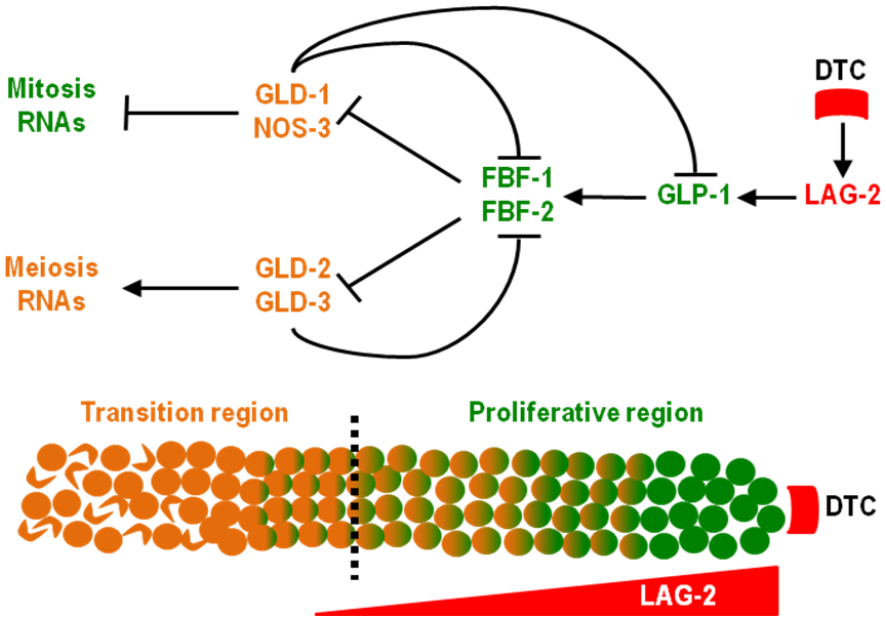


Figure 8. Regulation of proliferation and mitosis/meiosis transition in the germline.
The scheme shows the core of the pathway controlling mitosis/meiosis transition (Upper part). Bottom scheme shows the switch from mitosis to meiosis in the distal germline. Green: germ cells proliferate by mitotic division under the influence of the LAG-2 signaling (indicated by the red gradient) coming from the DTC. Orange: germ cells in meiotic division. Gradient from green to orange: germ cells switching from mitosis to meiosis (Modified from Kimble and Crittenden, 2007).

Complementing this core mechanism, there are many other genes involved in germline proliferation. For instance those associated with GLP-1 signaling (*ego, sog, sel* genes), P-granules components (PGL, GLH proteins), and other RNA regulators such as *mog* and *fog* genes (Kimble and Crittenden, 2005). The last two also regulate the sperm/oocyte switch.
An alternative pathway that functions by a mechanism distinct to GLP-1/Notch is the insulin/IGF receptor pathway (IIR), which promotes robust germline proliferation in L3–L4 stages. This mechanism is important because it establishes a link between germline proliferation and the developmental context, in this case the abundance of growth factors (Michaelson et al., 2010).

In summary, germline proliferation is regulated by a complex network of genes, most of them RNA regulatory proteins that work as post-transcriptional activators/repressors, that control three coupled and subsequent processes: germline proliferation, mitosis/meiosis transition and sperm to oocyte switch.

1.2.1.4 Embryonic development

C. elegans embryogenesis can be divided in two main steps: proliferation and organogenesis/morphogenesis. The proliferation stage is further divided in two phases. The first phase occurs inside the uterus and spans the time between zygote formation and the generation of embryonic founder cells (Figure 9). When the embryo has approximately 30 cells, it is expelled from the uterus and then the second phase of proliferation starts with gastrulation. This second phase encompasses very rapid cell divisions (from 30 to approx. 550 cells) with specific cell migrations. Conversely to what happens in the germline, all the cell divisions and migrations are stereotypical and invariant, which allows the accurate study of single cells fate and development. As in other metazoans, early embryonic divisions are fast, cycling between S and M phase without apparent gap phases (G1 and G2). In fact the first two divisions take between 20 and 25 min.

At the end of this proliferation stage, the embryo is a sphere of cells organized into three germ layers: ectoderm, which gives rise to hypodermis and neurons; mesoderm, which generates pharynx and muscle; and endoderm, which gives rise to germline and intestine. The organogenesis/morphogenesis stage is mainly accomplished by cell differentiation, migration and growth, without many additional cell divisions. At the end of this stage the newly formed worm has fully differentiated tissues and organs. At this point the embryo is ready to hatch (Altun and Hall, 2009).

During early embryogenesis, asymmetric cell division has an essential role in cell fate determination (Figure 9). At the first embryonic division, asymmetric cleavage of the zygote produces a larger anterior cell (AB) and smaller posterior cell (P1) (A/P polarity axis). Further asymmetric divisions generate six founder cells whose descendants will produce specific sets of cell types. Each type of founder cell displays a characteristic cell cycle rate that is related to its lineage (Bao et al., 2008). Therefore, the break of zygote symmetry occurring at the one cell stage not only defines the polarity axis (Anterior/Posterior) but is also essential for the cell fate of the daughter cells.

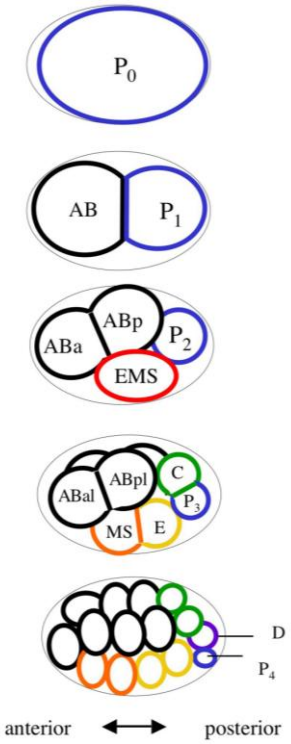


Figure 9. Generation of founder cells by asymmetric cell division in early embryos.
Asymmetric divisions in the early embryo generate different sets of founder cell (indicated with different colors). The founder cells each display a characteristic cell cycle rate and lineage. The AB lineage produces hypodermis, neurons, anterior pharynx and other cell types; MS produces the somatic gonad, muscle, the majority of the pharynx, neurons and gland cells; E produces all intestine; C produces muscle, hypodermis and neurons; D produces muscle; P4 is the germ-line precursor. (Taken from Gönczy and Rose, 2005).

1.2.1.5 PAR proteins and embryo polarity

The establishment of A/P polarity in *C. elegans* starts just after fertilization, when the centrioles donated by the sperm form the centrosomes that mark the posterior pole of the embryo. This promotes the reorganization of an actomyosin network that drives anterior-directed movement of the cortex, which is compensated for by posterior-directed cytoplasmic movements. These movements are important to establish distinct domains of localization of the PAR (partitioning defective) proteins, which reinforce the cortical flow. There are six PAR proteins that are required for A/P polarity establishment (Figure 10). PAR-3 and PAR-6 are PDZ-containing proteins that form a complex with the atypical protein kinase C, PKC-3. The distribution of this complex is restricted to the anterior cortex. On the other hand, PAR-2, a ring-finger protein, and PAR-1, a Ser/Thr kinase, localize in a reciprocal manner to the

posterior cortex. PAR-4, another Ser/Thr kinase, and PAR-5, a 14-3-3 protein, are present in a uniform manner throughout the cortex, as well as in the cytoplasm. Despite its uniform localization, PAR-5 is necessary for the asymmetric localization of other PAR proteins and the maintenance of distinct posterior and anterior domains (Morton et al., 2002). Furthermore, anterior and posterior PAR complexes repress each other to maintain their asymmetric localization.

Once polarity is established, interaction between the PAR complexes and other polarity mediators such as MEX-5/6 mediate the unequal segregation of cell fate determinants. Examples of these fate determinants are the P-granules; large ribonucleoprotein complexes destined to the germline, PIE-1; regulator of germline differentiation, and PAL-1; necessary to the fate of posterior blastomeres (Gonczy and Rose, 2005; Suzuki and Ohno, 2006). Therefore, dysfunction of PAR family members results in generation of daughter cells with altered fate, size, spindle orientation and cell cycle progression.

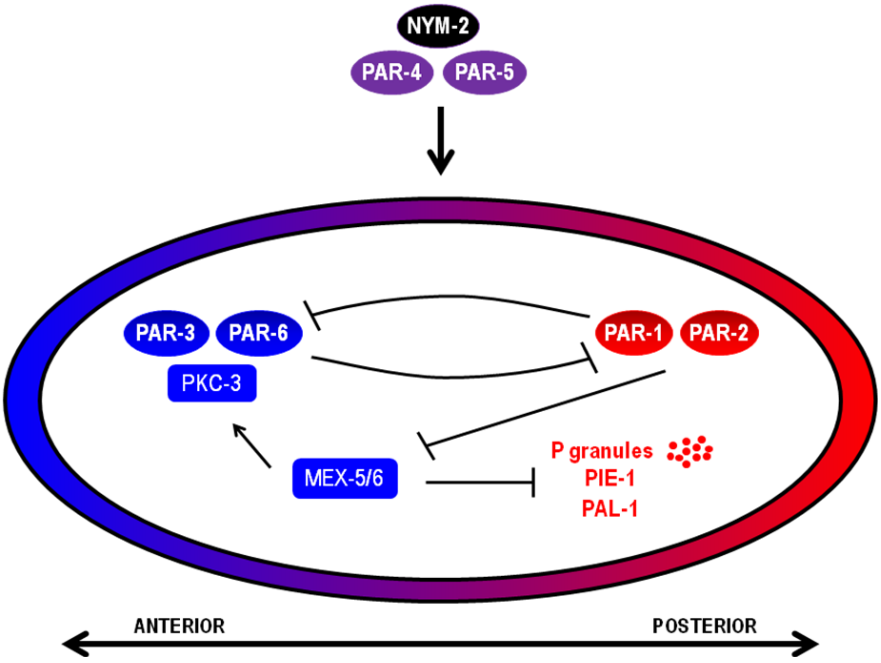


Figure 10 Anterior/Posterior polarity and PAR proteins in one-cell stage embryos.
Actomyosin (represented by NYM-2 proteins) directed movements break the symmetry of the zygote contributing the polarized localization of the PAR proteins. Proteins represented in red localize to the posterior part of the embryo, whereas proteins in blue localize to the anterior part. PAR-4 and PAR-5 are homogenously localized but they are required for the asymmetric distribution of the other PAR proteins. PAR-1/PAR-2 and PAR-3/PAR-6/PKC-3 complexes repress each other. In turn, PAR-1 prevents MEX-5/6 accumulation at the posterior. The presence of MEX-5/6 at the anterior prevents the presence of P granules, PIE-1 and PAL-1 on that side of the embryo, and also reinforces anterior PAR-3/PAR-6/PKC-3 distribution. The asymmetric distribution of all these components is required to define the fate of the daughter cells.

1.2.2 Cell cycle regulation in worms

Basic cell cycle regulation machinery is conserved in *C. elegans* relative to mammals. CDK/cyclin complexes work in the same manner, regulating the transition between the different cell cycle phases, although there are less cyclin isoforms in worms (Figure 11). Nevertheless, there are some differences between *C. elegans* and mammalian cell cycle core. For instance, regarding the CDK inhibitory family CIP/KIP, there is only one member (*cki-1*) in *C. elegans*, compared to three in mammals. Furthermore, homologs of the pINK family of CDK regulators have not been found in worms. Rb/E2F regulators are conserved; however, their function in G1/S transition is not essential as in mammals. In worms Rb plays a redundant role in this transition that only becomes indispensable in the absence of other genes such as cell cycle regulators like *cki-1* (Boxem and van den Heuvel, 2001; Ceron et al., 2007). Finally, only one of four members of the CDC25 family, *cdc-25.1,* has been implicated in cell cycle progression in *C. elegans* (van den Heuvel, 2005; Boxem, 2006).

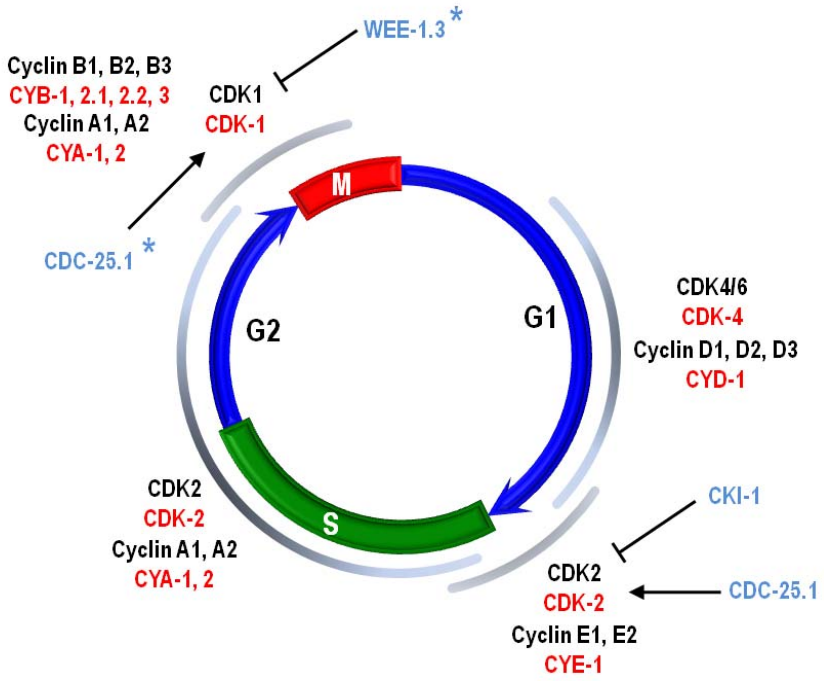


Figure 11. Cell cycle regulation in mammals versus *C. elegans*
CDK/cyclin complexes that regulates cell cycle progression are conserved between mammals and worms. Mammalian proteins are shown in black, whereas the known *C. elegans* orthologues are shown in red. Some of the proteins regulating CDKs function in *C. elegans* are shown in light blue. *Regulation of CDK-1 by WEE-1.3 and CDC-25.1 has been inferred from studies performed in the M phase of meiosis (Modified from van den Heuvel, 2005).

1.2.3 Checkpoint response in *C. elegans*

The *Caenorhabditis elegans* germline is a powerful model to study genes involved in cell cycle regulation and DNA damage response (DDR) (Gartner et al., 2000; Gartner et al., 2004). In the *C. elegans* germline, exposure to DNA damaging agents (e.g. IR, UV, Methyl Methane Sulfonate –MMS-, etc) and replicative stress (e.g. HU) trigger the checkpoint response through conserved pathways (Figure 12). This response leads to cell cycle arrest in the proliferative region and, in some cases (e.g. after IR, UV), also to an increase in the proportion of apoptotic cells in the late pachytene region of the germline. The underlying molecular pathway acts mainly through the ATL-1 kinase (ATR homologue) and other conserved proteins (Figure 12). The contribution of the different proteins to the checkpoint pathway has been characterized by their response to different types of DNA damage.

ATL-1 localizes at DNA damage sites, in RPA-1 dependent manner, including stalled replication forks following HU treatment, and DSBs induced by IR. Localization of ATL-1 to DSBs, depends on previous DSBs resection mediated by the MRE-11 nuclease (Garcia-Muse and Boulton, 2005). This indicates that the MRN complex (MRE11-RAD50-NBS1) function could be, at least in part, conserved in worms. However, the second member of the complex, RAD-50, is involved in meiotic DSB repair but not in response to exogenous insults like IR, and NBS-1 has no homolog in *C. elegans* (Hayashi et al., 2007). ATL-1 localization is important for its essential function in replication stress and IR-induced checkpoint responses (Garcia-Muse and Boulton, 2005). The 9-1-1 protein HUS-1 is also required for the response to DSBs. HUS-1 localizes at DSBs and this localization depends on the function of MRT-1 and HPR-9 (homologues of RAD1 and RAD9, respectively) (Hofmann et al., 2002). Furthermore, HUS-1 and MRT-2 are necessary for proper cell cycle arrest after IR and HU treatment, suggesting that the 9-1-1 complex is also implicated in DDR in worms (Hofmann et al., 2002; Boerckel et al., 2007). ATM-1 (ATM homologue), the other transducer of DNA damage in mammals, is involved in the checkpoint response following IR and UV (Stergiou and Hengartner, 2004; Garcia-Muse and Boulton, 2005; Lee et al., 2010). Therefore, ATM-1 and ATL-1 have redundant roles as primary transducers in response to DSBs, but response to stalled replication forks is dominated by ATL-1. Importantly for ATL-1 and ATM-1 functions, the Werner syndrome homolog WRN-1 plays a role in the proper localization and signaling of these transducers. WRN-1 function is required for robust recruitment of RPA-1, which in turn influences ATL-1 signaling and also mediates ATM-1 localization at DSBs

(Lee et al., 2010). Nevertheless, orthologues of other important mediators of ATR function in mammals, such as ATRIP, have not been found in worms.

Downstream of ATL-1 and ATM-1, CHK-1 is considered the main effector kinase (Kalogeropoulos et al., 2004). CHK-1 is phosphorylated at Ser345 upon DNA damage (by HU, IR of UV) in an ATL-1-dependent manner and is required for proper checkpoint response (Kalogeropoulos et al., 2004; Bailly et al., 2010; Lee et al., 2010). Another checkpoint kinase, CHK-2, is necessary for response to UV (Stergiou et al., 2007). As in mammals, the apoptotic branch of the pathway is dependent on the *cep-1* gene (p53 homologue) (Schumacher et al., 2001).

The repair proteins RAD-51 and its paralogue RFS-1 play major roles coupling the detection of DSBs and stalled replication forks with homologous recombination repair (Martin et al., 2005; Ward et al., 2007). BRC-1 and BRC-2 (BRCA1 and BRCA2 homologues) are also involved in DSBs repair by promoting loading of RAD-51 to the damaged sites (Martin et al., 2005; Polanowska et al., 2006). A partner of the BRCA proteins, FCD-2 (FANCD2 homologue), responds to DNA interstrand crosslinks, another specific type of DNA damage that blocks the replication forks and is detected by the checkpoint (Collis et al., 2006).

Checkpoint-directed regulation of the cell cycle proteins is thought to be conserved in *C. elegans*. Accordingly, CDK-1 phosphorylation (Tyr15) is induced after IR (Moser et al., 2009; Bailly et al., 2010). However, direct regulation of key checkpoint targets such as CDC-25 and WEE-1 has not been directly demonstrated.

In parallel with this canonical pathway there is another protein, RAD-5, that is involved in checkpoint responses. RAD-5 was firstly shown to be required for HU induced checkpoint response (Ahmed et al., 2001), and later was also implicated in sensing misincorporation of dUTP into DNA, a common endogenous lesion during DNA replication (Dengg et al., 2006). However, RAD-5 dysfunction leads to multiple phenotypes, such as early prophase arrest or delayed embryonic cell cycle, that are opposed to those observed in checkpoint mutants (Moser et al., 2009). Therefore, RAD-5 is considered part of the DDR, but the mechanism seems to be distinct from, or more complex at least, than the known checkpoint pathway. Similarly, GEN-1, a holliday junction resolvase, was recently found regulating DSBs and IR-induced mediated response in parallel to ATL-1/CHK-1 signaling (Bailly et al., 2010). Therefore, DDR pathways are still being elucidated in *C. elegans*.

Exogenous DNA damage (e.g. IR) is not the only source of DSBs. The meiotic compartment in the *C. elegans* germline has allowed the characterization of a checkpoint that monitors proper processing of DSBs generated during meiotic recombination. The core of the pathway is the same as explained above, but in this case, upon checkpoint activation the outcome seems to be biased towards apoptotic elimination of the damaged cells instead of cell cycle arrest. In hermaphrodite worms this would prevent accumulation of errors that could impair gamete viability (in male worms the apoptotic response is inhibited and the balance favors DNA repair instead of cell death) (Gartner et al., 2000; Jaramillo-Lambert et al., 2010). The meiotic checkpoint involves ATL-1 and CHK-1 signaling coupled to activation of CEP-1 and the apoptotic machinery (EGL-1, CED-13, CED-9, CED-4 and CED-3). In addition to the recombination checkpoint the existence of a chromosome pairing checkpoint has been reported, which prevents nondisjunction and also induces apoptosis (Bhalla and Dernburg, 2005).

The checkpoint machinery is also active in the *C. elegans* embryo. It has been shown that inter cellular differences in checkpoint activation contribute to asynchrony of cell division in the embryo (Brauchle et al., 2003). Therefore, the checkpoint seems to be developmentally activated. Furthermore, DNA replication inhibition (by HU, MMS or *div-1* mutations) induces a delay in embryonic cell cycle that is dependent on *atl-1* and *chk-1* genes (Encalada et al., 2000; Brauchle et al., 2003). However, checkpoint activation does not lead to complete cell cycle blockade upon DNA damage. This is because the embryo employs several mechanisms to prevent unscheduled checkpoint cell cycle activation (like the translesion polymerase *polh-1*) in order to maintain cell cycle timing that is essential for normal embryonic development (Holway et al., 2006).

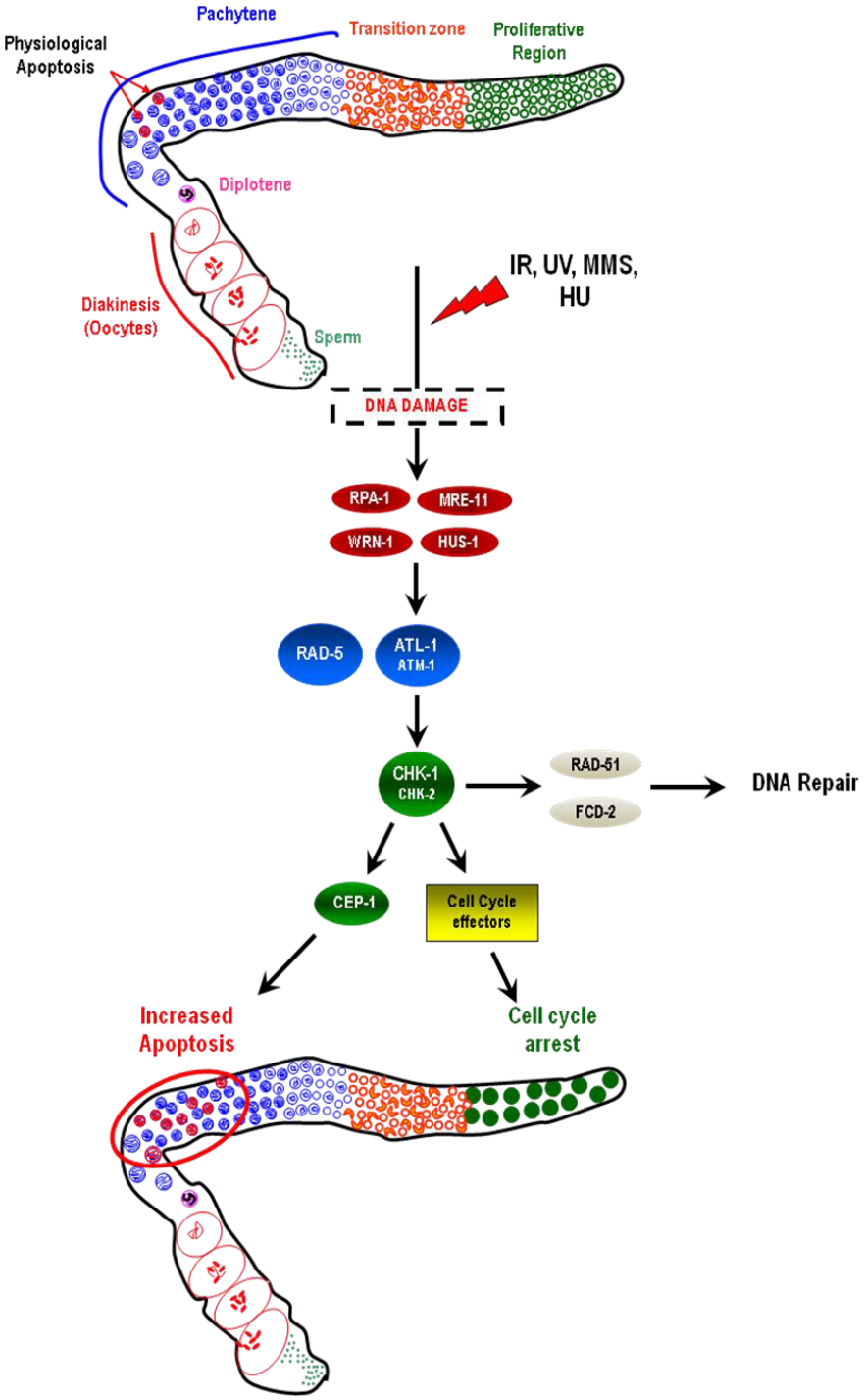


Figure 12. Checkpoint response in *C. elegans* germline.
In the distal germline, cells proliferate to produce new germ cell precursors. Next, cells abandon the proliferative region to pass into the transition zone before starting the meiotic phases. During development, a significant number of meiotic cells are eliminated by physiological apoptosis. After the induction of DNA damage by different agents, a checkpoint response is activated in the germline. DNA damage induces a molecular response pathway that includes several conserved transducer and effector proteins, as shown in the middle of the scheme. The activation of this pathway is reflected in two germline phenotypes: cell cycle arrest in the proliferative region and, in some cases, an increase in apoptotic cells in the pachytene region (bottom of the scheme).

1.2.4 14-3-3 proteins in *C. elegans*

14-3-3 proteins are involved in cell cycle arrest upon DNA damage in yeast, flies and mammals. However, the roles of the *C. elegans* 14-3-3 proteins in DNA damage response and cell cycle regulation have not been explored.

1.2.4.1 par-5 and ftt-2

In *C. elegans* there are two 14-3-3 genes, *par-5* and *ftt-2*, coding for proteins that share 86% of their amino acid sequence (Figure 13). The most divergent region is located in the C-terminus, and is distinct between the isoforms of different organisms and therefore could confer specificity. Furthermore, there is very little or no sequence similarity between the intron sequences, intron/exon boundaries and 5' and 3' non-coding genomic sequences (corresponding to putative UTRs and promoter), suggesting that different regulation could drive the expression of each gene. mRNA expression analysis during development has revealed that *par-5* and *ftt-2* expression is high in embryos but drops in L1. However, *par-5* transcripts increase during development, reaching their peak at L4 and maintaining their levels in adults, whereas *ftt-2* mRNA further decreases a little after L1 and thereafter is steadily expressed in lower levels (compared to its embryonic expression) (Wang and Shakes, 1997). Detailed study of embryo transcripts showed that *par*-5 is highly expressed in early proliferative stages, but decreases as embryo develops and by the middle of the organogenesis/morphogenesis stage, is no longer detectable. This pattern of expression suggests that *par-5* embryonic transcripts are maternal mRNAs. Conversely, *ftt-2* transcripts are not detected in one cell stage embryos, and its expression increases over embryonic development, remaining high in the older embryonic stages, implicating that is zygotically transcribed. According to their developmental and embryonic patterns, *par-5* mRNAs were found widely expressed in the germline while *ftt-2* transcripts were not detected in this tissue (Wang and Shakes, 1997). Therefore, in spite of their high protein homology, both genes are distinctly regulated through development, and only *par-5* is expressed in the germline.

Other studies using *par-5* and *ftt-2* transgenes fused to *gfp* have allowed the characterization of 14-3-3 proteins in somatic tissues. PAR-5::GFP is highly expressed in head neurons, ventral nerve cord and also intestine. FTT-2::GFP is also expressed in head neurons, weakly in intestine and highly expressed in pharynx (Wang et al., 2006). Therefore, in some somatic

tissues, 14-3-3 isoforms could function together. 14-3-3 proteins work as dimers, however it is not known if PAR-5 and FTT-2 are able to form functional dimers in those tissues where they are co-expressed.

Figure 13. *C. elegans* 14-3-3 proteins alignment.
PAR-5 and FTT-2 protein sequences were aligned using the CLC Sequence Viewer software. Divergent residues are colored in red.

C. elegans 14-3-3 proteins have been linked to life span extension and also to stress response, upon oxidative and heat stimuli. The underlying mechanism involves their interaction with SIR-2.1 deacetylase and the forkhead transcription factor DAF-16 (Berdichevsky et al., 2006; Wang et al., 2006). Upon nuclear translocation, DAF-16 activates transcription of several genes implicated in longevity and stress response (Lin et al., 1997). The role of 14-3-3 on DAF-16 regulation would be, at least in part, to modulate its cytoplasmatic/nuclear localization, but the mechanism would be different under distinct stress conditions, such as low insulin/IGF signaling, oxidative/heat stress or lack of germline (Berdichevsky and Guarente, 2006). However, this role, while well established for *ftt-2,* is still controversial for *par-5* and, additionally, other mechanisms independent of DAF-16 seem to be involved (Li et al., 2007; Araiz et al., 2008).

In addition, *ftt-2* overexpression partially alleviates the loss of neurons in a model of Parkinson disease that accumulates human α-synuclein, pointing to a role of *C. elegans* 14-3-3 proteins in neuronal cell death. Because *par-5* is also expressed in neurons it is expected to have a similar role, however this aspect has not been tested yet (Yacoubian et al., 2010).

1.2.4.2 par-5 roles in embryonic and post-embryonic development

As mentioned above (section 1.2.1.5), *par-5* belongs to the partitioning defective PAR family that regulates the first asymmetric cell division of the embryo. In this process, *par-5* is required for proper distribution of asymmetrically localized PAR proteins in the embryo. Other polarity mediators like MEX-5 and polarity targets as the P-granules are also mislocated in *par-5* mutant embryos. These defects results in nearly synchronous division at the two cell stage which further impairs embryo morphogenesis, particularly affecting intestinal and muscle cell fates (Morton et al., 2002). In early embryogenesis, *par-5* has also been shown regulating the asymmetric nucleic enrichment of POP-1 in anterior sister cells generated by A/P division of the blastomeres. POP-1 is a TCF/LEF transcription factor that contributes to endoderm fate determination (Lo et al., 2004).

Besides its essential role in embryonic development *par-5* is also involved in post-embryonic development. *par-5* mutants have reduced brood size, and those few individuals of the F1 that develop until adulthood are sterile. This was previously associated with the lack of gametes in the adult gonads of the F1 worms (Morton et al., 2002). In addition, *par-5* was identified as genetic interacting partner of *mpk-1*. In that study *par-5* RNAi enhanced the phenotype of a partial loss of function mutation of *mpk-1*. Additionally, *par-5* depletion lowered the MPK-1 active form, suggesting that *par-5* could be reinforcing MPK-1 activation. Moreover, *par-5* presents a putative docking site for MPK-1 phosphorylation. The phenotypes putatively regulated by this interaction are related to cellular organization and progression of the pachytene cells (Arur et al., 2009). Therefore, several lines of evidence indicate that *par-5* could function in germline development.

2 MOTIVATIONAL BASIS OF THE THESIS

14-3-3 proteins have been extensively studied from yeast to mammals, and are associated with multiple roles ranging from fundamental processes such as cell cycle, apoptosis and stress response to diseases such as neurodegeneration and cancer. Indeed, 14-3-3 proteins have been suggested as possible therapeutic targets in cancer treatment. There are seven 14-3-3 genes in mammals which share redundant functions and highly homologous sequences. This redundancy has hindered the study of the function of the individual genes and there is still little knowledge about the consequences of 14-3-3 dysfunction at the organism level. In *Caenorhabditis elegans*, there are only two 14-3-3 genes, *ftt-2* and *par-5*. The *ftt-2* gene is expressed only in somatic lineages, whereas *par-5* expression is detected in both soma and germline. Interestingly, *par-5* defective animals are sterile but its role in germ cells is unknown.

Previously in our lab, while exploring a set of candidate genes that could be participating in the DNA damage response in *C. elegans,* we found that *par-5* was possibly involved in the cell cycle arrest induced in germ cells in response to replication stress. This observation together with the sterility reported in *par-5* defective animals motivated the formulation of the aims of this thesis.

3 AIMS OF THE THESIS

The aims of this thesis are the following:

- To characterize the role of the 14-3-3 gene, *par-5*, in the *C. elegans* germline development.
- To determine the function of *par-5* in the DNA damage response.

4 MATERIALS AND METHODS

4.1 Worm strains and culture conditions

C. elegans strains were cultured and maintained using standard procedures (NGM plates and OP50 bacteria) and buffer formulations (Stiernagle, 2006). Bristol N2 was used as a WT strain. Alleles/transgenes used during the study are listed in Table 1. Transgenic lines expressing GFP fusion proteins were maintained and assayed at 25ºC in order to maximize transgene expression. The rest of the experiments were performed at 20ºC unless otherwise stated. Synchronization at L1 stage was carried out by extracting embryos from gravid adults using bleaching solution (2% sodium hypochlorite, 1M NaOH, diluted in M9 buffer) followed by 3 washes with M9 buffer (20 mM KH_2PO_4, 40 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$). Embryos were allowed to hatch at 20ºC in M9 for at least 24 hours to obtain a L1 larvae population.

In the case of experiments combining two RNAis (e.g. *cdc-25* and *wee-1.3* cossupression), the *rrf-3(pk1426)* strain was used as a background. This strain presents a mutation in a RNA-directed RNA polymerase (RRF-3) that promotes enhanced sensitivity to RNAi (Simmer et al., 2002). In addition, we used *rrf-1(pk1417)* for the study of somatic influence over the *par-5* RNAi phenotype. This strain shows resistance to RNAi suppression in somatic genes, due to the requirement for RRF-1 in the production of secondary siRNA triggers (Sijen et al., 2001).

Allele / transgene	Source	Strain code
atl-1(tm853)	CGC	DW101
hus-1(op241) opIs34 [HUS-1:GFP]	CGC	WS1433
par-5(it55)	CGC	KK299
rrf-1(pk1417)	CGC	NL2098
rrf-3(pk1426)	CGC	NL2099
par-5 promoter:5'UTR:GFP:par-5genomic:3'UTR	JC*	
unc-119(ed3) III; axIs1608 [pie-1 prom:GFP:histone H2B:par-5 3'UTR , unc-119(+)]	CGC	JH2220

Table 1. List of strains used in this study.
CGC: Caenorhabditis Genetics Center; JC*: Dr. Julián Cerón's lab

4.2 RNAi

RNAi-mediated knockdown was carried out by feeding worms with bacteria clones producing dsRNA targeting the respective genes (cloned in the L4440 vector). The RNAi clones used for the experiments were obtained from either the ORFeome library (Rual et al., 2004) (*par-5, mpk-1, cdc-25.1, wee-1.3*) or the Ahringer library (Kamath et al., 2003) (*par-2, par-3*). For each experiment, the corresponding RNAi clone was picked from glycerol stocks and grown in LB medium supplemented with 100 μg/mL ampicillin and 12.5 μg/mL tetracycline for 16 hours. RNAi induction was performed by seeding clones onto plates supplemented with ampicillin, tetracycline and 3 mM IPTG (American Bioanalytical, Cat. # AB00841). Plates were dried for at least 24h to allow RNAi production. RNAi plates were used to feed WT synchronized L1 worms, unless another stage is stated. All RNAi clones were verified by sequencing using the L4440 primer (Table 3). Previously reported phenotypes were also checked to confirm RNAi efficiency. One clone carrying the L4440 empty vector was used as an RNAi negative control during the experiments. To perform RNAi co-suppression of *cdc-25.1* and *wee-1.3* genes, liquid cultures with equal optical density (OD) of the corresponding RNAi clones were mixed in a 1:2 proportion respectively, before being seeded onto the RNAi plates. The same proportion was used to mix *cdc-25.1* with *L4440* and *par-2* RNAi clones.

4.3 Germline dissection and quantification

For DAPI staining of germlines, worms were immobilized in Levamisole 0.3 mM diluted in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Gonads were dissected out by cutting the worms at the pharynx or tail level. After 10 minutes fixation (3% methanol, 75% K_2HPO_4, 6.2 mM solution) the gonads were washed three times using PBS 0.05% Tween. DAPI 0.6 μg/mL was added for the final wash. The stained gonads were photographed using a Leica DM5000B microscope. Digital pictures were used for subsequent germ cell quantification. To quantify cells in the proliferative region, we counted the germ cells present in a single Z stack within 50 μm of the distal end of the gonad. For the germline time course experiment, germ nuclei from the distal part to the bend of the gonad were scored in a single Z stack. At least 15 germlines were quantified for each experiment.

4.4 Determination of apoptosis by Syto-12 staining

To stain apoptotic bodies in the germline we used Syto-12 (Invitrogene, Cat. # S7574), a fluorescent nucleic acid dye. Worms were stained by incubating in 33 μM Syto 12 solution diluted in M9 buffer for 4 hours at 20ºC. Then, they were transferred to a plate seeded with bacteria, to allow the elimination of stained bacteria. After 1 hour, worms were immobilized (in Levamisole), mounted in agar pads and observed by fluorescence microscopy using the GFP filter. Bright apoptotic corpses present at the gonad bend were scored in at least 40 germlines for each genotype/RNAi.

4.5 DNA damage response assays

To perform the cell cycle arrest assays, L4 stage worms (42–46 hours post-L1 at 20ºC) of the corresponding genotypes/RNAi were treated with different DNA-damaging agents.

Hydroxyurea assay: worms were transferred onto plates containing 25 mM HU (Sigma, Cat. # H8627) for 20–24 hours before dissection. HU plates were prepared one day before the treatments by adding 500 μL of 0.5 M HU diluted in M9 buffer to plates containing 10 mL of medium seeded with the corresponding RNAi clone.

Camptothecin assay: worms were transferred onto plates containing 40 μM CPT (Sigma, Cat. # C9911) or DMSO 0.1% for 20–24 hours. CPT solution dissolved in DMSO (40 mM), or DMSO alone, was added to autoclaved medium at ~60ºC before pouring the plates (1 μL of CPT stock or DMSO for each mL of medium). CPT plates were prepared 1 day before seeding the RNAi clones.

Ionizing Radiation assay: worms were irradiated with γ-rays (120 Gy) using a Cesium137 source (model IBL-437-C H) in the animal facility of the Barcelona Biomedical Research Park (PRBB). Dissection and germline staining were performed 12 hours post-irradiation.

4.6 Embryo cell cycle timing

Embryos for video recordings were obtained from worms treated as follows: L4 stage worms, grown at 20°C, were transferred onto plates containing the indicated RNAi or L4440 vector. After 24 hours, half of the adult worms were transferred onto plates containing HU (75 mM).

The other half was used as a control. Early embryos were obtained by dissecting immobilized adults on cover slips. Immediately, after extraction, embryos were mounted in agar pads and placed in a Nomarski inverted microscope (NIKON eclipse Ti-S) for video recording. HU-treated embryos were recorded from ~5 to ~10 hours after HU treatment. Video recordings were performed using the 100X objective of the microscope and continuous video acquisition at one frame per second. The temperature of the microscope room was maintained at 21ºC. The videos covered the time from approximately the pseudocleavage stage to the end of the first division. The cell cycle timing of the first embryonic division was determined as described by Holway (Holway et al., 2006). P0 division was defined as follows: S-phase, time from pro-nuclei first contact until they become fused in the middle of the embryo (NEBD); M-phase, time from NEBD until the beginning of the furrowing (P0 cytokinesis).

4.7 Immunostaining

For immunostaining, adult worms were immobilized in Levamisole. Next, gonads were dissected and fixed in a manner appropriate for the primary antibody (Table 2). Incubations with primary and secondary antibodies were performed overnight at 4ºC and for 2 hours at room temperature, respectively. Antibody dilutions were done in PBS 0.05% Tween 1% BSA (Table 2). After fixation and antibody incubations, gonads were washed three times with PBS 0.05% Tween. Alexa 568-conjugated goat anti-rabbit (Molecular probes) was used to label the primary antibodies, except CHK-1. All samples were counterstained with DAPI (0.6 µg/mL) in order to visualize the nuclei. CHK-1 staining conditions and antibody labeling were carried out using the Tyramide Signal Amplification kit (Invitrogen) following the manufacturer's instructions.

Antibody	Source	Fixation / time	Dilution
RAD-51	*AG	2% PFA / 10 minutes	1:200
P-H3 (Ser10)	Millipore, Cat. # 04-817	3% FA / 10 minutes	1:1000
CDK-1(pTyr15)	Calbiochem, Cat. # 219440	3% FA / 10 minutes	1:50
PAR-5	**AG	2% PFA / 30 minutes MeOH / 5 minutes	1:800
CHK-1 (pS345)	Cell signaling, Cat. # 2348	3% FA / 1 hour MeOH / overnight -20ºC	1:15

Table 2. Antibodies used in this study.
PFA: paraformaldehyde; FA: formaldehyde; MeOH: methanol
*AG: Generously provided by Dr. Anton Gartner
**AG: Generously provided by Dr. Andy Golden

4.8 Generation of the GFP::PAR-5 transgenic strain.

To generate a vector expressing the PAR-5 protein fused to GFP, we used the MultiSite Gateway Three-Fragment Vector Construction Kit (Invitrogen). This technology allows the combination of three pENTRY clones (5' element, middle element and 3' element) to generate expression vectors by using *att* flanked sequences and recombinase reactions (Figure 14). BP and LR clonase enzymes were purchased directly from Invitrogen for recombination reactions. All reactions were done according to manufacturer's instructions. We combined three entry vectors: pENTRY 1, containing the *par-5* promoter and the 5'UTR (1620 bp upstream the ATG of the *par-5* gene); pENTRY 2, containing the *gfp* sequence and pENTRY 3, containing the full *par-5* genomic sequence and the 3'UTR (from ATG until 600bp downstream of the termination codon). pENTRY 1 (pM117.2_93) was purchased from the promoterome (distributed by Lifesciences), and pENTRY 2 (pCM1.53 from Addgene) was a gift from Dr. Ben Lehner. To generate pENTRY 3 we used an adaptor strategy to add *att* sites to the genomic sequence of *par-5*. Two rounds of PCR were used (Figure 14A). First, genomic DNA was amplified using primers (attB2r-3' par5-ORF+5'UTR FW and attB3-3' par-5 ORF+5'UTR RV) containing half of the *att* sites and a specific sequence for the genomic *par-5*. The resulting PCR product was used as a template for the second amplification using the adaptor primers (attB2r-5' adapter FW and attB3-5' adapter RV) to

complete the *att* sites (Figure 14A). Phusion High-Fidelity polymerase (Finnzymes) was used for both amplifications, following the manufacturer's instructions. Genomic DNA was extracted from N2 worms using the PureLink Genomic DNA Mini kit (Invitrogen). After the second amplification, the resulting *par-5* genomic sequence flanked by *att*B2r and *att*B3 sites was inserted into the pDONR P2R-P3 using BP recombination, generating the pENTRY 3 (Figure 14B). This vector was transformed in One ShotR TOP10 Chemically Competent bacteria (Invitrogen) and later purified using the Miniprep kit (Qiagen). Proper recombination was confirmed by digestion with EcoRV enzyme. Purified pENTRY 3 vector was sequenced using M13 primers. To obtain the GFP::PAR-5 expressing vector (containing *par-5* promoter:5'UTR:GFP:*par-5*genomic:3'UTR) pENTRY 1, pENTRY 2 and pENTRY 3 vectors were combined with the pCFJ150 vector (Frokjaer-Jensen et al., 2008) using a multiple fragment LR recombination (Figure 14C). The final expression vector was transformed in Library Efficiency DH5α competent bacteria (Invitrogen), purified and verified by sequencing using the M13 primers. Primer sequences are listed in Table 3.

The expression construct "*par-5* promoter:5'UTR:GFP:*par-5*genomic:3'UTR" was used to bombard N2 animals using a gene gun provided by Dr. Ralph Schnabel. In brief, N2 worms were grown until they were adults with few embryos inside. Prior to bombardment, 20 µL of worm pellet was transferred to ice-cold 35 mm plates containing a dry and thin bacterial layer (~20 µL). DNA-coated gold particles were prepared by mixing respectively 1 mg of Gold (Chempur, 0,3-3 um diameter) with 100 µL of 50 µM spermidin (Sigma, Cat. # S-0266 in deionised water, sterile filtered) and 16 µg of DNA. Precipitation was performed by adding 100 µL of 1 M $CaCl_2$ solution. Before resuspension with 0.1 mg/mL polyvinylpyrolidon (dissolved in ethanol) (Sigma, Cat. # P-5288), gold particles-DNA were washed three times with 96% ethanol. Eight plates were shot and the agar cut into six pieces, each being put onto a fresh 90 mm plate and incubated at 20ºC. Stable transgenic worms were selected by checking GFP expression in the F1.

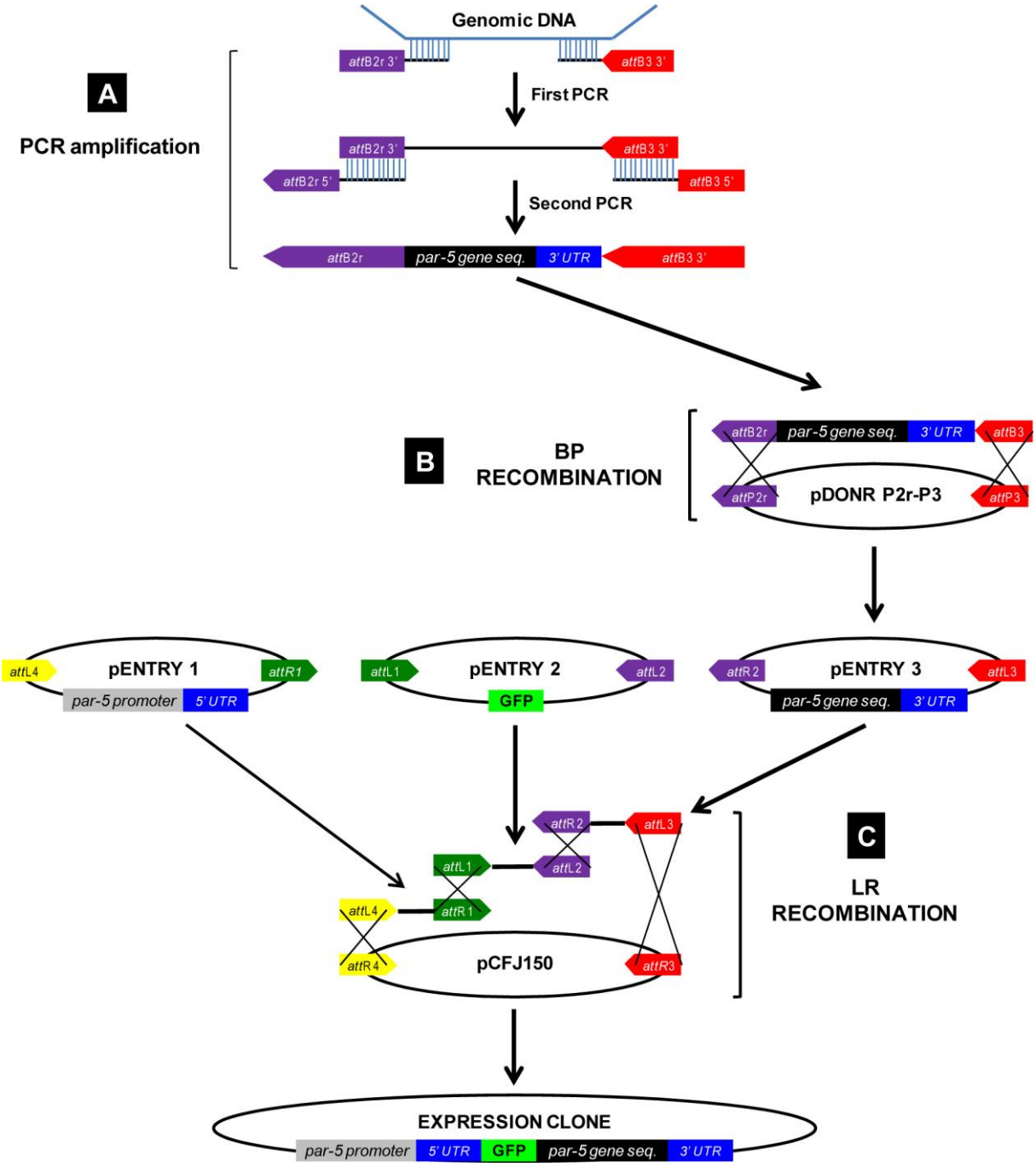


Figure 14. Cloning strategy for the GFP::PAR-5 expressing vector construction.
See text for details.

4.9 Western blotting

Adult worms were washed off plates with M9 buffer and rocked for 30 minutes. Later, they were washed twice with M9, and the pellets were mixed with lysis buffer 2X (4% SDS, 100 mM Tris HCl pH 6.8, 20% glycerol, 1X protease inhibitor cocktail -Calbiochem-, 1 mM orthovanadate, 2 mM NaF, 10 mM glycerol 2-phosphate disodium, 500 nM sodium pyrophosphate). Once mixed, the pellets were incubated in boiling water for 15 minutes. The obtained lysates were quantified, electrophoresed on SDS 12% polyacrylamide gels and electroblotted onto nitrocellulose membranes. 5% Non- fat milk diluted in TBS-Tween buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 0.05% Tween) was used to block the membranes. Blotting was carried out using primary antibodies for PAR-5 (from Andy Golden) and tubulin (DSHB, Cat. # E7) incubated overnight at 4ºC and diluted 1:4000 and 1:10000 respectively. Secondary antibodies, HRP conjugated anti-rabbit and anti-mouse (DAKO), were incubated for 1 hour at room temperature. All the antibodies were diluted in TBS-Tween. Autoradiography-films were used to detect protein bands.

4.10 Quantitative RT-PCR

Adult worms were washed off plates with M9 buffer and rocked for 30 minutes. Then, they were washed twice with M9, and the pellets were mixed with TRI REAGENT (MRC) to extract RNA following the manufacturer's instructions. RNA integrity was checked by agarose electrophoresis and fluorometric methods. For cDNA synthesis, a High Capacity Retro Transcription kit (Applied Biosystems) was used. SYBR-GREEN (Applied Biosystems) reagent was used to perform cDNA amplification reactions followed by real-time quantification using the ABI PRISM 7500 system. The fold change expression of the corresponding genes was based on the ddCT method and normalized relative to the amplification obtained using *act-1* (actin) primers. Primer sequences are listed in Table 3.

Primer	Sequence	Application
attB2r-3' par5-ORF+5'UTR FW	5' TTGTACAAAGTGGAAATGTCCGATACCGTGGAAGAGCTC 3'	GFP::PAR-5 transgene construction
attB3-3' par-5 ORF+5'UTR RV	5' TATAATAAAGTTGATTCGGACAACCGGAAAAGTTCAC 3'	GFP::PAR-5 transgene construction
attB2r-5' adapter FW	5' GGGGACAGCTTTCTTGTACAAAGTGG 3'	GFP::PAR-5 transgene construction
attB3-5' adapter RV	5' GGGGACAACTTTGTATAATAAAGTTG 3'	GFP::PAR-5 transgene construction
M13 FW	5′ GTAAAACGACGGCCAG 3′	GFP::PAR-5 transgene construction
M13 RV	5′ CAGGAAACAGCTATGAC 3′	GFP::PAR-5 transgene construction
L4440 FW	5' GTTTTCCCAGTCACGACGTT 3'	RNAi clones confirmation
par-5 FW	5' ACCGCGTCAAGGTTGAGCAAGA 3'	qRT-PCR
par-5 RV	5' ACAACGGCAGCGCGATCCTC 3'	qRT-PCR
ftt-2 FW	5' TCCGGAGACGACAGAAACTCGGT 3'	qRT-PCR
ftt-2 RV	5' CTGGCAAGCCTTGTCCGGGG 3'	qRT-PCR
act-1 FW	5' CCGCTCTTGCCCCATCAACCA 3'	qRT-PCR
act-1 RV	5' CGATGGATGGGCCGGACTCG 3'	qRT-PCR

Table 3. List of primers used in this study

5 RESULTS

5.1 *par-5* is required for proper germline development

In order to investigate the role of *par-5* in the adult germline we studied phenotypes in *par-5*(*RNAi*) and *par-5(it55)* worms. The *it55* mutation corresponds to a single amino acid substitution (from Alanine to Valine) that reduces the expression level of the PAR-5 protein (Morton et al., 2002). Since *it55* is a hypomorphic allele rather than null, we also used RNAi by feeding to knockdown the PAR-5 protein and analyze the consequence of stronger *par-5* inactivation in germ cells.

5.1.1 Germ cells proliferation depends on *par-5* function

As a first approach, by observing DAPI stained germlines at one-day adult stage, we found that the number of germ cells and gonad size were reduced in the *par-5* mutant strain. Because the molecular consequences of the *it55* mutation were uncharacterized, we wanted to explore whether this allele showed a temperature dependent phenotype. We studied germ cell proliferation in *par-5(it55)* worms at different temperatures but the reduction in germline proliferation was not significantly different. However, germ cells number was slightly increased at 15ºC, compared to 20ºC and 25ºC (Figure 15). The germline proliferation defect was more dramatic in *par-5* RNAi-fed worms (Figure 16A). In contrast to wild type (WT) and *par-5* mutants, *par-5*(*RNAi*) gonads showed a rather small and misshapen morphology with fragmented nuclei, indicating not only a failure in proliferation but also mitotic catastrophe and genome instability in the proliferative region. Furthermore, we were able to modulate *par-5* phenotypes by diluting or administrating the RNAi feeding clone at different stages. This implies that the observed germline defects depend on *par-5* levels.

By performing a time course analysis of the germline development, we found that the proliferative defect in *par-5*-defective worms is evident at the L4 stage when hypercondensed and fragmented nuclei are detected. After this stage, the number of germ cells decays in *par-5*(*RNAi*) germlines in contrast to the continuous proliferation observed in the WT and *par-5(it55)* (Figure 16B). Despite the significant reduction in germ cells in *par-5(it55)* worms, nuclei fragmentation was not as abundant in *par-5* mutants as it was in *par-5*(*RNAi*) animals

(Figure 16A). The difference between *par-5(it55)* and *par-5*(*RNAi*) phenotypes implies that the *it55* allele is hypomorphic rather than null. To corroborate this, we observed that *par-5(it55)* fed with *par-5* RNAi presented a *par-5*(*RNAi*) phenotype (Figure 16A).

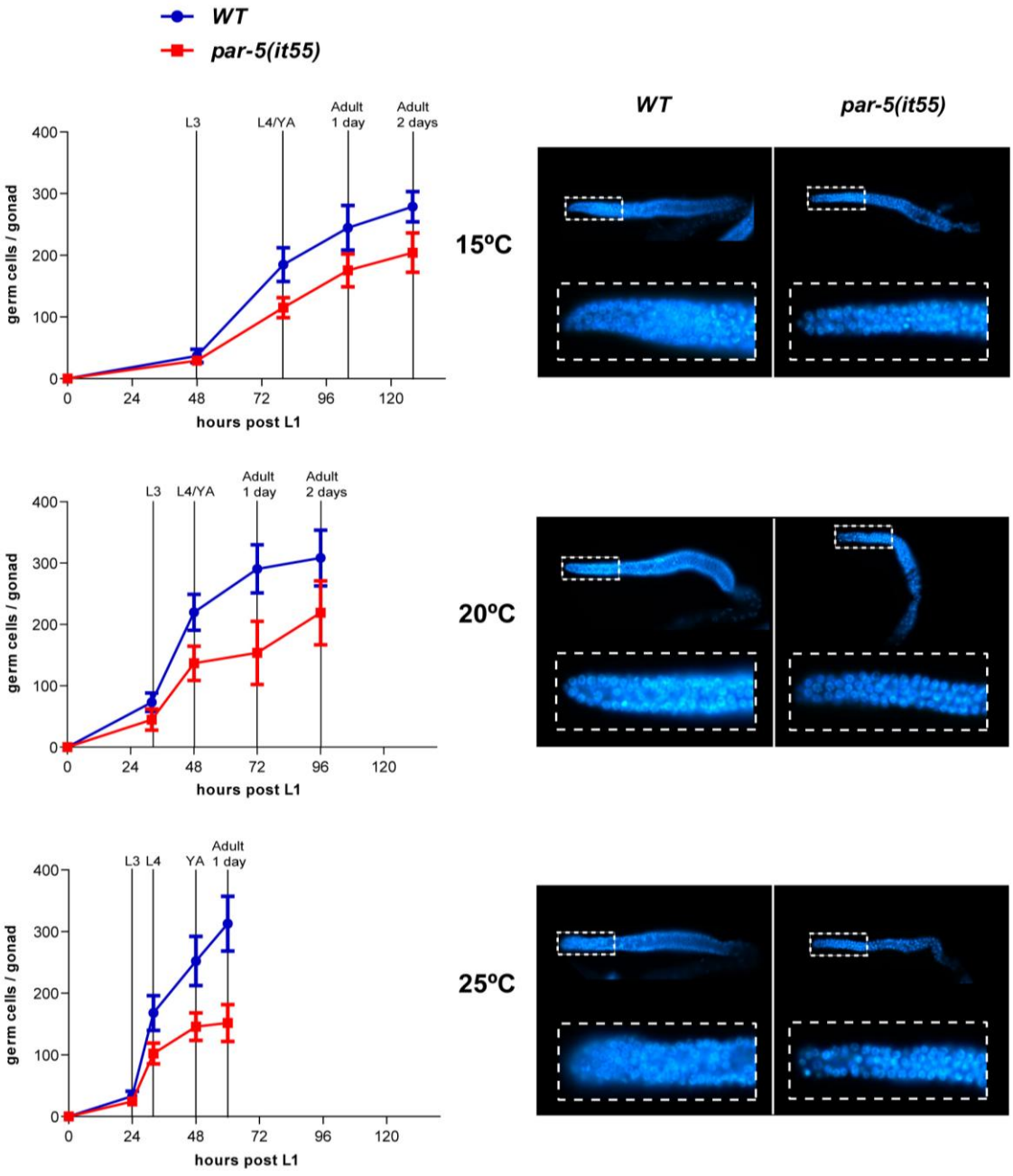


Figure 15. ***par-5(it55)*** **germline development at different temperatures**.
Graphs represent the number of germ cells per gonad at different developmental stages of WT and *par-5(it55)* animals. Synchronized L1 larvae were grown at 15°C, 20°C or 25°C, and then fixed and stained with DAPI at the indicated times. Representative images of DAPI-stained germlines from WT or *par-5(it55)* worms (one-day adults) at the indicated temperatures are shown on the right side. The proliferative regions of germlines are highlighted and magnified.

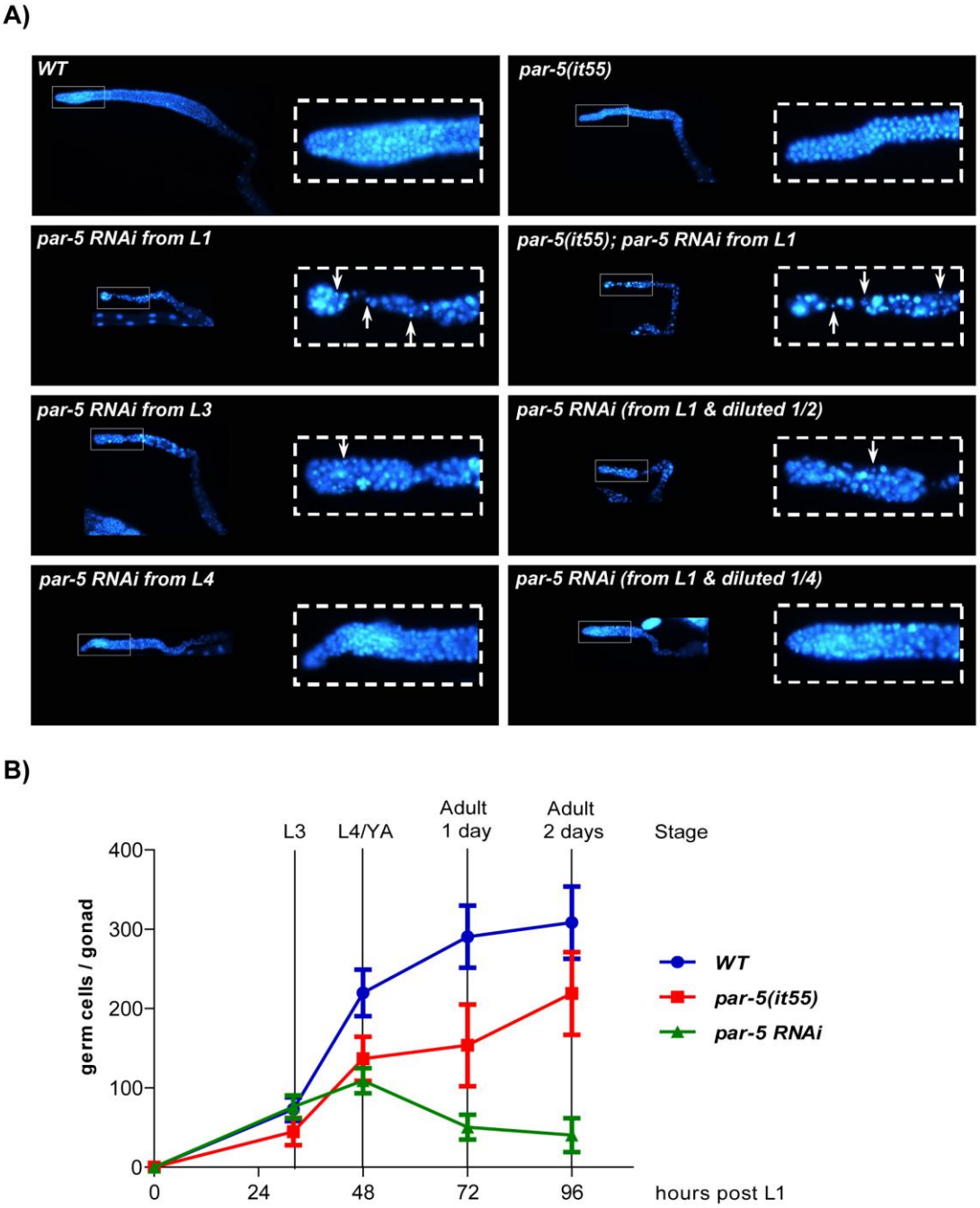


Figure 16. *par-5 (RNAi)* causes a more penetrating germline proliferation defect than the *par-5(it55)* mutation.
A) Representative images of DAPI stained germlines (1 day old adults) from WT or *par-5(it55)* worms (1 day adults) fed with the *par-5 RNAi* from the indicated larval stages and the *par-5* RNAi clone diluted at the indicated proportions with the control L4440 clone. Proliferative regions of germlines are shown enlarged in rectangles. Arrows indicate hypercondensed and fragmented nuclei. **B)** Graph showing the number of germ cells per gonad at different developmental stages for WT, *par-5(it55)* and *par-5* RNAi-fed worms. Synchronized L1 larvae grown at 20ºC were fixed and stained with DAPI at the indicated times. Error bars indicate standard deviations.

5.1.2 Meiotic progression is affected upon *par-5* depletion

In addition to the phenotypes in the proliferative region, most of the *par-5*(*RNAi*) gonads show either a reduction in the number or an absence of pachytene meiotic cells which are characterized by a "spaghetti-like" morphology (reflecting the chromosome recombination occurring at this stage) (Figure 17). This defect was clearly observed in the vicinity of the gonad bend, where germ cells normally progress from pachytene to diplotene to produce oocytes. Consequently, most of *par-5(RNAi)* germlines present lack of properly differentiated oocytes, suggesting that *par-5* is implicated not only in germline proliferation but also in meiotic progression (Figure 17). Interestingly, *par-5(RNAi)* animals present apparently normal sperm cells (Figure 17), indicating that meiotic progression is particularly impaired in oocytes when *par-5* expression is compromised. As a consequence, *par-5* RNAi produced more that 80% of sterile adults.

These meiotic defects where not observed in the *par-5(it55)* strain, demonstrating again that this allele produces a mild defect in the protein function, rather than being a null allele. In agreement with previous studies reported in the literature, we found that *par-5(RNAi)* and *par-5(it55)* animals show 100% embryonic lethality, which is related with the already known role of *par-5* in the early embryonic development (Morton et al., 2002).

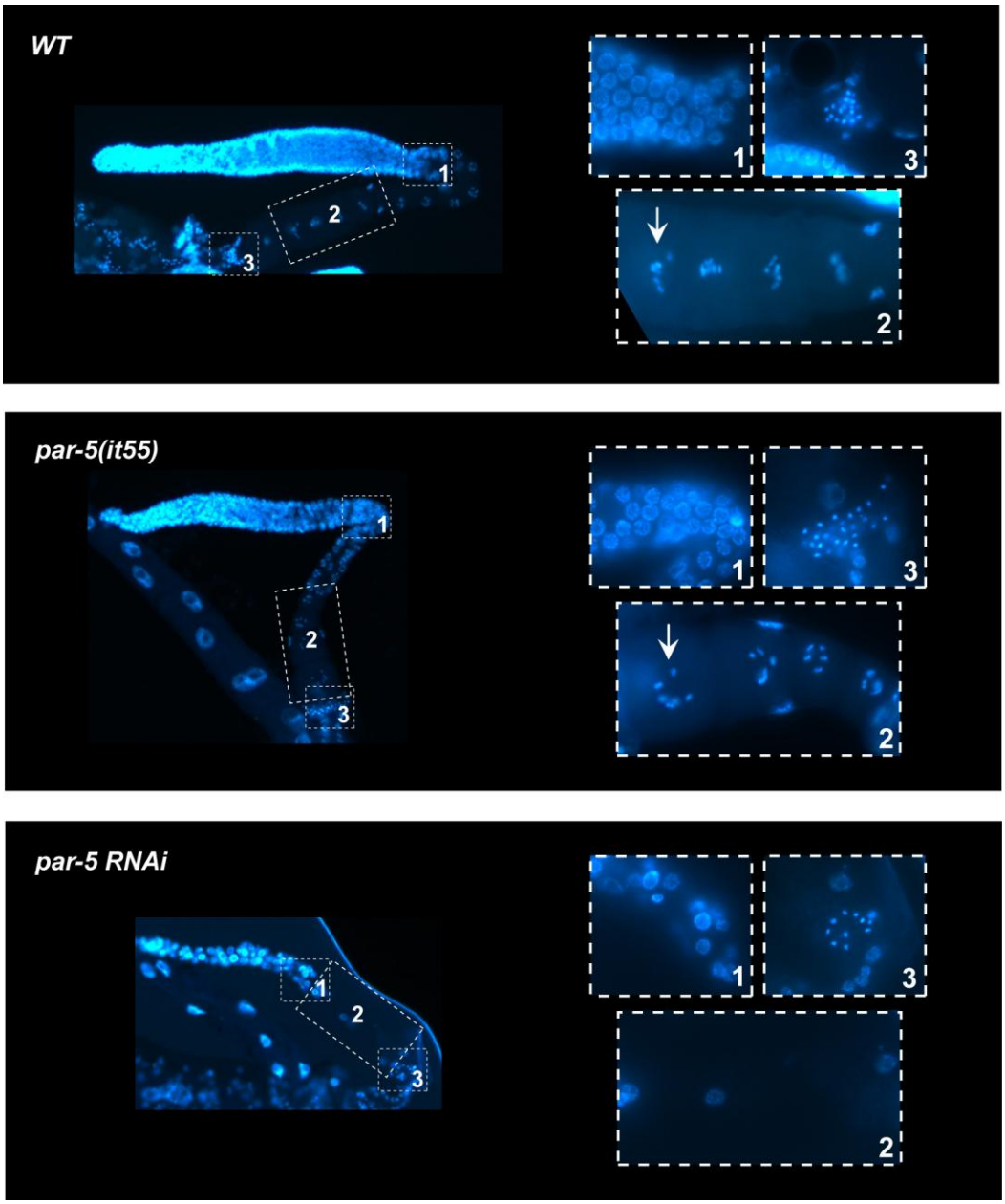


Figure 17. *par-5* meiotic phenotypes.
Representative images of DAPI stained germlines for WT, *par-5(it55)* and *par-5(RNAi)* worms. Meiotic cells at the gonad bend (1), mature oocytes in the proximal region (2) and sperm (3) are shown at higher magnification. Arrows point the last oocyte of the gonad before the spermatheca.

5.1.3 Germline phenotypes in *par-5* defective worms are not caused neither by somatic defects nor altered apoptosis

The *par-5* gene is also expressed in somatic tissues, therefore, the germline proliferation defect observed after *par-5* knockdown could be explained by the influence of the somatic gonad on germline proliferation (Killian and Hubbard, 2005). However, *par-5* RNAi treatment in the *rrf-1(pk1417)* background (strain with defective RNAi in somatic cells) showed the same germline phenotype as that in WT animals (Figure 18). Therefore, the *par-5* knockdown effect on the germline is independent of the somatic functions of *par-5*.

The reduction of germ cells in the *par-5* defective animals could be also explained by an increase in the number of cells undergoing physiological apoptosis. In the course of the germline development, almost half of the mature meiotic cells are eliminated by this mechanism (Gartner et al., 2008). Nevertheless, we discarded this hypothesis since *par-5* suppression does not increase the number of apoptotic corpses at the gonad bend, where the physiological apoptosis takes place (Figure 19).

Observations from the last 3 sections indicate that *par-5* function is required for the proliferation and meiotic progression of the germ cells. Defects in these processes results in the sterility of *par-5* depleted animals.

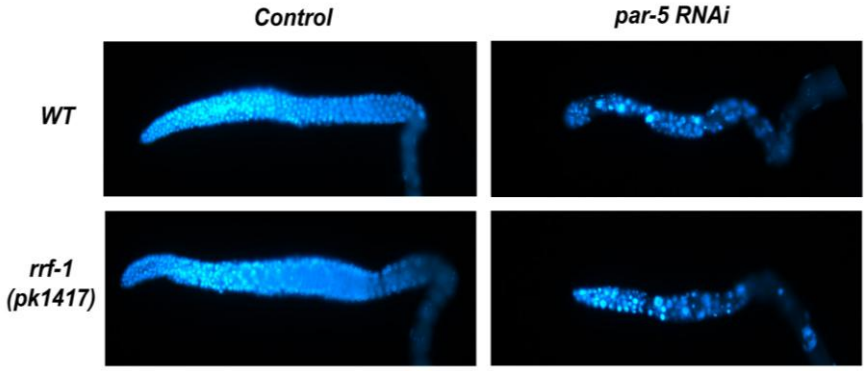


Figure 18. *par-5* RNAi is not affecting germline proliferation through somatic regulation.
Representative images of germlines from WT or *rrf-1(pk1417)* mutants worms (1 day adults) fed with the L4440 vector or the *par-5* RNAi and stained with DAPI.

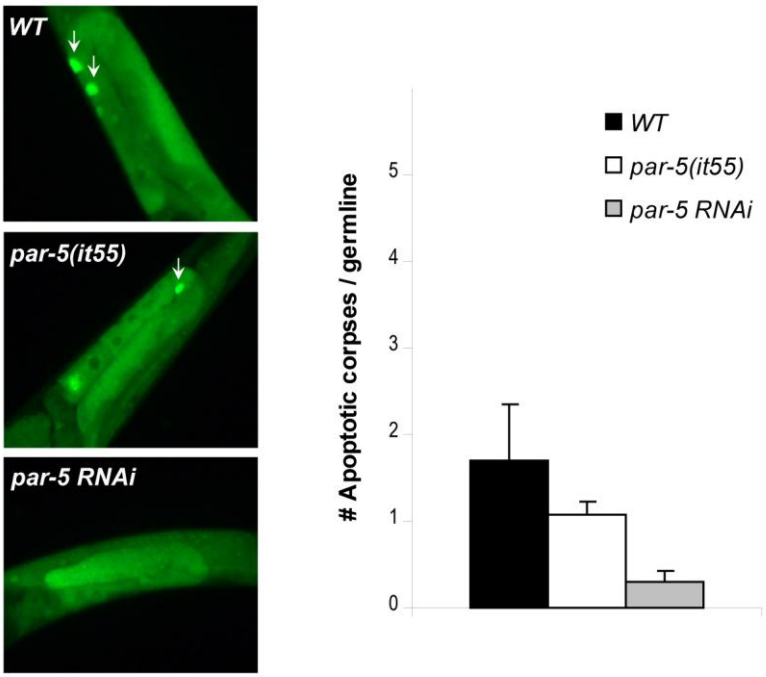


Figure 19. **Physiological apoptosis is not increased by *par-5* suppression.**
Representative images of germlines from WT, *par-5(it55)* or *par-5* RNAi fed worms (1 day adults) stained with Syto-12. The graphic shows the quantification of positive Syto-12 corpses (pointed by arrows) at the gonad bend. Error bars indicate standard deviation of mean from three experimental replicates.

5.2 *par-5* RNAi does not affect *ftt-2* levels

par-5 shares ~80% sequence homology with *ftt-2*, which is the other 14-3-3 *C. elegans* gene. Because we were using an RNAi clone targeting the whole *par-5* ORF sequence, we were concerned about the specificity of the gene knockdown. To test whether the observed RNAi phenotype was *par-5* specific, we quantified *par-5* and *ftt-2* transcript levels using quantitative RT-PCR after *par-5* RNAi treatment. This experiment showed that *par-5* RNAi depleted *par-5* mRNA, whereas *ftt-2* transcript levels were unaffected (Figure 20). Therefore, the germline defects observed in *par-5(RNAi)* animals are independent of *ftt-2* function. This was anticipated because *ftt-2* is not expressed in the germline (Wang and Shakes, 1997).

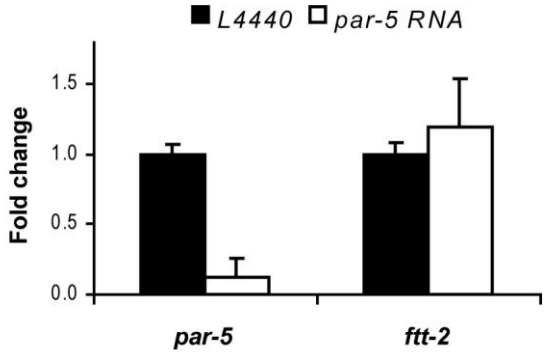


Figure 20. *par-5* RNAi does not affect *ftt-2* mRNA levels.
mRNA quantification (using quantitative RT-PCR) of *par-5* and *ftt-2* transcripts in WT worms fed with *par-5* RNAi or the L4440 control vector. The fold change expression is expressed relative to the control. Error bars indicate the standard deviation of the mean from three independent experiments.

5.3 Inactivation of *par-5* promotes endogenous DNA damage accumulation

Because we found a reduced number of germ cells and DNA fragmentation after *par-5* inactivation by RNAi (Figure 16), we wanted to further investigate the role of *par-5* in the maintenance of DNA stability. RAD-51 protein is involved in DNA repair by homologous recombination and is a marker of processed double-strand breaks (DSBs) and stalled replication forks (Alpi et al., 2003; Ward et al., 2007) Therefore, we examined the abundance of RAD-51 foci. Interestingly, we observed a 10-fold increase in the number of RAD-51 foci at the proliferative region of *par-5*(*RNAi*) worms (Figure 21). This increase is similar to that obtained with the checkpoint defective strain *atl-1(tm853)*, used as a positive control of genomic instability. A raise in RAD-51 foci was also observed in the *par-5(RNAi)* meiotic cells, however it was less evident and difficult to score since the WT strain already present many foci, as a result of meiotic recombination. The *par-5(it55)* strain does not present such accumulation of DNA damage in the proliferative region, although there was a slight increase of RAD-51 foci compared to the WT germline (Figure 21B).

To corroborate the role of *par-5* in preserving genomic stability, we used a transgenic strain expressing the fusion protein HUS-1::GFP, which is a DNA damage sensor protein that forms defined foci at DSBs (Hofmann et al., 2002). The meiotic region of WT animals showed a few HUS-1::GFP foci as a result of transient DSBs that occur during meiotic recombination.

However, *par-5(RNAi)* meiotic germ cells showed a marked increase in the number of HUS-1::GFP foci, indicating higher accumulation of DSBs (Figure 22A).

In addition to the increase in DNA damage markers (RAD-51 and HUS-1 foci), *par-5*(*RNAi*) worms showed a constitutive phosphorylation (at Serine 345) of the checkpoint kinase CHK-1 in germ cells localized at the proximal part of the proliferative region (Figure 22B). This modification has been associated with recombination defects that trigger meiotic checkpoint activation (Jaramillo-lambert et al., 2007). Notably, the same pattern was also observed in the *atl-1(tm853)* strain, whereas this phenotype was rarely present in WT worms and *par-5(it55)* mutants. Therefore, the RNAi depletion of *par-5* seems to cause pre-meiotic checkpoint activation similar to the effect of inactivating genes that control DNA stability such as *atl-1*.

Altogether, these results suggest that *par-5* is necessary for proper DNA maintenance, since its inhibition promotes DNA damage accumulation both in proliferating and in meiotic germ cells. In addition, DNA instability in meiotic cells of *par-5(RNAi)* animals seems to trigger a constitutive activation of the DNA damage response, by phosphorylating the CHK-1 kinase.

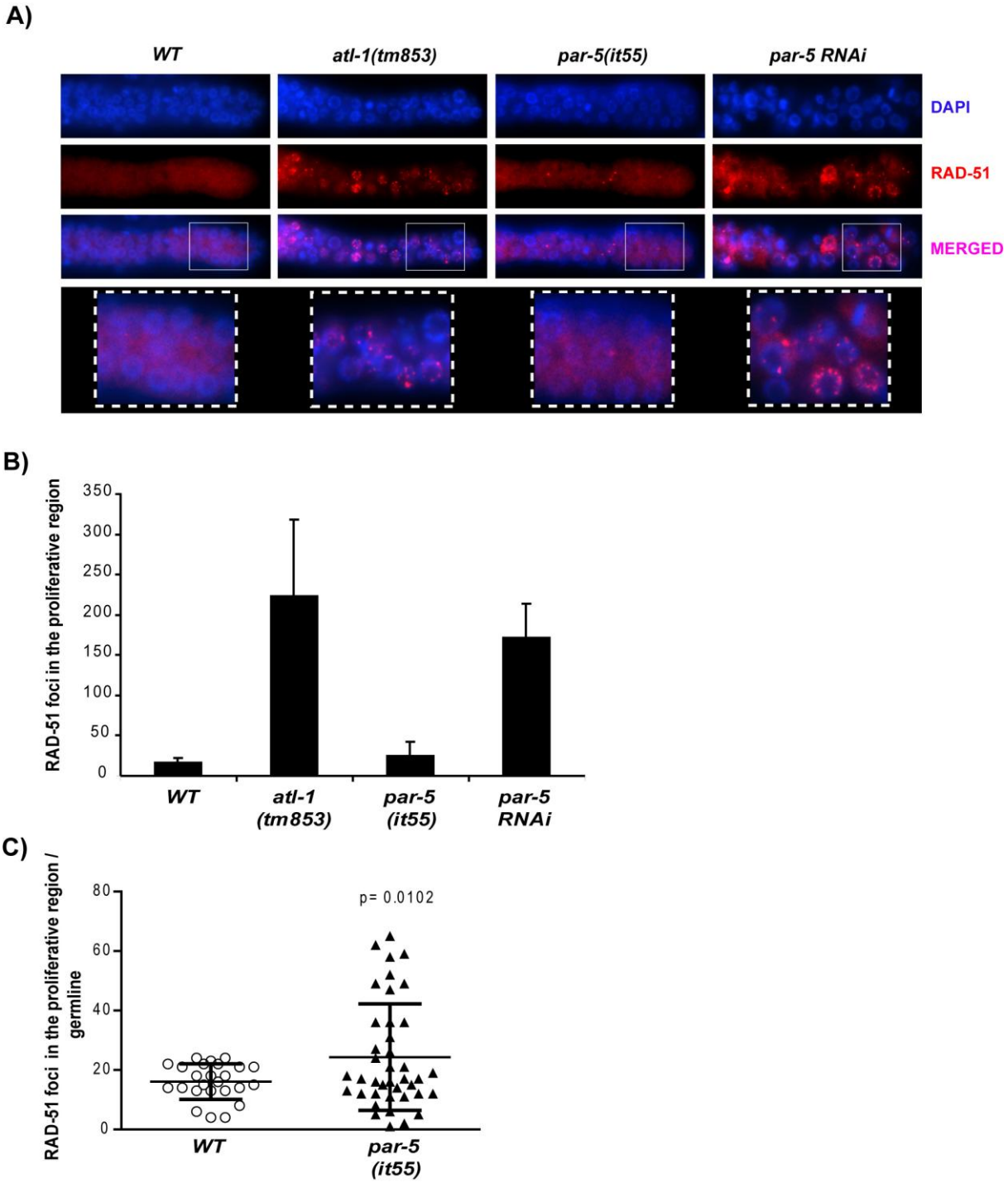


Figure 21. *par-5* inactivation promotes RAD-51 accumulation.
A) Representative images of germline proliferative regions from worms of the indicated genotypes/RNAi, immunostained with a RAD-51 antibody and counterstained with DAPI. Distal proliferative regions enlarged in squares show RAD-51 foci nuclear localization. **B)** Graph showing RAD-51 foci quantification in all the optical stacks within 30 μm of the distal end of the gonad. Error bars indicate standard deviation of the mean for at least 15 germlines. **C)** Graph showing the distribution of RAD-51 foci quantification per gonad in WT and *par-5(it55)* animals. p-value corresponds to a t-student test.

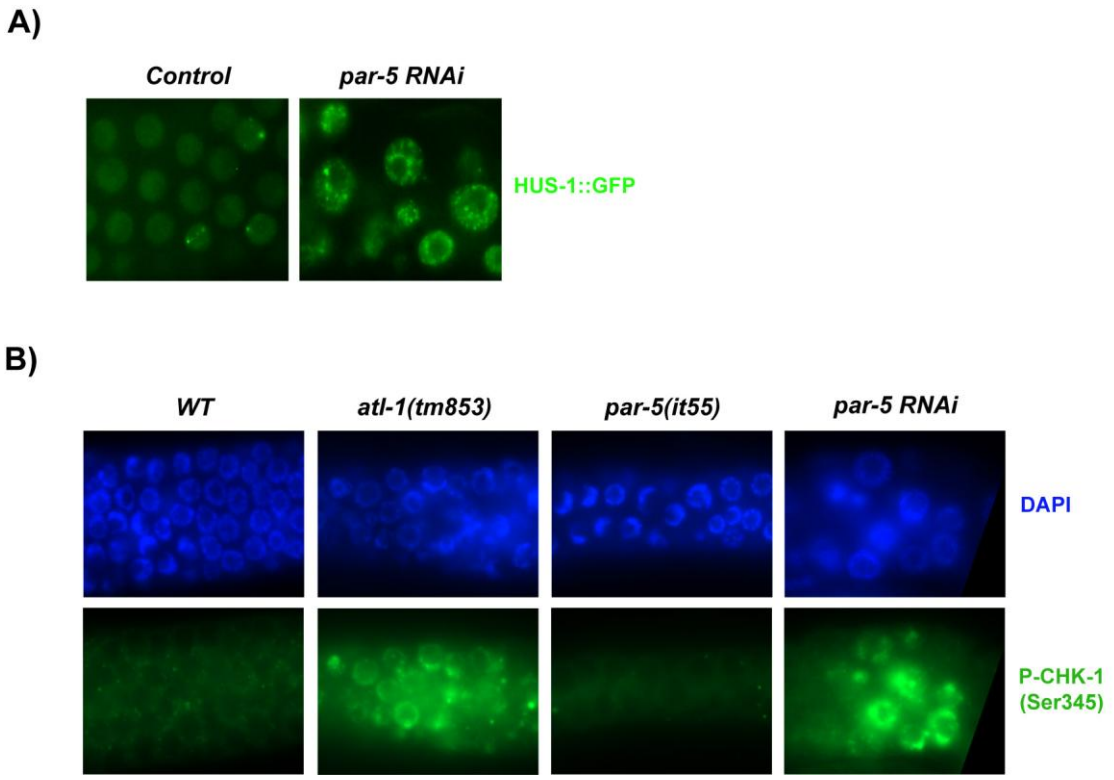


Figure 22. DNA damage accumulation in *par-5(RNAi)* meiotic cells.
A) HUS-1::GFP foci increase after *par-5* knockdown. Representative images of the meiotic germ cells from a transgenic strain expressing the HUS-1::GFP fusion protein with or without *par-5* RNAi treatment**. B) CHK-1 phosphorylation is detected in pre-meiotic germ cells after *par-5* RNAi knockdown**. Representative images of the pre-meiotic germ cells (cells between the proliferative and the transition region) from worms of the indicated genotypes/RNAi. Germlines were immunostained with a P-CHK-1 (Ser345) antibody and counterstained with DAPI. The percentage of germlines positively stained (at least 4–5 stained germ cells/gonad) with P-CHK-1 was: 5% for WT, 50% for *atl-1(tm853),* 10% for *par-5(it55)* and 75% for *par-5(RNAi).*

5.4 *par-5* is necessary for the cell cycle arrest induced by the checkpoint response

5.4.1 Role of *par-5* in the germline checkpoint

5.4.1.1 S phase checkpoint defect after par-5 *suppression upon replicative stress*

Nuclei fragmentation (Figure 16) and RAD-51 foci accumulation (Figure 21) observed in the proliferative region of *par-5* RNAi germlines resemble the effect of mutations on genes of the checkpoint pathway such as *atl-1*, *clk-2/rad-5* and *chk-1* (Kalogeropoulos et al., 2004; Garcia-

Muse and Boulton, 2005). Thus, we tested whether *par-5* was actively implicated in the DDR under replicative stress induced by HU. HU inhibits the activity of the ribonucleotide reductase enzyme, causing the depletion of dNTP levels and thereby hampering DNA replication (Kim et al., 1967). After HU treatment, cells in the proliferative region of the germline arrest in S-phase as a result of checkpoint activation. This cell cycle arrest is evidenced by fewer nuclei with larger sizes (Gartner et al., 2004). Interestingly, after HU treatment, these checkpoint response marks were absent in *par-5*(*RNAi*) worms and *par-5(it55)* mutants (Figure 23). Instead of big arrested cells, *par-5* defective gonads showed fragmented and hypercondensed nuclei. Such incapacity to arrest the cell cycle after HU treatment was also observed in mutants for the checkpoint gene *atl-1*.

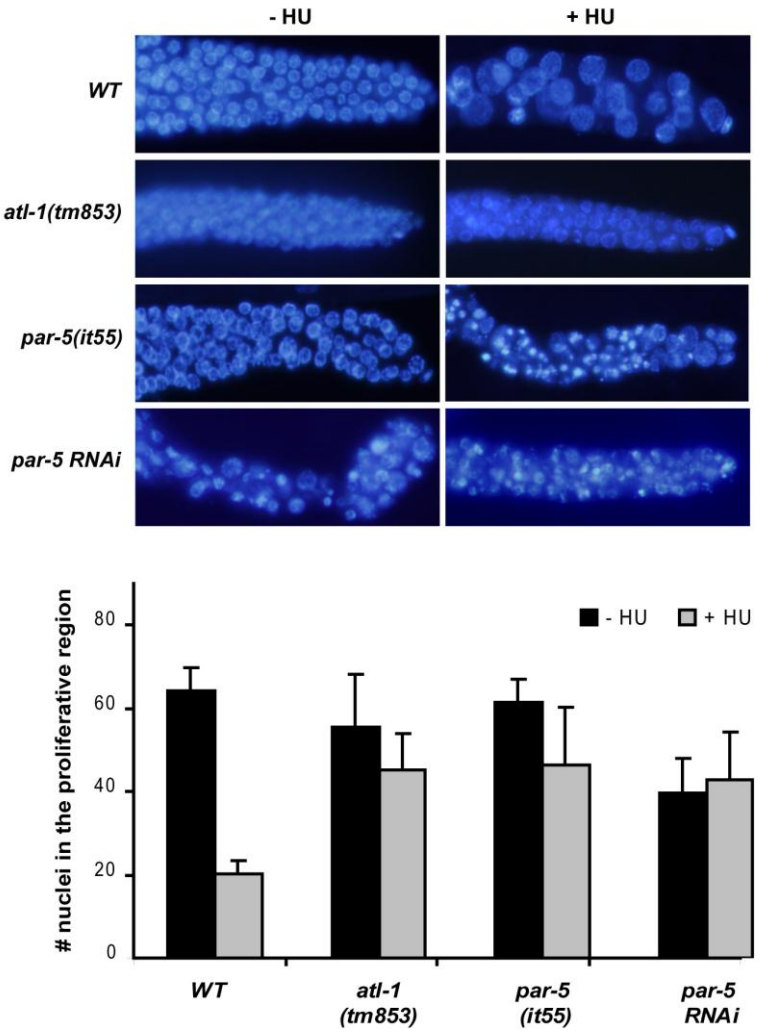


Figure 23. *par-5* is required for HU-induced cell cycle arrest.
Representative images of germline proliferative regions from worms of the indicated genotypes/RNAi, treated with or without HU and stained with DAPI. The graph shows germ nuclei quantification. Error bars indicate standard deviations.

5.4.1.2 par-5 *is also required for the G2/M checkpoint in response to DSBs*

To investigate whether the checkpoint role of *par-5* was exclusive for the S-phase, we examined its implication in the G2/M checkpoint. Similar to HU, IR induces a cell cycle arrest in the proliferating region of the germline. However, IR-induced DSBs not only promote S phase but also G2 cell cycle arrest. *par-5*(*RNAi*) and *par-5* mutant germ cells bypass the cell cycle arrest induced by IR, which results in DNA fragmentation (Figure 24). This suggests that the *par-5* role in checkpoint is also necessary for the G2/M arrest in response to DSBs.

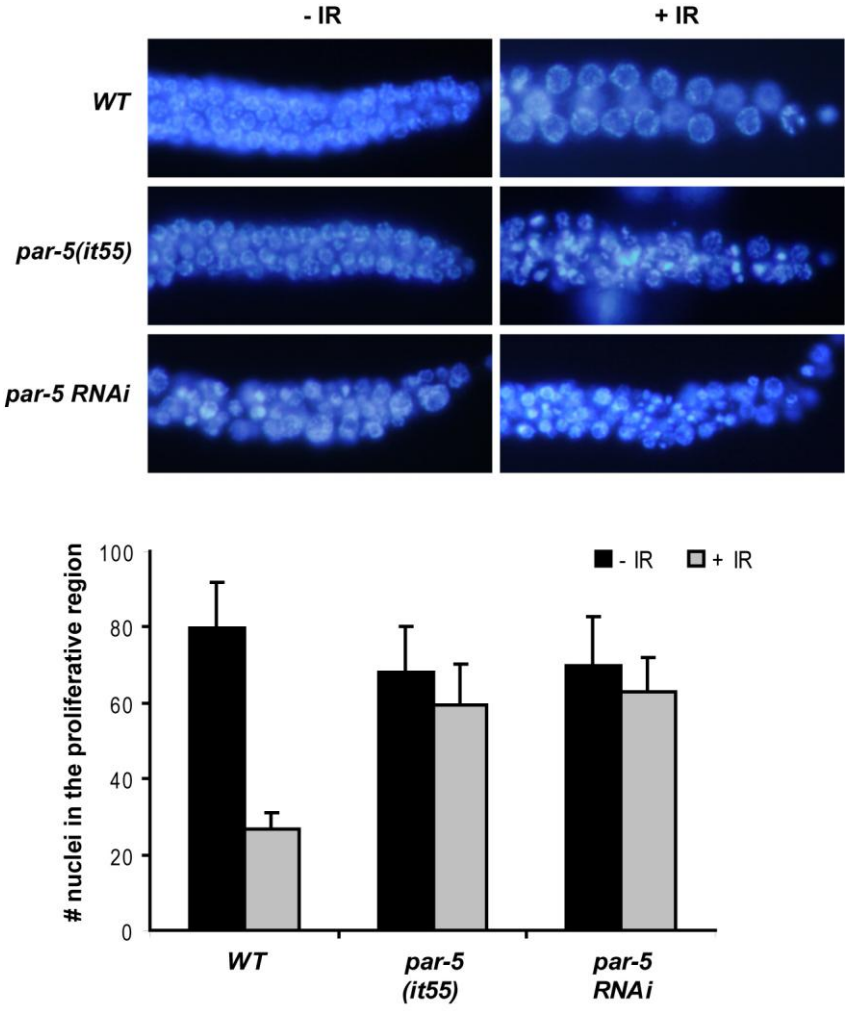


Figure 24. ***par-5*** **is necessary for IR-induced response.**
Representative images of germline proliferative regions from worms of the indicated genotypes/RNAi, irradiated (+IR) or not irradiated (-IR) with γ-rays. The graph shows germ nuclei quantification and error bars indicate standard deviations.

These experiments indicate that *par-5* is an essential gene to arrest the cell cycle in response to diverse exogenous insults, participating in both the S and the G2/M checkpoints.

5.4.1.3 Checkpoint function is not common for the PAR family.

PAR-5 belongs to the PAR family, which controls the first asymmetric cell division in the embryo. This process includes the tight regulation of the cycling time in the posterior and anterior cells (Suzuki and Ohno, 2006). Indeed, *par-1, par-2, par-3* and *par-4* have been shown required for the asymmetric distribution of the cell cycle regulators *cdc-25.1* and *plk-1* in the embryo. For this reason, we wondered whether the involvement in checkpoint was a common feature of the PAR family members. However, worms fed with RNAi against *par-2* and *par-3* (members of the anterior and posterior complexes that drive asymmetry in the embryo) showed normal cell cycle arrest after HU treatment in the germline (Figure 25).

5.4.1.4 **par-5** *checkpoint functions do not depend on ERK pathway*

par-5 has been also related to ERK pathway. This pathway coordinates distinct biological processes in the germline, ranging from developmental switches such as male germ cell fate specification, to other processes such as membrane organization and morphogenesis of pachytene cells. Specifically, *par-5* has been shown to enhance *mpk-1* activity in the germline, in regulating pachytene cellular organization and progression (Arur et al., 2009). Therefore, we checked whether *par-5* role in DDR was related to activation of the *mpk-1* pathway. However, we observed that inhibition of *mpk-1* did not affect cell cycle arrest, although the worms were sterile (Figure 25).

These results suggest that the role of *par-5* in checkpoint response is not a common feature of the PAR family. Furthermore, the *mpk-1* pathway is not related with the DDR pathway.

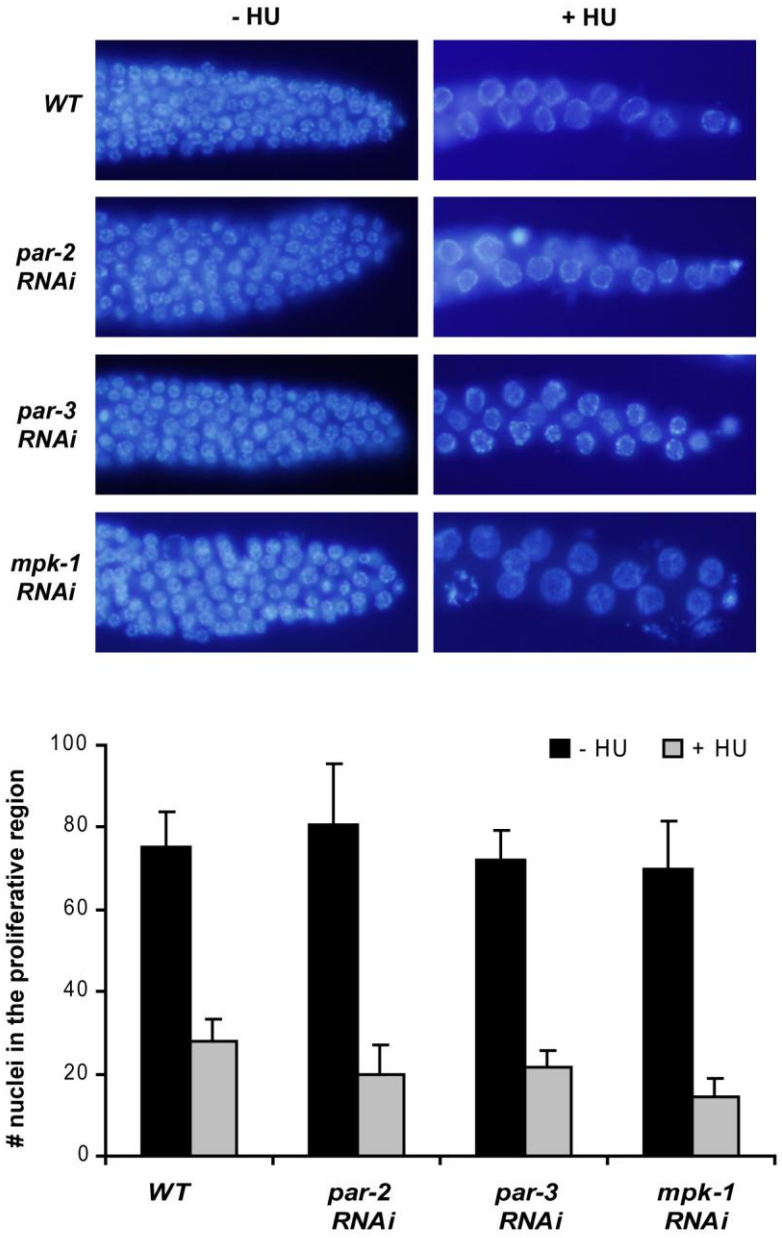


Figure 25. ***par-2, par-3*** **and** ***mpk-1*** **are dispensable for the S-phase checkpoint.**
Representative images of the germline proliferative regions from WT worms fed with the indicated RNAi, treated with or without HU and stained with DAPI. The graph shows germ nuclei quantification with the corresponding standard deviations.

5.4.2 Role of *par-5* in embryonic DDR

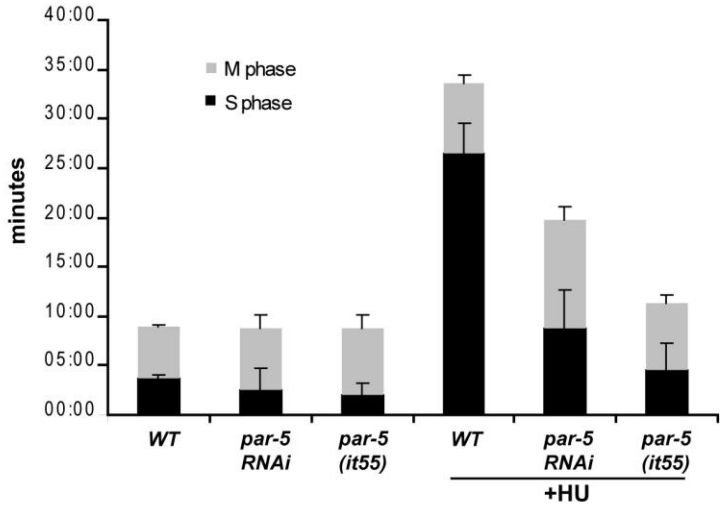
The *C. elegans* embryo is another context where checkpoint response has been widely studied. In particular, replicative stress mediated by either mutation in DNA replication genes (like *div-1*) or treatment with DNA replication inhibitors (like HU) causes a delay in the mitotic entry at the first embryonic division, as a consequence of a longer S-phase. This cell cycle delay is mediated by activation of the checkpoint pathway since *atl-1* or *chk-1* defective embryos present nearly normal cell cycle timing upon replication stress (Brauchle et al., 2003).

Because *par-5* is highly expressed in the embryo (Wang and Shakes, 1997), we addressed whether *par-5,* besides its described role in embryo asymmetric division, was also regulating the cell cycle delay in response to replication stress. Through several video recordings of the first embryonic division, we observed that *par-5*(*RNAi*) and *par-5*(*it55*) embryos significantly rescued the HU-induced cell cycle delay (Figure 26). HU treatment provoked a 7 fold increase in the S phase length of WT embryos, while this delay was of 3,5 fold and 2,2 fold in *par-5(RNAi)* and *par-5(it55),* respectively. Contrary to the previous results in the germline, *par-5(it55)* mutants showed a stronger phenotype compared to *par-5(RNAi)*. However, in this case the *par-5* RNAi was administrated from L4 instead of L1 to reduce the RNAi efficiency, allowing the production of embryos in the otherwise sterile *par-5(RNAi)* animals.

Interestingly, in the absence of HU, we found that *par-5* defective embryos presented a slightly shorter S phase and also a longer M phase compared to WT. To check whether this cell cycle pattern was common for the PAR family we analyzed video recordings of embryos lacking other PAR genes (video recordings were obtained from the phenobank) (http://www.worm.mpi-cbg.de/phenobank/cgi-bin/MenuPage.py). By performing cell cycle timing quantification in the first embryonic cell division we found that only *par-5* suppressed embryos showed a shorter S-phase accompanied by a delayed M-phase (Figure 27).

Therefore, *par-5* is required for the embryonic DNA replication checkpoint and probably for normal cell cycle progression in the embryo.

A)



B)

	-HU	
	S phase	**M phase**
WT	03:43 +/- 00:17	05:10 +/- 00:18
par-5 RNAi	02:27 +/- 02:17	06:23 +/- 01:21
par-5(it55)	01:57 +/- 01:16	06:51 +/- 01:14

	+HU	
	S phase	**M phase**
WT	26:29 +/- 02:58	07:05 +/- 00:49
par-5 RNAi	08:42 +/- 04:00	11:00 +/- 01:25
par-5(it55)	04:29 +/- 02:44	06:45 +/- 00:57

C)

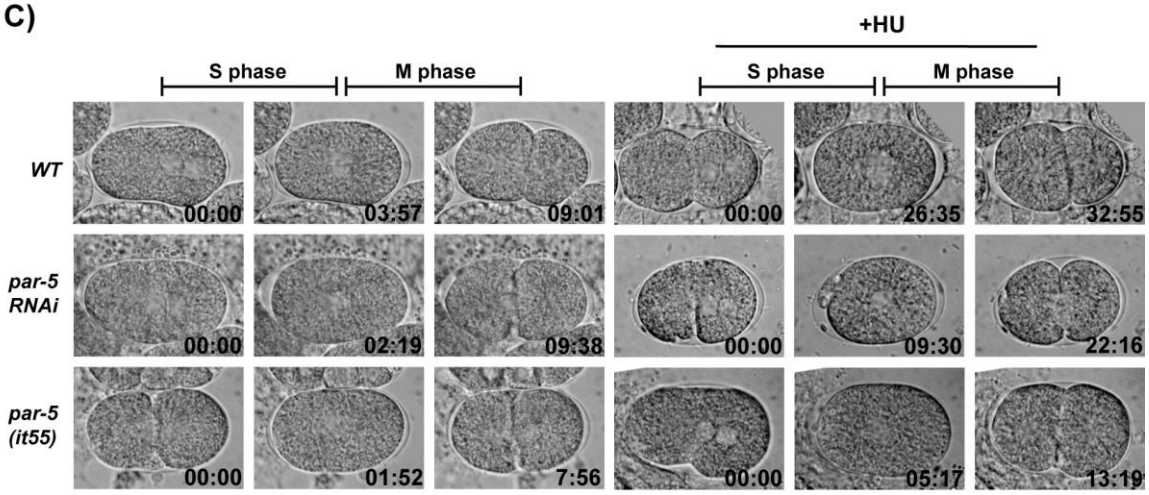


Figure 26. *par-5* is required for replication stress-induced cell cycle delay in the first embryonic division.

A) S and M-phase timing quantification corresponding to the first embryonic cell cycle (P0) of WT, *par-5(it55)* or *par-5(RNAi)* embryos from worms treated with or without HU. At least five embryos were recorded for each experimental point. Error bars indicate standard deviations. **B)** Table showing absolute values of timing quantification from A. **C)** Representative photos of embryos at the indicated times of video recordings, corresponding to the beginning of the S- and M-phases and the initial furrowing (end of the M-phase).

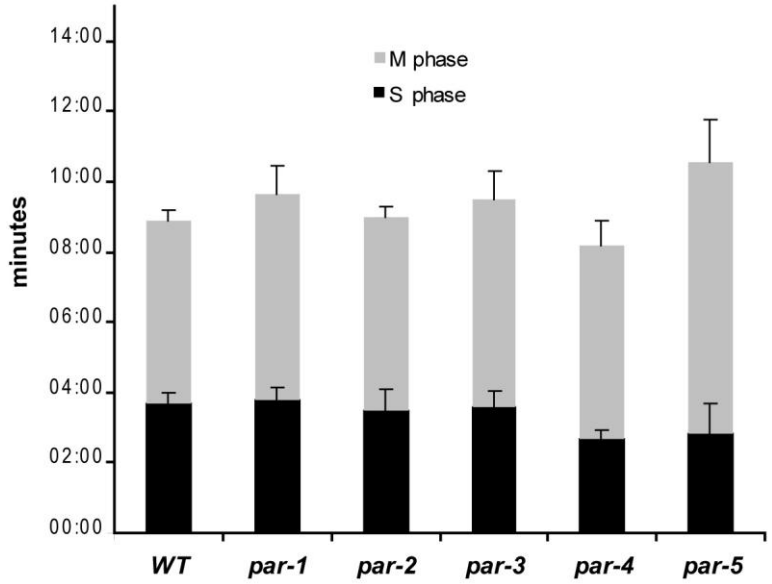


Figure 27. P0 cell cycle timing in *par* genes defective embryos.
The graph shows the S and M phases timing quantification corresponding to the first embryonic cell cycle (P0) of embryos treated with the indicated RNAi. Embryos video recordings were taken from the Phenobank.

5.5 *par-5* prevents premature entry into mitosis

While testing the germline response to HU and IR after *par-5* inhibition, we observed many germ nuclei that presented hypercondensed chromatin and reduced size. This effect, observed both in *par-5*(*RNAi*) and in *par-5*(*it55*) animals, was likely due to cells entering prematurely into mitosis before the DNA was properly replicated, thereby causing DNA fragmentation. To study this phenotype we used an antibody against phosphorylated histone 3 (P-H3) as a mitotic marker (Figure 28). Although the number of mitotic germ cells was reduced in WT animals as a result of the S-phase checkpoint activation, the inactivation of *par-5* (either by RNAi or mutation) caused an increase in the number of mitotic cells after HU treatment. Therefore, this result indicates that HU-treated germ cells, in which *par-5* function is impaired, are able to enter mitosis, thereby bypassing the S-phase checkpoint. Consistently, a similar phenotype was also observed in the *atl-1(tm853)* strain.

Although *par-5* activity in controlling premature mitotic entry becomes obvious after HU treatment, we also observed a slight increase in the number of P-H3-positive cells in *par-*

5(*RNAi*) and *par-5(it55)* unchallenged worms (Figure 28). Using a time course experiment, we detected an increase in the percentage of P-H3 cells and DNA fragmentation at L4 stage, which is the developmental stage chosen to expose worms to HU in our checkpoint assays (Figure 29).

All these results suggest that *par-5* is required to prevent premature entry into mitosis, both upon replicative stress and during normal germ cell proliferation. Such a function is a hallmark of checkpoint genes.

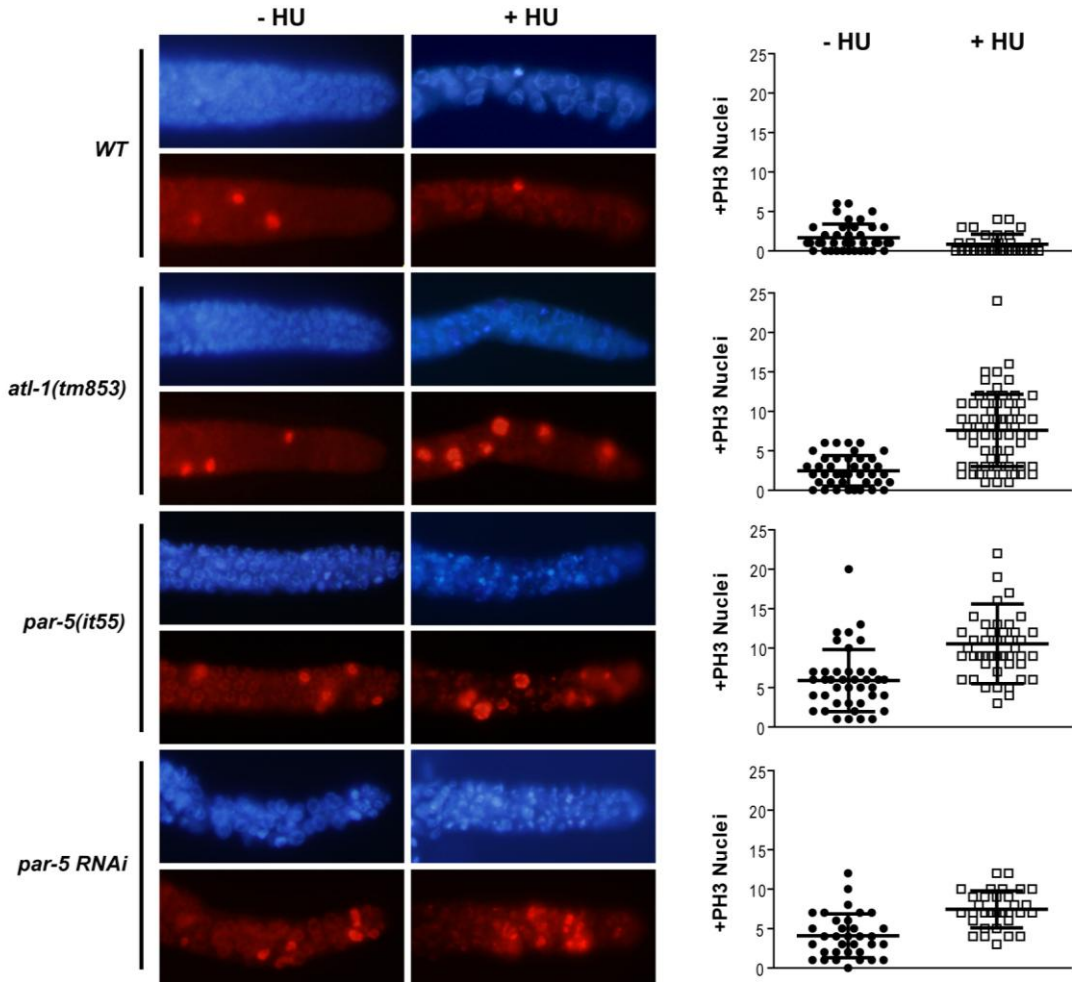
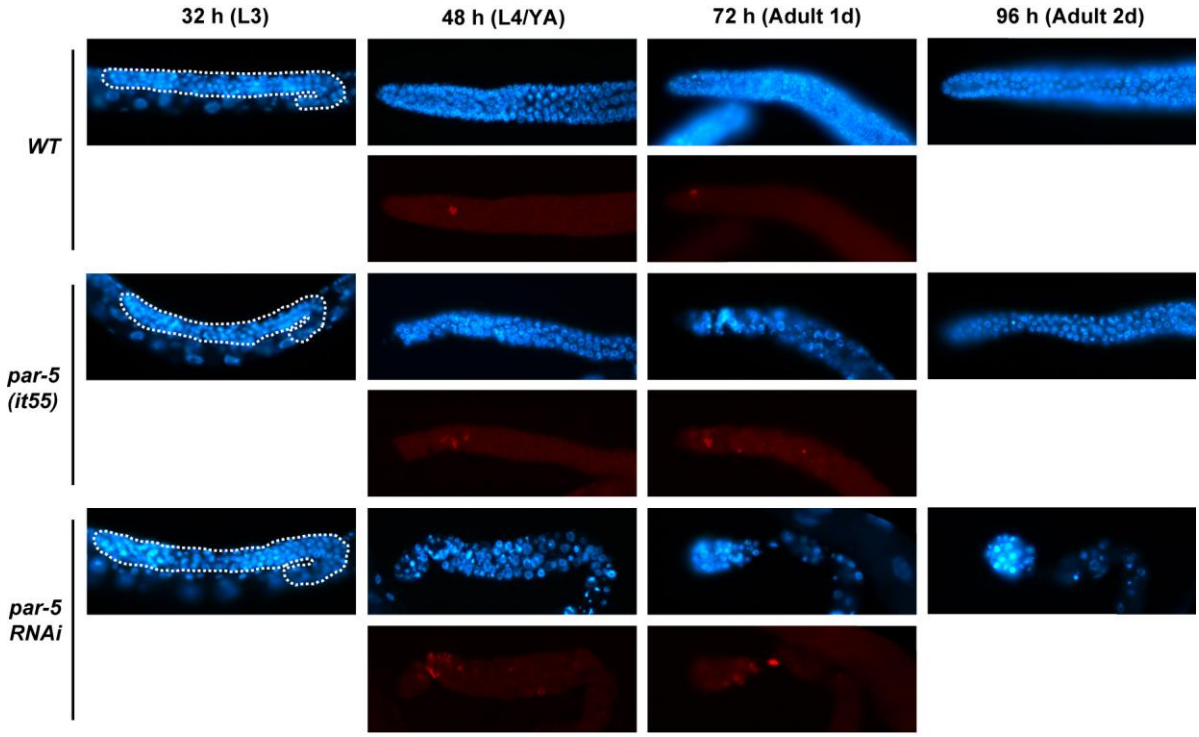


Figure 28. *par-5* inactivation leads to premature mitotic entry.
Worms were treated with HU and then the germlines were immunostained with a P-H3 antibody and counterstained with DAPI. The graph shows the quantification of P-H3-positive cells in all the optical stacks within 50 μm of the gonad distal end. Error bars indicate the standard deviation of the mean from at least 30 germlines for each experiment.



	% of PH3 positive cells	
	48h (L4/YA)	72h (Adult 1 day)
WT	1.5	0.8
par-5(it55)	3.6	3.2
par-5 RNAi	6.7	9.2

Figure 29. *par-5* defective germlines show increased number of cells in mitosis.
Representative images of germline proliferative regions from WT, *par-5(it55)* and *par-5(RNAi*) worms at different developmental stages. Synchronized L1 larvae were grown at 20°C. At the indicated times, worms were fixed and stained with DAPI or the P-H3 antibody and DAPI. The table on the bottom shows the percentage of P-H3 cells relative to the total number of germ cells in the proliferative region at the indicated stages.

5.6 *par-5* expression

5.6.1 PAR-5 expression in somatic cells.

In order to study *par-5* expression, we generated a transgenic strain expressing the PAR-5 protein fused to GFP. To do this, we cloned the complete genomic sequence of *par-5* (coding sequence, introns and UTRs) under the control of the *par-5* promoter (Figure 30A). Since this transgene included all the putative regulatory sequences, we hoped it would give us precise

information about endogenous regulation of *par-5* expression. By examination of this transgenic strain at one-day adult stage we noticed that the GFP::PAR-5 fusion protein was widely expressed in most of the worm tissues including neurons and intestine (Figure 30B). However, we did not observe any GFP signal coming from the gonad, indicating the transgene was probably silenced in the germline (attempts to integrate this transgene by using either gen gun or single insertion did not work). As expected for a 14-3-3 protein, this somatic expression indicates that *par-5* plays additionally roles other than germline regulation.

A)

B)

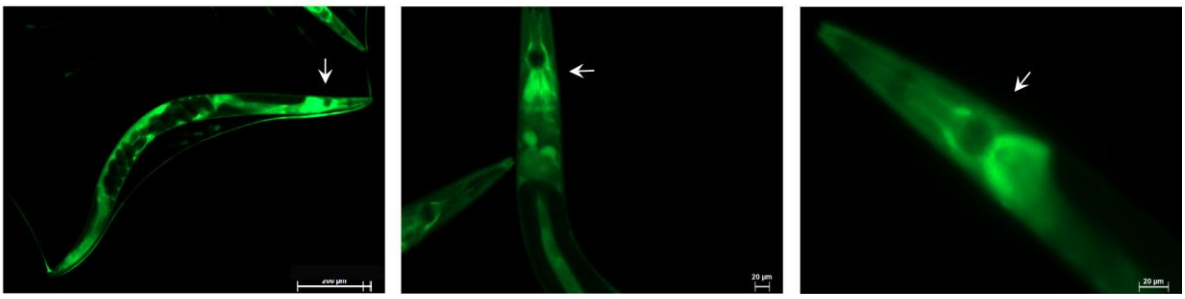


Figure 30. GFP::PAR-5 transgene is widely expressed in somatic tissues.
A) Scheme of the transgene bombarded in the worms. The GFP coding sequence was fused to the full genomic sequence of *par-5* gene (from ATG until 600bp downstream of the termination codon) and expressed under the control of the *par-5* promoter (containing also the 3'UTR). **B)** Representative images of adult transgenic worms expressing the GFP::PAR-5 construct. Images were taken at different magnifications (10X, 20X and 40X from left to right respectively). The transgene seems to be widely expressed in somatic lineages but silenced in the germline as in embryos. Worm pharynx is pointed by arrows.

5.6.2 PAR-5 expression in the germline

5.6.2.1 The par-5 *3' UTR do not restrict* par-5 *expression in the germline*

It has been described that gene expression in the gonad is mainly regulated via post-transcriptional control mediated by the 3'UTR sequences (Merritt et al., 2008). We took advantage of a transgenic strain generated by Merrit and collaborators to study whether *par-5*

was regulated at the post-transcriptional level. This strain carries the GFP sequence linked to the Histone H2B ORF and the 3'UTR of *par-5,* all under the control of the *pie-1* promoter (Figure 31A). *pie-1* promoter drives constitutive transcription in the germline, therefore the construct allowed us to check whether *par-5* 3'UTR mediated an additional post-transcriptional regulation generating an specific pattern of expression in some particular types of germ cells. However, we observed H2B::GFP expressed in all the germ nuclei from the distal proliferative region until the oocytes in the proximal region (Figure 31B). Furthermore, we used this strain to analyze the expression pattern under replication stress, but no changes were observed (Figure 31).

These results suggest *par-5* 3' UTR sequence does not restrict *par-5* expression to a specific region of the germline. In addition, no further 3'UTR-mediated post-transcriptional regulation is promoted upon replication stress.

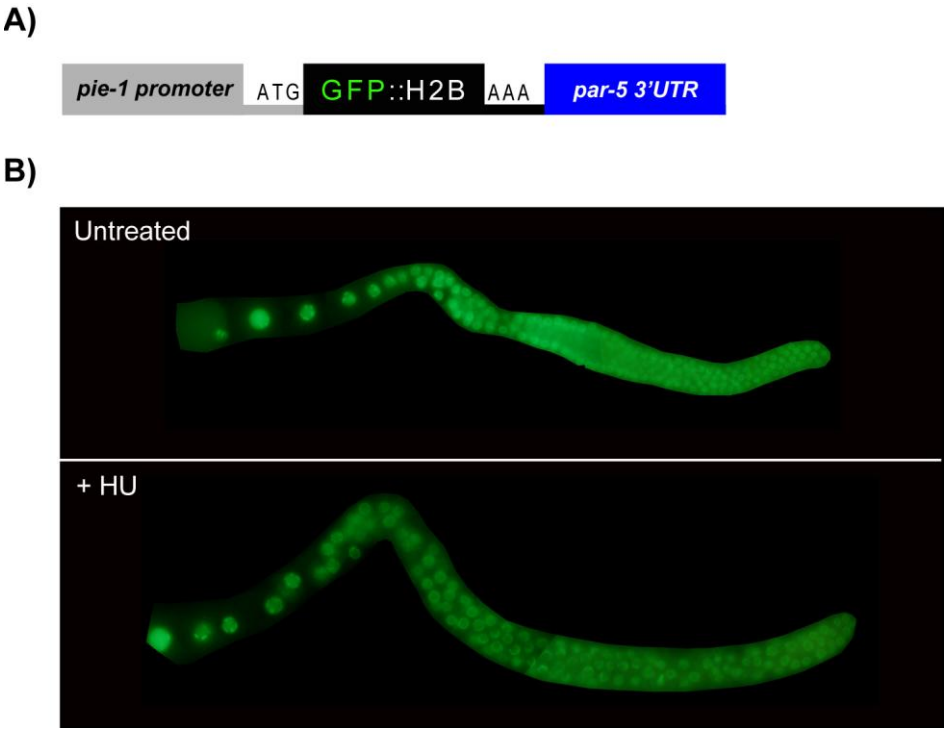


Figure 31. *par-5* expression is not regulated through its 3'UTR.
A) Scheme of the transgene used. The *pie-1* promoter drives the expression of GFP fused to the Histone H2B coding sequence and the *par-5* 3'UTR. **B)** Representative images of gonads from transgenic worms treated with or without HU. As expected, GFP::H2B was localized in the germ cell nuclei. *par-5 3' UTR* promoted expression in all the germ cells.

5.6.2.2 PAR-5 is expressed in all the germ cells

Previously, Golden and collaborators made an antibody against PAR-5 to study its role in embryonic development (Morton et al., 2002). This antibody was generated using the C-terminal of the PAR-5 protein as antigen. This region is the most divergent in respect to FTT-2 (Figure 13) therefore it should be specific for PAR-5. We used this antibody to study PAR-5 endogenous expression in dissected gonads of WT animals. In agreement with our evidence showing *par-5* involvement in several processes of germline development, we found that PAR-5 was present throughout the gonad from distal to proximal regions. Additionally, PAR-5 localizes outside germ nuclei in the gonad syncytium. We confirmed that this pattern was specific for PAR-5 since *par-5* RNAi feeding resulted in depletion of the antibody staining (Figure 32A). It is noteworthy that PAR-5 expression was lower in the mature sperm compared to the rest of the germ cells. However, expression in the sperm precursors at L4 stage was comparable to that observed in the oocytes precursors in the adult (Figure 32B).

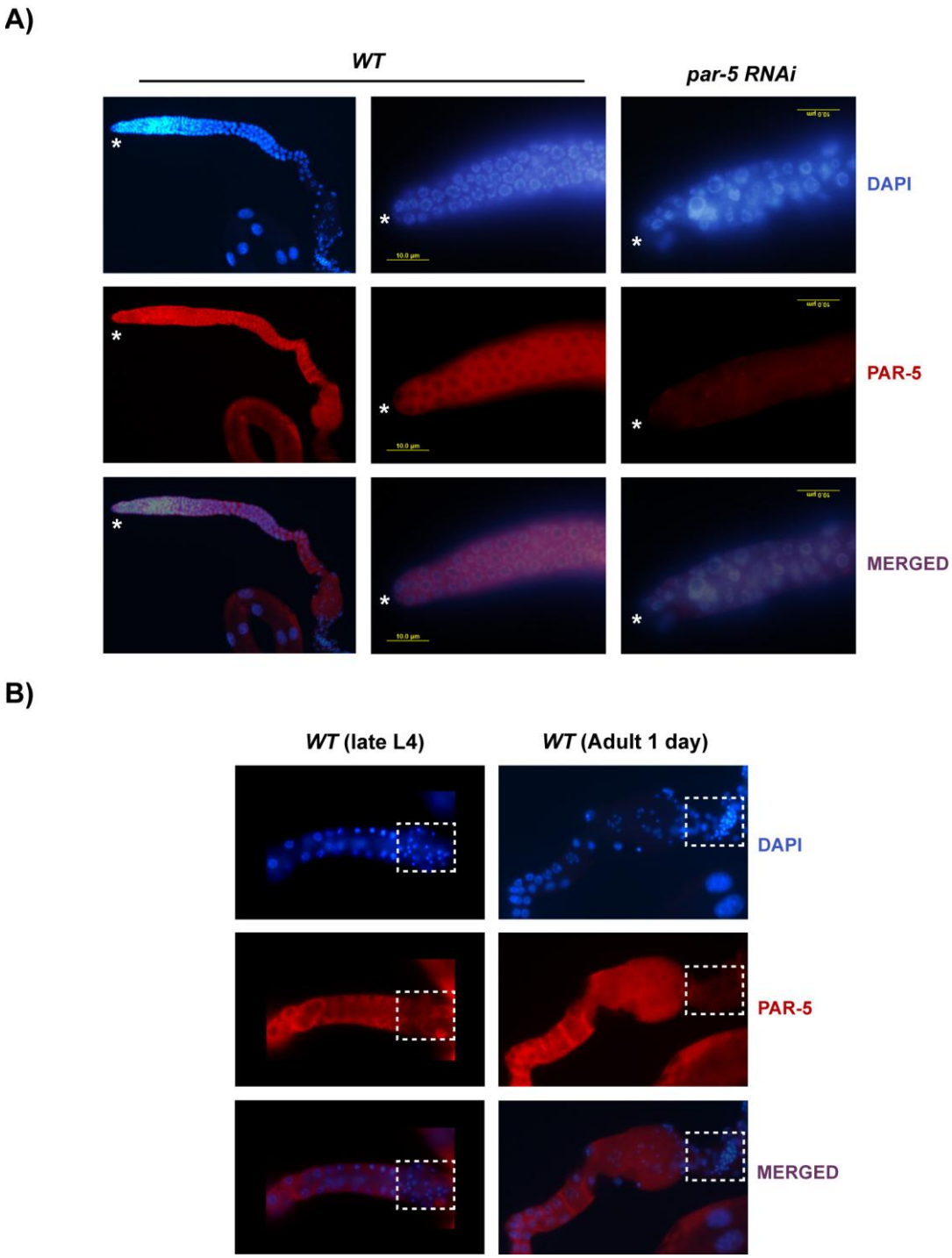


Figure 32. PAR-5 expression in the germline.
A) Representative images of germlines (column 1) or magnified proliferative regions (columns 2, 3) from WT and *par-5* RNAi-fed worms stained with the PAR-5 antibody and counterstained with DAPI. Distal end of the germlines are indicated by asterisks. **B)** Magnified proximal regions of WT germlines at the indicated developmental stage. Regions inside the squares correspond to the sperm.

5.6.2.3 PAR-5 accumulates in germ cell nuclei after checkpoint activation

To investigate the mechanism by which *par-5* acts in the checkpoint response, we examined PAR-5 expression and localization upon DNA damage. To check whether there was an increase in the protein expression levels upon replication stress, we used whole protein lysates of worms treated with and without HU to carry out western blot analysis. As shown in Figure 33A, no changes in protein levels were observed after HU treatment. Therefore, PAR-5 expression is not induced after replication stress. Nevertheless, this experiment confirmed the high efficiency of our *par-5* RNAi protocol, since the band corresponding to PAR-5 disappeared in the samples from *par-5* RNAi-fed animals.

14-3-3 proteins regulate the subcellular localization of their substrates in response to DNA damage. Specifically, 14-3-3 proteins seems to control the transport between nucleus and cytoplasm of several interaction partners (Lopez-Girona et al., 1999; Dunaway et al., 2005). Consequently, we further analyzed PAR-5 subcellular localization by confocal microscopy in normal and HU-treated germlines. Previous studies and the results shown in section 5.6.2.2 indicate that PAR-5 is expressed in the germline syncytium (Morton et al., 2002). In agreement with this, by analyzing different planes at various depths (Z stacks) of confocal images from WT germlines we confirmed that PAR-5 was localized around the nuclei of germ cells. Interestingly, after HU treatment we observed a significant amount of PAR-5 protein inside the large S-phase-arrested nuclei (Figure 33B), suggesting a change in PAR-5 localization upon DNA damage.
Therefore, nuclear localization rather than expression induction of PAR-5 could be important for its role in the checkpoint.

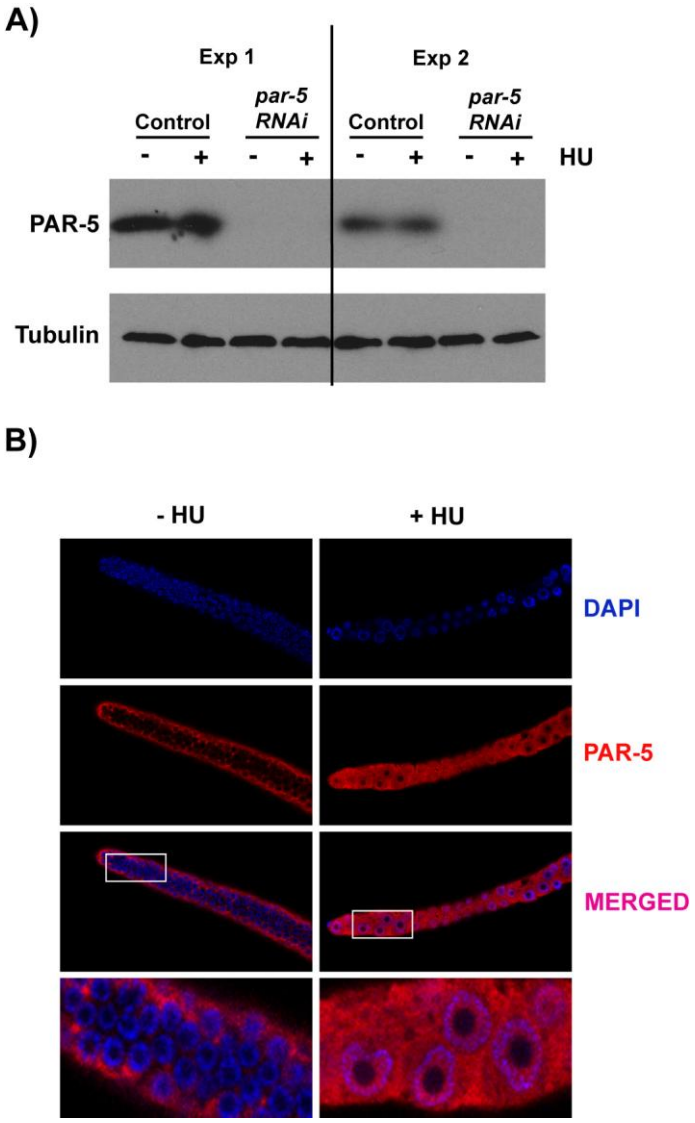


Figure 33. PAR-5 expression and subcellular localization after replication stress induced by HU. **A)** Protein extracts from WT worms fed with *par-5* RNAi or the L4440 vector (control) and, treated with (+) or without (–) HU were blotted using a PAR-5 antibody. The blotting was performed using extracts from two experimental replicates. **B)** Representative confocal images showing a single Z stack of germlines from WT worms treated with or without HU, immunostained with the PAR-5 antibody and counterstained with DAPI.

5.7 *par-5* is required for CDK-1 regulation after DNA damage

It has been demonstrated that 14-3-3 homologs in yeast, flies and mammals regulate G2/M transition through interactions with the cell cycle regulator proteins WEE1, CDC25 and CDK1. As a canonical cell cycle progression mechanism, CDC25 dephosphorylates CDK1 to

allow entry into mitosis. However, after DNA damage, CDK1 and CDC25 are inactivated by phosphorylation by kinases WEE1 and CHK1 in a checkpoint-dependent manner, leading to cell cycle arrest. In *C. elegans,* CDK-1 is also phosphorylated in the Tyr15 inhibitory residue upon DNA damage (Moser et al., 2009; Bailly et al., 2010). Therefore, we investigated the relationship between *par-5* function and these cell cycle regulators, in order to understand how *par-5* suppression disrupts the cell cycle arrest induced by the DDR pathway.

5.7.1 CDK-1 inhibitory phosphorylation depends on *par-5* function

Because we observed premature entry into mitosis in *par-5*(*RNAi*) and *par-5(it55)* worms after DNA damage, we checked whether *par-5* inactivation affected CDK-1 phosphorylation. Similar to HU, treatment with Camptothecin (CPT) induces DNA damage response in the worm. CPT treatment promotes CDK-1 phosphorylation and the consequent cell cycle arrest in the proliferative region of the WT germline (Figure 34). However, after *par-5* RNAi knockdown, P-CDK-1 staining was strongly reduced in the proliferative region. The same effect was observed in the *atl-1(tm853)* strain, suggesting that lack of P-CDK-1 is a consequence of deficient checkpoint activation. *par-5* mutants presented some germ cells with P-CDK-1 staining after CPT treatment, reflecting the milder *par-5* inactivation compared with the *par-5*(*RNAi*) animals (Figure 34). These results indicate that *par-5* function is necessary for CDK-1 phosphorylation upon DNA damage.

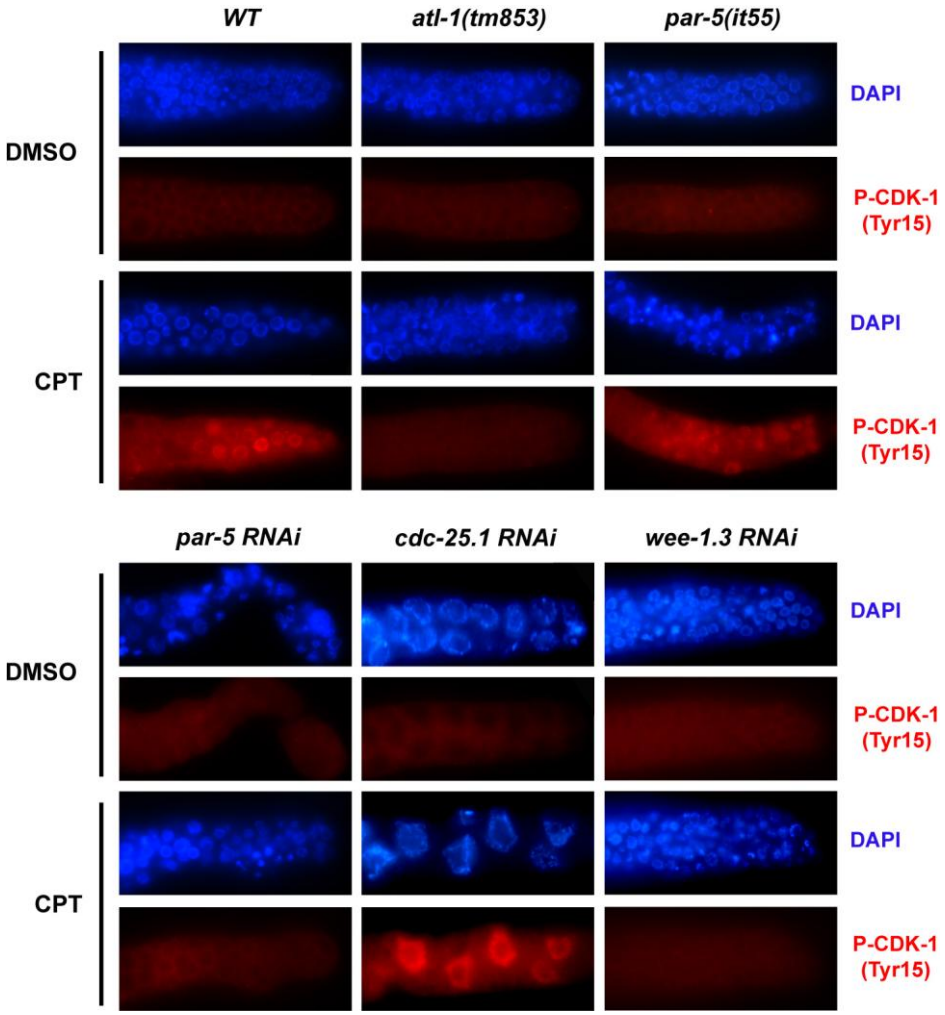


Figure 34. *par-5* is required for CDK-1 phosphorylation after DNA damage.
Representative images of germline proliferative regions from worms of the indicated genotypes/RNAi, treated with CPT or vehicle control (DMSO) immunostained with a P-CDK-1 (Tyr15) antibody and counterstained with DAPI.

5.7.2 *par-5* counteracts *cdc-25.1* phosphatase function

To further investigate the link between PAR-5 and CDK-1 phosphorylation, we examined the functional relationship between *par-5* and *cdc-25.1*. In yeast and mammals, CDC25 phosphatase removes the CDK1 inhibitory phosphorylation (Tyr15) in order to promote mitotic entry. There are four *cdc-25* phosphatases in worms but only *cdc-25.1* has been found important for germline proliferation (Ashcroft and Golden, 2002). Accordingly, we observed that *cdc-25.1* suppression by RNAi enhanced both CDK-1 phosphorylation and cell cycle arrest upon CPT treatment. This was evident by the bigger size of the nuclei and stronger

signal of P-CDK-1 in *cdc-25.1(RNAi)* germ cells compared to WT (Figure 34). This phenotype is opposite to the one observed after *par-5* suppression suggesting an antagonist functional relationship between *par-5* and *cdc-25.1* upon DNA damage (Figure 34).

As was previously reported in the literature, we found that *cdc-25.1* RNAi produces cell cycle arrest in the proliferative region of the germline. Since *cdc-25.1* must be inactivated to prevent cell cycle progression, the *cdc-25.1* RNAi phenotype mimics the checkpoint response. Interestingly, the cell cycle arrest induced by *cdc-25.1* RNAi during germline development was rescued in the *par-5(it55)* background (Figure 35), reinforcing the notion of opposite functions for *par-5* and *cdc-25.1* in cell cycle control.

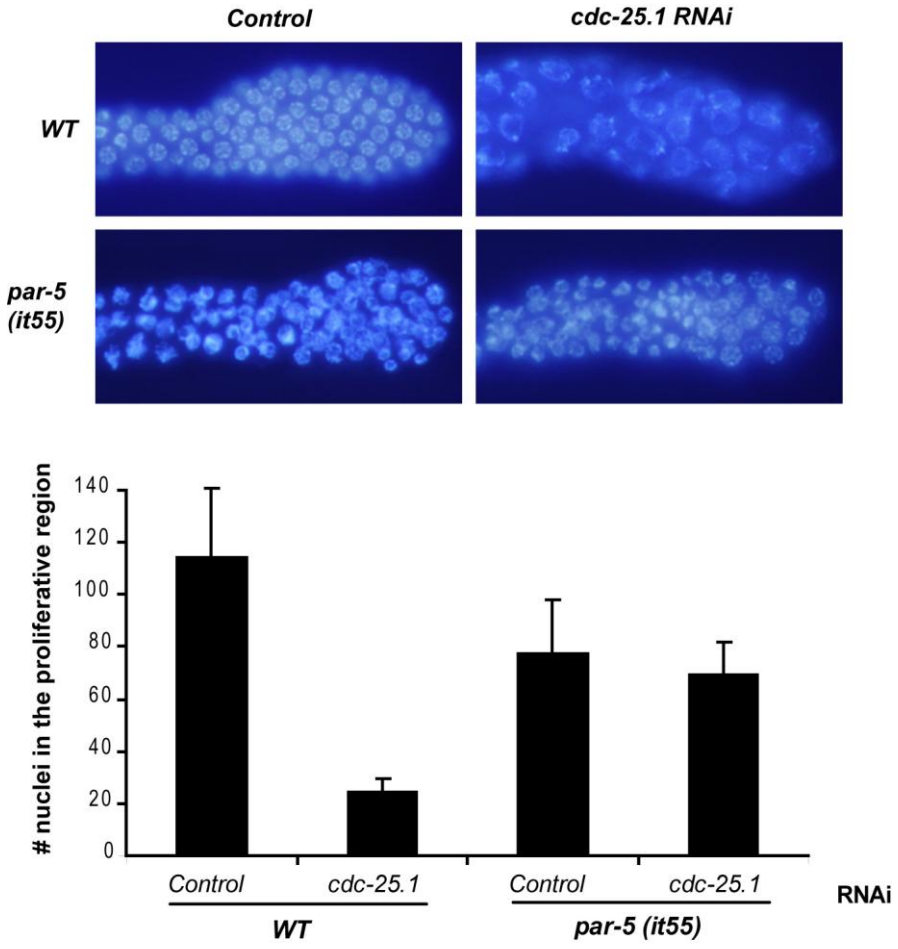


Figure 35. *par-5* counteracts *cdc-25.1* function in cell cycle control.
Representative images of the proliferative region of germlines from WT or *par-5(it55)* worms (one-day adults) fed with the L4440 vector or *cdc-25.1* RNAi (fed from L3 stage) stained with DAPI. The graph shows germ nuclei quantification. Error bars indicate standard deviations.

5.7.3 *par-5* acts in the same pathway as *wee-1.3* to promote CDK-1 phosphorylation

In yeast, Cdk1 phosphorylation relies on the balance between the activities of the Wee1 kinase and the Cdc25 phosphatase (Raleigh and O'Connell, 2000). Consequently, we assessed whether *par-5* could be acting in the same pathway as *wee-1* in order to counteract *cdc-25.1* function. In *C. elegans,* there are two *wee-1* genes, *wee-1.1* and *wee-1.3.* It has been described that *wee-1.3* regulates *cdk-1* function during oocyte maturation in the germline (Burrows et al., 2006), but nothing is known about their interaction in germ cell proliferation. By RNAi mediated co-inactivation, we observed that *wee-1.3* partially suppresses the *cdc-25.1* RNAi arrest phenotype (Figure 36). Even though the rescue was partial in terms of total germ cells at the proliferative region when compared to *cdc-25.1 RNAi*, the increase in the number of mitotic cells observed after *wee-1.3* and *cdc-25.1* co-suppression was remarkable (Figure 36C). *cdc-25.1* and *par-2* RNAi combination did not show such rescue, proving that the rescued phenotype was not the result of the *cdc-25.1* clone dilution when mixed with another RNAi clone. This indicates that *wee-1.3* counteracts *cdc-25.1* function in controlling entry into mitosis. Supporting this hypothesis, we observed that *wee-1.3* RNAi results in the suppression of CDK-1 phosphorylation upon DNA damage (Figure 34).

Since we found similar antagonist functions between *par-5* and *wee-1.3* with respect to *cdc-25.1*, we wondered whether *wee-1.3,* like *par-5,* was necessary for HU-induced cell cycle arrest. Similar to *par-5* RNAi, *wee-1.3* knockdown inhibited the checkpoint induced by replication stress, leading to aberrant mitosis and nuclei fragmentation (Figure 37A). However, *wee-1.3* depletion, in contrast to *par-5*, does not seem to affect germline proliferation in the absence of HU (Figure 37B). Therefore, *par-5* functions in the germline are not necessary coupled with *wee-1.3*.

Taken together these results suggest that upon DNA damage PAR-5 might control entry into mitosis in the same manner as WEE-1.3, in order to promote CDK-1 phosphorylation and counteract CDC-25.1 function. Such a model would place PAR-5 within the checkpoint pathway as part of the effector proteins required for DNA damage-induced cell cycle arrest.

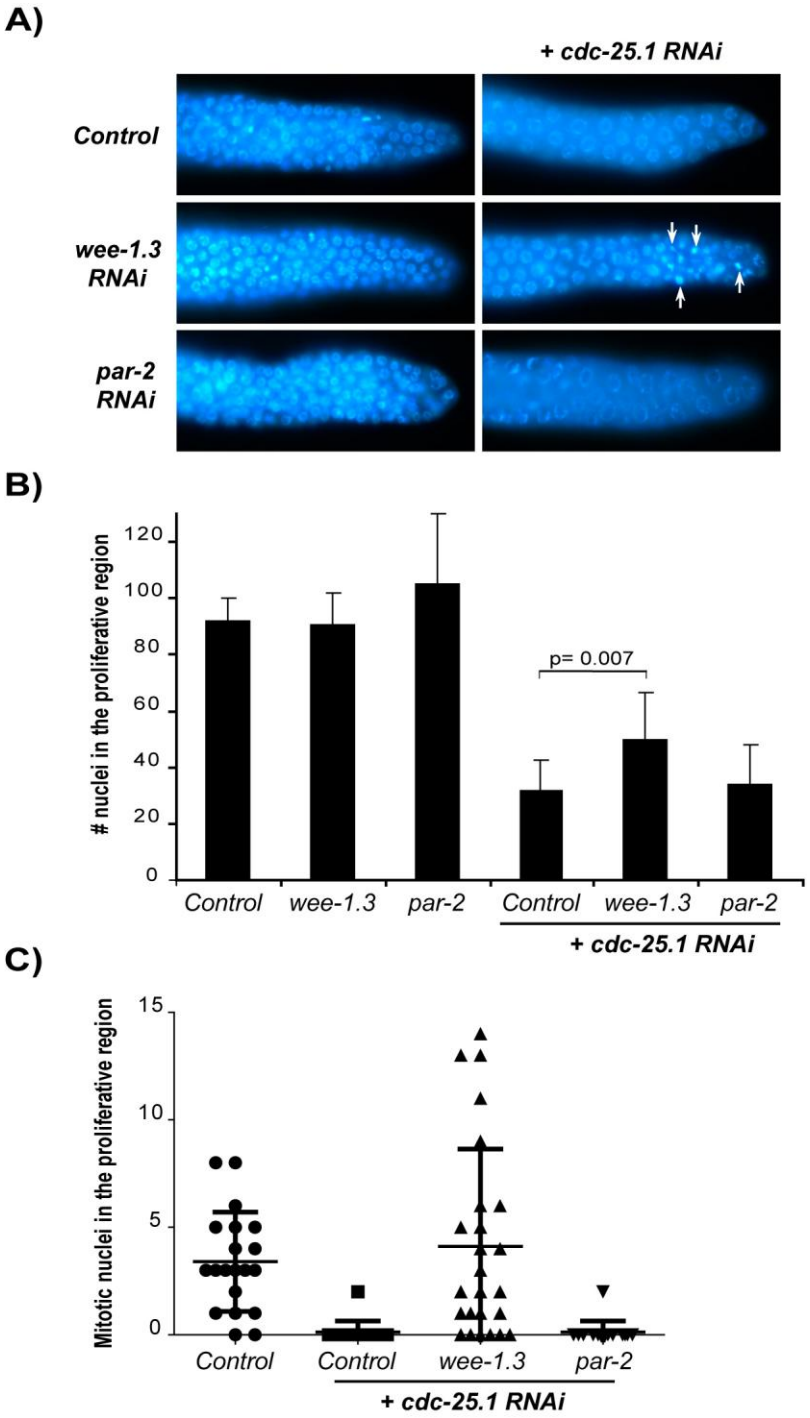


Figure 36. *wee-1.3* antagonizes *cdc-25.1* in mitotic entry.
A) Representative images of DAPI-stained proliferative regions of germlines from *rrf-3(pk1426)* worms fed with RNAi empty vector (control), *wee-1.3* or *par-2* RNAi alone or in combination with *cdc-25.1* RNAi (from L3 stage). *cdc-25.1* RNAi produces cell cycle arrest, however in combination with *wee-1.3* RNAi, germ cell arrest is partially rescued as evidenced by the presence of mitotic nuclei (pointed out by arrows). *par-2* RNAi was used as control to prove that the rescued phenotype was not the result of the *cdc-25.1* clone dilution when mixed with another RNAi clone. **B)** The graph shows germ nuclei quantification in the proliferative region of worms described in A. Error bars indicate standard deviations. **C)** Graph showing the quantification of mitotic figures observed in the proliferative regions of the germlines showed in A.

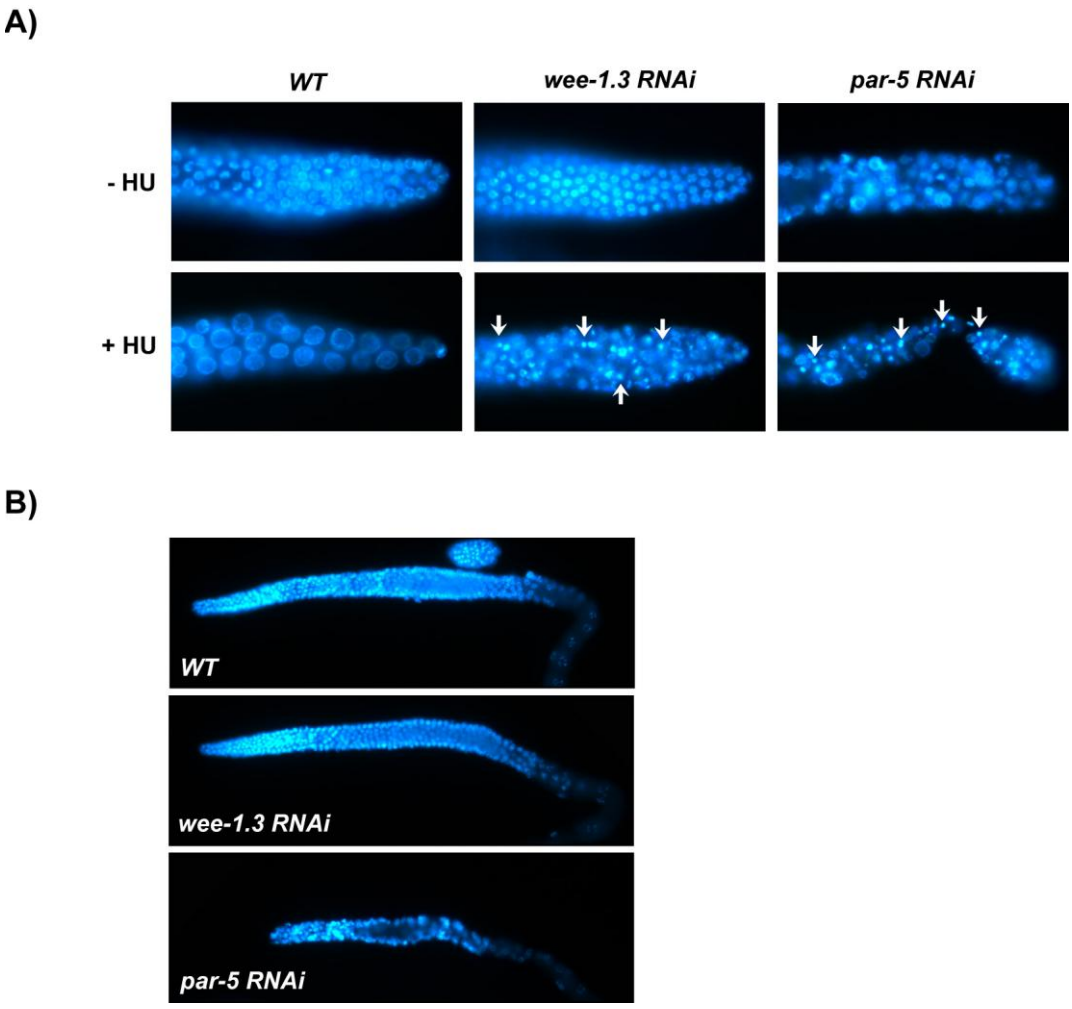


Figure 37. Role of *wee-1.3* in germline DDR and proliferation.
A) *wee-1.3* suppression mimics *par-5* RNAi phenotype upon HU treatment. Representative images of germline proliferative regions from WT worms fed with *wee-1.3* or *par-5* RNAi, treated with or without HU and stained with DAPI. Nuclear fragmentation (indicated by arrows) was observed in 90% of the *wee-1.3* and *par-5* RNAi-treated germlines. **B) *wee-1.3* is not required for germline proliferation.** Representative images of germlines from WT worms (one-day adults) treated with *wee-1.3*, *par-5* RNAi or the RNAi empty vector and stained with DAPI.

6 DISCUSSION

The capability of 14-3-3 proteins to physically interact with many proteins offers PAR-5 the potential to be involved in several developmental processes. In this study, we elucidated two separate functions for *par-5* in the germline, one in germ cell proliferation and another in response to DNA damage. Although both functions might be associated with the role of *par-5* in preventing premature mitotic entry, the pathways regulating these two processes, and the levels of *par-5* required, may be different.

6.1 *par-5* and germline development

6.1.1 *par-5* mediated regulation of the germline proliferation

The decrease in the number of germ cells in *par-5*-defective animals could be at least partially explained by abnormal and uncontrolled entry into the M-phase, which leads to mitotic defects (Figure 28). After *par-5* knockdown, we detected some nuclei that showed hypercondensed and fragmented chromatin. These cells probably suffered mitotic catastrophe and therefore, were unable to continue dividing, contributing to the strong decrease in germ cell precursors after *par-5* RNAi administration (Figure 16). This phenotype was rarely observed in *par-(it55)* animals in which, although the proliferation rate was affected, reduced PAR-5 levels are sufficient to maintain germ cell divisions without mitotic catastrophe. The nuclear fragmentation observed in *par-5*(*RNAi*) germ cells was accompanied by an accumulation of RAD-51 foci in the proliferative region of the germline (Figure 21). Both phenotypes have previously been related to defects in the maintenance of replication stability and the consequent aberrant mitosis, which has also been observed after the suppression of key checkpoint genes such as *atl-1*, *wrn-1* and *chk-1* (Kalogeropoulos et al., 2004; Garcia-Muse and Boulton, 2005; Lee et al., 2010). In addition, budding yeast 14-3-3 proteins negatively regulate Exo1 nuclease activity, which is involved in the pathological process of stalled replication forks that produces the accumulation of single-strand DNA gaps (Engels et al., 2011). However, we observed that nuclei fragmentation in *par-5* depleted animals is not

alleviated by *exo-1* knockdown (data not shown). Therefore, DNA instability observed in *par-5* suppressed animals is independent of *exo-1* activity.

Interestingly, although *par-5* suppression resulted in reduced proliferation, we observed an increase in the number of cells in mitosis (Figure 28). Consequently, this higher mitotic index is probably caused by a delay in mitosis, which can be explained by mitotic problems due to a premature entry into mitosis. However, we cannot rule out the possibility of *par-5* being also required for mitotic progression. In agreement with this hypothesis, 14-3-3 proteins have been associated with cytokinesis and mitotic exit (Wilker et al., 2007; Saurin et al., 2008).

There are other pathways that could be modulated by *par-5* which are also involved in germline proliferation. *par-5* has been functionally related with *daf-16* (Berdichevsky et al., 2006; Wang et al., 2006). This transcription factor translocates to the nucleus and activates genes implicated in stress response and lifespan, and its nuclear/cytoplasmic localization seems to be regulated by the 14-3-3 proteins, PAR-5 and FTT-2, depending on the context (Berdichevsky and Guarente, 2006). Interestingly, *daf-16* is repressed by the insulin pathway, which recently has been shown to be required for robust germline proliferation in larval stages. Specifically, *daf-2* suppression (the insulin receptor) provokes a decrease in proliferating germ cells at L3 and L4 stages, and this phenotype is suppressed in a *daf-16* mutant background. This implies a role of *daf-16* in inhibiting germline proliferation (Michaelson et al., 2010). Since this function depends on *daf-16* activation in the germline, *par-5* may be necessary to inhibit *daf-16* during germ cell proliferation at the larval stages. This interaction could help to explain the decrease in germline proliferation observed after L3 in *par-5* suppressed animals.

In addition, preliminary results from a proteomic screening (using mass spectrometry to identify proteins co-precipitated with a PAR-5 antibody) allowed us to identify some of the PAR-5 putative interactors in WT worms. Among these, we found two proteins related with the mitochondrial stability maintenance, PHB-1 and PHB-2. Interestingly, knockdown of these genes produces mitochondrial dysfunction associated with higher production of ROS (Reactive oxygen species) and germline defects such as reduced germ cell number and defective differentiation (Artal-Sanz et al., 2003). However, the possible interaction of PAR-5 with these proteins needs further validation.

6.1.2 *par-5* and meiotic progression.

The reduction in pachytene cells observed in *par-5*(*RNAi*) worms also highlighted that *par-5* plays a role in meiotic progression. There are several factors that could hamper meiotic transition in *par-5* suppressed worms.

Firstly, the germline proliferation defect in *par-5(RNAi)* produces reduced germline elongation. Since mitotic/meiosis transition is inhibited by the signaling coming from the DTC (see section 1.2.1.3), germ cells must reach a certain distance from the DTC to be able to enter into meiosis (Killian and Hubbard, 2005; Kimble and Crittenden, 2007). This normally occurs by L3/L4 in WT animals. However, in the absence of robust germline proliferation, which is the case of *par-5* animals, most of the germ cells would remain under the DTC signaling effect (LAG-2/GLP-1) and therefore the transition to meiosis would not be favored. Accordingly, we observed that meiotic transition and gametogenesis is delayed in *par-5(RNAi)* animals. At early L4 stage, 60 % of the WT animals have already started spermatogenesis, whereas none of the *par-5(RNAi)* worms presented sperm at this stage. Secondly, most of the *par-5(RNAi)* animals show a drastic reduction in the number of mature oocytes. This was observed even in adults of two days, suggesting that is not due to the delayed entry into meiosis mentioned above. Furthermore, the sperm in *par-5(RNAi)* adults is comparable to that observed in WT animals (Figure 17), suggesting that oocyte progression is specifically impaired. One possible explanation could be related to the low levels of PAR-5 protein observed in mature sperm of WT adult animals, suggesting *par-5* is not required for sperm differentiation. Nevertheless, PAR-5 is highly expressed in sperm precursors at the L4 stage (Figure 32B). Another possibility is that *par-5* is required for the spermatogenesis to oogenesis switch that occurs at L4. However, this is not likely to be the case, because *par-5(RNAi)* worms do not present an excess of sperm, as happens in mutants where the switch to oogenesis is suppressed. Moreover, some of the *par-5(RNAi)* worms showed few abnormal oocytes suggesting the switch is indeed occurring, but the oocytes are not properly maturated.

Interestingly, after *par-5* knockdown we observed an evident increase in HUS-1::GFP foci, reflecting the accumulation of unrepaired DSBs in the pachytene region. This observation, together with the accumulation of RAD-51 foci in proliferating cells, suggests that *par-5* could be required to repair endogenous DNA damage. However, we cannot rule out the possibility that *par-5* depletion causes additional DNA damage through a different direct or

indirect mechanism. The increase in DNA damage in the germlines of *par-5*(*RNAi*) worms was also accompanied by a constitutive phosphorylation of CHK-1 (Ser345) in pre-meiotic germ cells. This modification is activated as a consequence of recombination errors or elevated DNA damage in meiotic cells (Bhalla, 2010; Jaramillo-Lambert et al., 2010). Both the accumulation of DSBs (HUS-1::GFP foci) and constitutive meiotic CHK-1 activation, could contribute to the absence of oocytes after *par-5* RNAi, since the damaged meiotic cells may not further progress to reach proper oocyte differentiation. Moreover, oocyte maturation requires cell cycle arrest in meiotic prophase one. Similarly to the G2/M arrest in mitotic cells, this is dependent on the inhibition of the CDK-1 (Boxem et al., 1999; Burrows et al., 2006). Because *par-5* is required for CDK-1 and G2/M regulation (see next section), it is possible that CDK-1 dysregulation could also account for the oocyte defects observed in *par-5(RNAi)* worms.

Therefore, alterations in germline proliferation and cell cycle, together with the accumulation of DNA damage, might both contribute to the meiotic defects observed in *par-5* defective worms.

6.2 Embryonic cell cycle regulation by *par-5*

When we studied the cell cycle of the first embryonic division, we found that *par-5*-defective embryos presented a shorter S-phase and a longer M-phase, implying *par-5* is also regulating the embryonic cell cycle. This was anticipated since PAR-5 belongs to the PAR family, which controls the asymmetric first cell division in the embryo. However, by analyzing videos from the Phenobank, such cell cycle alterations seem to be unique among PAR family members (Figure 27). Interestingly, the shorter S phase and longer M phase observed in the embryo supports the evidence found in the germline pointing towards a role of *par-5* in controlling the mitotic entry.

Furthermore, we showed that *par-5* is required for checkpoint mediated delay of the first embryonic cell cycle in response to replication stress. The checkpoint contributes to asynchronous cell division occurring at the two cell stage (Brauchle et al., 2003) whereas *par-5* suppression produces synchronous cell division (Morton et al., 2002), therefore, it is tempting to hypothesize a double role for *par-5* in embryonic development. The first one, already known, is the regulation of the other PAR proteins to promote proper asymmetric

distribution of cytoplasmatic components at the one cell stage. The second, related to the uncovered role of *par-5* in checkpoint response, would be the regulation of cell cycle timing during early embryonic divisions. Therefore, PAR-5 could be the link between the PAR family function and cell cycle regulation, which is required for proper determination of cellular fate in the embryo. In addition, it will be interesting to address if DNA damage accumulation is also contributing to the defects observed in *par-5* defective embryos.

6.3 Function of *par-5* within the DDR pathway

We have demonstrated that the *C. elegans* 14-3-3 gene *par-5* is required to promote proper cell cycle arrest after DNA damage. Interestingly, although only *par-5(RNAi)* worms showed endogenous DNA damage accumulation and nuclei fragmentation, both *par-5(it55)* and *par-5*(*RNAi*) worms presented similar checkpoint defects in response to exogenous DNA damage. Therefore, taking into account the fact that the mutant strain retains some protein expression (Morton et al., 2002), it is clear that a mild decrease in PAR-5 levels is enough to affect the extrinsic DNA damage-induced checkpoint response, whereas a stronger depletion of the protein (as showed in our RNAi experiments, Figure 33A) affects germ cell cycle progression and DNA stability.

The main function of *par-5* uncovered in this study is the regulation of entry into mitosis. We observed that *par-5* suppression compromises the mitotic entry blockade promoted during both S and G2 phases in response to replication stress and DSBs induction, respectively. Furthermore, hypercondensed and bright nuclei observed in *par-5(RNAi)* worms (Figure 16) resemble the phenotype of premature chromosome condensation which is associated with defects in the so called S/M checkpoint that prevents entry into mitosis when DNA replication is not finished (Nghiem et al., 2001; Niida et al., 2005). However, one of our results raises the possibility of *par-5* also acting in the G1/S transition. When we used *cdc-25.1* RNAi, we observed cell cycle arrest in the proliferative region, which was rescued in the *par-5(it55)* background (Figure 35). However, *cdc-25.1(RNAi)* germ cells showed two important differences compared to WT cells arrested upon CPT or HU treatment. First, their nuclei were strikingly bigger, and second they did not show CDK-1 phosphorylation (Figure 34). Since this modification is promoted by the S and G2/M checkpoints, these results suggest that *cdc-25.1(RNAi)* germ cells could be arrested in G1/S. Accordingly, *cdc-25.1* has been also

implicated in the entry into S phase in intestinal cells (Kostić and Roy, 2002). Therefore, by counteracting *cdc-25.1* function, *par-5* may be a general regulator of progression through the different phases of the cell cycle, which is the case of the 14-3-3 proteins in other organisms. However although PAR-5 is expressed in the intestine (Figure 30), there were no obvious defects in the morphology or number of the intestinal nuclei in *par-5* defective worms, indicating tissue specific roles for *par-5* in cell cycle regulation.

6.3.1 Functional interaction of *par-5* with diverse checkpoint and cell cycle regulators

Several 14-3-3 protein partners that could help to explain the role of *par-5* in DNA damage-induced cell cycle arrest have been identified in diverse organisms. These interactions, together with functional evidence provided in this study, are compiled and depicted in Figure 38. In yeast, 14-3-3 proteins interact with Chk1 to regulate cell cycle arrest upon DNA damage (Dunaway et al., 2005). Chk1 phosphorylates Wee1, which in turn phosphorylates Cdk1 (Tyr15) in order to stop cell cycle (O'Connell et al., 1997), and 14-3-3 proteins are required for proper Chk1 nuclear localization and function (Chen et al., 1999; Dunaway et al., 2005). Therefore, the hypothesis that PAR-5 is necessary for CHK-1 function could explain the defect in CDK-1 phosphorylation and cell cycle arrest after *par-5* knockdown. However, it seems that PAR-5 is not strictly necessary for CHK-1 activation because we observed the active form CHK-1 (phosphorylated at Ser345) and its proper nuclear localization in *par-5 (RNAi)* worms (Figure 22B). Nevertheless, as this observation was carried out in pre-meiotic cells, we cannot rule out a functional interaction between PAR-5 and CHK-1 in proliferating germ cells. Because we observed the same phenotype in *atl-1(tm853)* mutants, CHK-1 phosphorylation is probably mediated by ATM-1 instead of by ATL-1. In agreement with this hypothesis, it has been suggested that *atm-1* and *atl-1* could have redundant roles in meiotic checkpoint activation (Bhalla, 2010; Jaramillo-Lambert et al., 2010). Downstream of Chk1, 14-3-3 proteins have been shown to interact with Cdc25 phosphatase, preventing its interaction with Cdk1 (Peng et al., 1997; Lopez-Girona et al., 1999; Zeng and Piwnica-Worms, 1999). Cdc25 eliminates the Cdk1 (Tyr15) inhibitory phosphorylation (executed by Wee1), thereby allowing Cdk1 to promote progression into mitosis. Therefore, Cdk1 phosphorylation and activity depend on the kinase and phosphatase activities of Wee1 and Cdc25, respectively (O'Connell et al., 2000). Accordingly, our results are compatible with the

idea of *par-5* collaborating with *wee-1.3* and counteracting *cdc-25.1* in order to promote proper cell cycle arrest upon DNA damage. However, *wee-1.3* depletion, in contrast to *par-5*, does not seem to affect germline proliferation in the absence of HU (Figure 37). Therefore, *par-5* functions in the germline are not always coupled with *wee-1.3* and CDK-1 phosphorylation.

Finally, 14-3-3 proteins have been shown to directly regulate Cdk1 localization and function (Chan et al., 1999; Laronga et al., 2000; Su et al., 2001). In mammals, phosphorylated CDK1 is sequestered in the cytoplasm upon DNA damage in a 14-3-3-dependent manner, in order to prevent mitotic catastrophe (Chan et al., 1999). However, in *C. elegans* (similar to fission yeast), phosphorylated CDK-1 is located inside the nucleus (Boxem et al., 1999). Therefore, if PAR-5 regulates CDK-1 function, the mechanism is likely to be different from that of cytoplasmic sequestration. Moreover, we showed that PAR-5 is localized in the nucleus upon replication stress, suggesting that the relevant interactions for DDR take place inside the nucleus (Figure 33), for instance with WEE-1.3 or CDK-1 itself. Interestingly, cell cycle arrest is inhibited in *par-5(it55)* worms although the CDK-1 is partially phosphorylated (Figure 34), suggesting that P-CDK-1 is not enough to promote cell cycle arrest in absence of *par-5*. Therefore, PAR-5 binding to phosphorylated CDK-1 could be necessary to block the interaction of CDK-1 with its targets and hence the entry into mitosis. Further experiments need to be carried out in order to identify PAR-5 interactions and their impacts on checkpoint responses and germline proliferation.

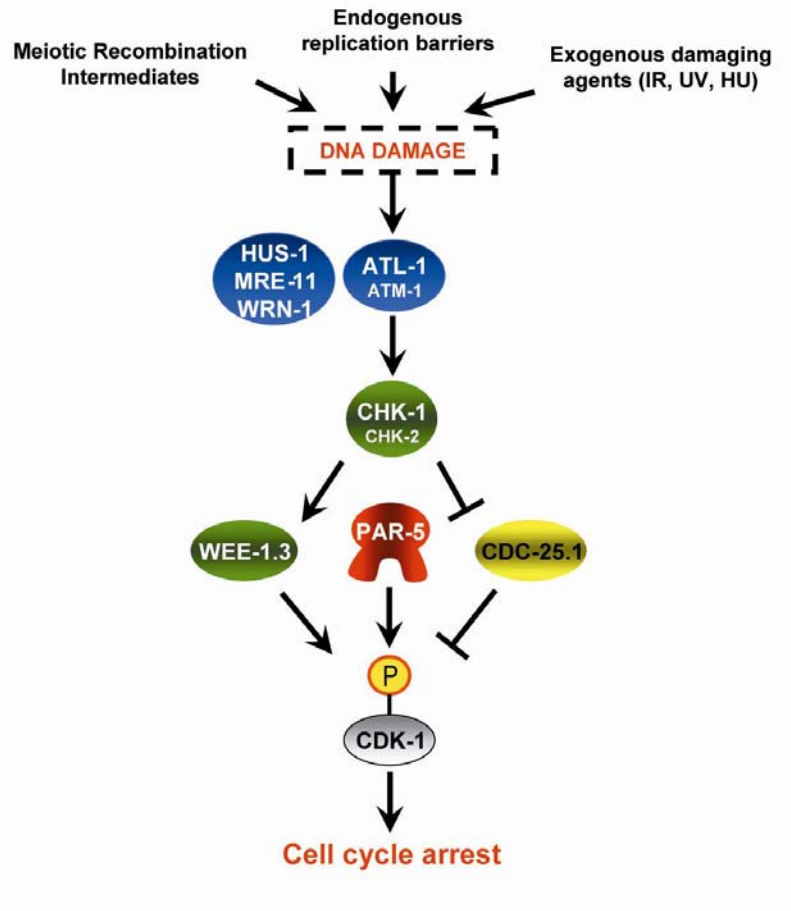


Figure 38. Model for *par-5* function within DNA damage-induced cell cycle arrest.
After the detection of endogenous or exogenous DNA damage, checkpoint sensor proteins (such as HUS-1 and MRE-11) activate ATL-1 and ATM-1, which in turn phosphorylate CHK-1 and CHK-2 kinases. The contribution of ATL-1/ATM-1 and CHK-1/CHK-2 to the response depends mainly on the type of DNA damage that triggers the response. However, ATL-1 and CHK-1 are considered to be the main players in the pathway. Downstream of CHK-1, the cell cycle can be arrested by promoting CDK-1 inactivation by phosphorylation. According to our results, CDK-1 phosphorylation status relies on the balance between the activities of WEE-1.3 kinase and CDC-25.1 phosphatase. Therefore, checkpoint signaling would favor WEE-1.3 activation and CDC-25.1 inhibition (likely to be by CHK-1-mediated phosphorylation). In this context, we propose that PAR-5 is necessary to promote and/or maintain CDK-1 phosphorylation (inactive form), and therefore is required to induce cell cycle arrest upon DNA damage.

6.4 *C. elegans* as a model to study 14-3-3 regulation and function

Even though mammalian 14-3-3 homologs have diverged into seven genes, we verified that the basic functions of 14-3-3 in cell cycle control have been conserved in *C. elegans*. Indeed, the mitotic catastrophe observed in *par-5*(*RNAi*) worms has already been noted in human cells

lacking 14-3-3σ after the induction of DNA damage (Chan et al., 1999). However, *C. elegans*, in contrast to mammals, has only one 14-3-3 protein (PAR-5) expressed in the germline. That could explain why *par-5* is essential to maintain the proliferation and genomic stability of the germline. By contrast, the single knockdown of mammalian 14-3-3 has less influence on the cells in the absence of exogenous DNA damage, probably because of functional redundancy among family members (Hermeking and Benzinger, 2006).

DNA fragmentation in the germ cells of *par-5* knockdown worms treated with different DNA-damaging agents (CPT, HU or IR) implies increased sensitivity of proliferating cells to these agents. This observation is in agreement with multiple reports showing that 14-3-3 overexpression is related to chemotherapy resistance in cancer cell lines, and also that 14-3-3 downregulation sensitizes cells to therapy-induced cell death. Indeed, 14-3-3 proteins have been suggested as possible therapeutic targets in cancer treatment (Porter et al., 2006; Tzivion et al., 2006; Neal and Yu, 2010; Bergamaschi et al., 2011; Hodgkinson et al., 2012).

Even though plenty of studies on 14-3-3 proteins have been published, few have shown the effects of 14-3-3 up/downregulation in animal models, and most have been focused on one isoform (14-3-3σ) (Porter et al., 2006). Hence, the present study paves the way to use *C. elegans* as a model to study 14-3-3 functions and expression regulation, as well as a platform to perform genome-wide RNAi screening in order to identify new 14-3-3 interactors and suppressors. Furthermore, taking into account the high number of putative 14-3-3 interactors that have been already identified in mammalian cells using proteomic methods (Jin et al., 2004; Meek et al., 2004; Pozuelo Rubio et al., 2004; Benzinger et al., 2005; Ge et al., 2010; Pozuelo-Rubio, 2010), *C. elegans* represents a powerful model to test the functional relevance of these interactions, as well as the genetic interactions and pathways involved.
Furthermore, *C. elegans* could be used as a high-throughput platform to test new drugs targeting 14-3-3 proteins. The multiple roles uncovered for *par-5* in this study allow the identification of drugs/peptides that inhibit some specific functions of the 14-3-3 proteins, for example those related with chemotherapy resistance. Nevertheless, extrapolation of results obtained in *C. elegans* regarding 14-3-3 function must be carefully analyzed due to the divergence of the 14-3-3 family in mammals.

7 CONCLUSIONS

In this study we examined the role of the 14-3-3 gene, *par-5*, in the *C. elegans* germline by using RNA interference that resulted in total protein depletion, and a hypomorphic allele that provokes a partial loss of function. This approach allowed the identification of several essential roles of *par-5* in worm development and the DNA damage response which are summarized as follows:

- Depletion of *par-5* severely impairs proliferation and meiotic progression of germ cells. Defects in these processes result in the sterility of *par-5* defective animals.
- *par-5* is necessary for DNA maintenance. Its inactivation promotes nuclei fragmentation and DNA damage accumulation in proliferating and meiotic germ cells.
- *par-5* is essential for the cell cycle arrest in response to diverse exogenous insults, participating in both the S and the G2/M checkpoints. This function is unique for a PAR family member.
- Additionally to its known role in embryonic asymmetric division, *par-5* is required for the DNA replication checkpoint, and also for cell cycle progression in the embryo.
- *par-5* function prevents premature entry into mitosis, both upon exogenous DNA damage and during germ cell proliferation.
- PAR-5 protein is widely expressed in somatic and germ cells. Although its subcellular localization is predominantly cytoplasmatic, *par-5* is localized in the nucleus of germ cells upon DNA damage.
- As part of the checkpoint response induced by DNA damage, *par-5* blocks entry into mitosis by regulating CDK-1 phosphorylation in the same manner as *wee-1.3* and counteracting *cdc-25.1* function.
- Given the relevance of 14-3-3 proteins in human diseases, this study presents *C. elegans* as a suitable model to identify new genes or to test drugs regulating 14-3-3 activity.

8 SUMMARY IN SPANISH (RESUMEN EN ESPAÑOL)

8.1 INTRODUCCIÓN

En presencia de daño genómico, las células responden promoviendo mecanismos de supervivencia con el objetivo de mantener la estabilidad genómica. Para ello, activan cascadas de transducción de señales que constituyen la llamada respuesta al daño genómico o DDR (de la sigla en inglés *DNA damage response*). Estos mecanismos se encargan principalmente de parar el ciclo celular, promover la reparación de los daños ocasionados en el ADN y en algunos casos inducir la apoptosis de la célula afectada. Como parte de la DDR, tanto la integridad del ADN como de la funcionalidad de los elementos requeridos para la división celular, son verificados en diferentes puntos a lo largo del ciclo celular, llamados *checkpoints.*

Las proteínas 14-3-3 conforman una familia conservada a través de la evolución que está implicada en múltiples procesos celulares. Las proteínas 14-3-3 actúan como dímeros que se unen principalmente a motivos fosforilados de Serina y Treonina presentes en otras proteínas. Su función es la de regular la localización, estabilidad e incluso la actividad de las proteínas a las que se unen (van Heusden, 2005). Como consecuencia de la interacción con múltiples proteínas, las proteínas 14-3-3 se han relacionado con varias enfermedades entre ellas la neurodegeneración y el cáncer (Tzivion et al., 2006; Morrison, 2008; Steinacker et al., 2011). En mamíferos existen siete proteínas 14-3-3 correspondientes a isoformas codificadas por diferentes genes (β, γ, ε, η, σ, τ, ζ). Esta redundancia ha dificultado el estudio de sus funciones celulares y aún existe poco conocimiento acerca de las consecuencias de la disfunción de las 14-3-3 a nivel del organismo.

Las proteínas 14-3-3 son necesarias para el arresto del ciclo celular en respuesta al daño genómico. Esta función se ha descrito en levadura, *Drosophila* y mamíferos, y depende de la interacción de las 14-3-3 con varias proteínas implicadas en la regulación del ciclo celular como Chk1, Cdc25 y Cdks (Chen et al., 1999; Kumagai and Dunphy, 1999; Lopez-Girona et al., 1999; Laronga et al., 2000; Dunaway et al., 2005). El rol de las proteínas 14-3-3 en el *checkpoints* fue descrito por primera vez en levadura de fisión (*Schizosaccharomyces pombe*), donde dos proteínas 14-3-3, Rad24 y Rad25, regulan el *checkpoint* de G2/M mediante el control de la localización subcelular de Cdc25 y Chk1 (Ford et al., 1994; Lopez-Girona et al.,

1999; Dunaway et al., 2005). En *Drosophila melanogaster*, las proteínas 14-3-3 (ζ and ε) regulan el ciclo celular durante el desarrollo. Específicamente, dichas proteínas inhiben la entrada en mitosis por medio de la inactivación de Cdk1 (Su et al., 2001). Esta función de las 14-3-3 en el control de la entrada en fase M, esta conservada en mamíferos. No obstante, la contribución de cada isoforma por separado se encuentra aún en exploración.

Caenorhabditis elegans se ha convertido en un modelo eficaz para el estudio de la función de los genes involucrados en el ciclo celular y la respuesta al daño genómico (Gartner et al., 2004). En la línea germinal de *C. elegans,* la exposición a agentes que dañan el ADN (radiación ionizante -IR-) o inhiben la replicación (Hidroxiurea -HU-), desencadena una respuesta del *checkpoint.* Dicha respuesta promueve una detención del ciclo celular en las células de la región proliferativa y en algunos casos, un incremento en la apoptosis de las células en meiosis. La vía molecular, conservada de levadura hasta mamíferos, actúa a través de las quinasas ATL-1/ATM-1 (homólogos de ATR y ATM, respectivamente) (Garcia-Muse and Boulton, 2005), así como otras proteínas sensores tales como RPA-1, WRN-1, MRE-11 o HUS-1 (Hofmann et al., 2002; Garcia-Muse and Boulton, 2005; Lee et al., 2010). Las quinasas CHK-1 y CHK-2 son los principales transductores de la vía (Kalogeropoulos et al., 2004; Stergiou et al., 2007; Bailly et al., 2010; Lee et al., 2010), pero también existen otras proteínas como CLK-2/RAD-5 que actúan en paralelo a esta vía canónica y promueven las respuestas del *checkpoint* (Ahmed et al., 2001; Collis et al., 2007).

En *C. elegans* existen dos genes 14-3-3, *par-5* y *ftt-2*, que codifican para proteínas cuya identidad es del 90%. A pesar de esta homología tan alta, su patrón de expresión es diferente y sólo *par-5* se encuentra expresada en la línea germinal. En *C. elegans* las proteínas 14-3-3 se han visto implicadas en longevidad y respuestas de estrés (oxidativo y calórico) mediante su interacción con la deacetilasa SIR-2.1 y el factor de transcripción DAF-16 (Berdichevsky et al., 2006). Sin embargo, esta función ha sido atribuida principalmente a *ftt-2*, mientras el papel de *par-5* sigue en exploración (Li et al., 2007). *par-5* pertenece a la familia PAR (*partitioning defective*) que regula la asimetría de la primera división embrionaria. En este proceso, *par-5* se requiere para la distribución asimétrica de las proteínas PAR. Adicionalmente, *par-5* también se expresa en la línea germinal del adulto y gusanos deficientes para *par-5* presentan menos progenie y un fenotipo de esterilidad (Morton et al., 2002). No obstante la función de *par-5* en la línea germinal del adulto no ha sido explorada hasta ahora.

Previamente en nuestro laboratorio, mediante la exploración de una serie de genes candidatos que podían participar en la respuesta al estrés replicativo, se observó que *par-5* podía estar implicado en el arresto del ciclo celular inducido en células de la línea germinal en respuesta al daño genómico. Teniendo en cuenta estos resultados previos y el fenotipo de esterilidad reportado previamente en los gusanos deficientes para *par-5* se decidió estudiar la función de *par-5* en la línea germinal de los gusanos adultos.

8.2 OBJETIVOS

- Caracterizar el papel del gen *par-5* en el desarrollo de la línea germinal de *Caenorhabditis elegans*.

- Determinar la función de *par-5* en la respuesta al daño genómico.

8.3 RESULTADOS

Para investigar el papel de *par-5* en la línea germinal, se estudiaron los fenotipos en una cepa mutante para *par-5,* (*it55*). Este alelo presenta un cambio de una amino ácido, lo que resulta en una reducción del nivel de expresión de la proteína (Morton et al., 2002). Para complementar el estudio también se analizó el fenotipo de gusanos alimentados con un RNA interferente (RNAi) que inhibe la expresión de *par-5*.

8.3.1 *par-5* es requerido para el desarrollo de la línea germinal

En los gusanos mutantes *par-5(it55)* en estadio de adulto de 1 día, se observó una reducción en el número de células germinales y en el tamaño de la gónada. Este defecto fue aún más pronunciado en gusanos alimentados con el RNAi de *par-5* (Figura 16). Adicionalmente y en contraste con los gusanos del tipo salvaje (*wild type*) y los mutantes *par-5(it55)*, las gónadas de los gusanos *par-5(RNAi)* presentaban núcleos pequeños fragmentados, sugiriendo catástrofe mitótica e inestabilidad genómica en las células germinales de la región proliferativa. La reducción en el número de células germinales empieza en el estadio L4 coincidiendo con la aparición de los núcleos hipercondensados y fragmentados. A diferencia de los gusanos *par-5(RNAi),* este fenotipo de inestabilidad genómica fue raramente observado en los gusanos *par-5(it55)*. Las diferencias entre los gusanos *par-5(it55)* y *par-5(RNAi)* implican que el alelo *it55* es hipomórfico. De hecho, los gusanos *par-5(it55)* alimentados con el RNAi contra *par-5*, presentan un fenotipo más pronunciado que los mutantes alimentados con un RNAi control (Figura 16). Además del defecto en proliferación, la mayoría de las gónadas de los gusanos *par-5(RNAi)* presentaban una reducción o ausencia de ovocitos. Esta observación sugiere que *par-5* también está implicado en la progresión a través de meiosis (Figura 17).

El defecto en la proliferación de la línea germinal observado luego de la inhibición de *par-5* podía estar relacionado con la influencia que tiene la gónada somática sobre la proliferación de la línea germinal. Sin embargo, gusanos de la cepa *rrf-1(pk1417)* (deficientes para el RNAi en tejidos somáticos) alimentados con el RNAi contra *par-5*, mostraron el mismo fenotipo que los gusanos salvajes alimentados con dicho RNAi. Por lo tanto, la reducción de la línea germinal observada en los gusanos *par-5(RNAi)* no está relacionado con un defecto en los

tejidos somáticos (Figura 18). Así mismo, se constató que las gónadas de los gusanos *par-5(it55)* y *par-5(RNAi)* no presentan un aumento en el número de células que sufren apoptosis en la región meiótica (Figura 19). Por tanto la reducción de ovocitos no está relacionada con un aumento de la apoptosis.

Por otra parte, para confirmar que el fenotipo obtenido con el RNAi era específico de *par-5*, se cuantificaron los niveles de tránscritos de *par-5* y *ftt-2* luego de alimentar a los gusanos con el RNAi de *par-5*. Usando RT-PCR cuantitativa se observó que el RNAi de *par-5* inhibe casi el 90% del mRNA de *par-5*, mientras que el mRNA de *ftt-2* no se ve afectado de manera significativa (Figura 20).

8.3.2 La inactivación de *par-5* promueve la acumulación de daño genómico endógeno

Debido a la observación de fragmentación genómica luego de la inhibición de *par-5* mediada por RNAi, se decidió profundizar en el papel de *par-5* en el mantenimiento de la estabilidad genómica. Por lo tanto, se examinó la abundancia de focos de la proteína RAD-51 en las células germinales. RAD-51 participa en la reparación del ADN promoviendo la recombinación homologa (Alpi et al., 2003; Ward et al., 2007). Utilizando un anticuerpo contra esta proteína, se observó un incremento de 10 veces en el número de focos de RAD-51 en las células germinales proliferativas de los gusanos *par-5(RNAi)* (Figura 21). La cepa *par-5(it55)* no presentó tal acumulación de daño, aunque mostró un pequeño incremento en los focos de RAD-51 comparando con la cepa salvaje. Así mismo, también se analizó la abundancia de focos de la proteína HUS-1, la cual actúa como sensor de daño genómico, principalmente en sitios de roturas de doble cadena. Los focos de HUS-1 se vieron aumentados en la región meiótica de la línea germinal luego del tratamiento con el RNAi de *par-5*, sugiriendo una acumulación de roturas de doble cadena (Figura 22A).

En los gusanos *par-5(RNAi)*, la acumulación de marcadores de daño genómico también se vio acompañada por una fosforilación constitutiva de la quinasa CHK-1 en las células ubicadas entre la zona proliferativa y la zona meiótica (zona de transición) (Figura 22B). Esta modificación se ha asociado con defectos que activan el *checkpoint* meiótico. Todos estos resultados sugieren que *par-5* previene la acumulación de daño genómico tanto en células germinales en proliferación como en células en meiosis.

8.3.3 La función de *par-5* es necesaria para la activación del checkpoint en las fases S y G2/M

Luego de observar que la inhibición de *par-5* produce una acumulación de daño genómico endógeno, se decidió investigar si *par-5* estaba implicado en la respuesta al daño genómico inducido por agentes genotóxicos exógenos como la Hidroxiurea (HU). La Hidroxiurea inhibe la enzima ribonucleótido reductasa, lo cual resulta en una disminución de los niveles de dNTPs y por tanto la inhibición de la replicación (estrés replicativo). El tratamiento con HU causa que las células germinales de la región proliferativa se arresten en la fase S como resultado de la activación del *checkpoint*. Este arresto celular se manifiesta en la disminución del número y el aumento del tamaño de los núcleos de las células germinales. Sin embargo, estas marcas de arresto no se observaron en los gusanos *par-5(it55)* y *par-5(RNAi)* luego del tratamiento con HU (Figura 23). La incapacidad para arrestar el ciclo celular en los gusanos deficientes para *par-5* no solo se observó en respuesta al estrés replicativo sino también en respuesta a radiación ionizante, la cual también activa el *checkpoint* de G2/M (Figura 24). En ambos casos, los núcleos de las células germinales *par-5(RNAi)* y *par-5(it55)* presentaron fragmentación y núcleos condensados en respuesta al daño inducido. Esto implica que las células germinales en gusanos deficientes para *par-5* son capaces de eludir el *checkpoint* y probablemente entran en mitosis de manera aberrante, como consecuencia del daño inducido.
Notablemente, *par-5* también se requiere para el *checkpoint* de replicación que se activa en el embrión en respuesta al estrés replicativo. El tratamiento con HU en la cepa salvaje produce un incremento en la duración de la fase S de aproximadamente siete veces la duración normal como consecuencia de la activación del *checkpoint*. Sin embargo, este retraso disminuye de manera significativa en los gusanos *par-5(RNAi)* y *par-5(it55)* (Figura 26). Por lo tanto el rol de *par-5* en el *checkpoint* no está restringido a la línea germinal.

8.3.4 *par-5* previene la entrada prematura en mitosis

La observación de fragmentación nuclear e hipercondensación de cromatina observada en gusanos deficientes para *par-5* después del tratamiento con agentes genotóxicos, sugería que en ausencia de *par-5* las células germinales entraban en mitosis de manera aberrante. Para estudiar este fenotipo se uso un anticuerpo que reconoce la Histona 3 fosforilada en la Serina

10 (P-H3) como marcador de mitosis. En la cepa salvaje el tratamiento con HU produce una reducción en el número de células en mitosis. No obstante, esta reducción no se observó en gusanos *par-5(RNAi)* y gusanos *par-5(it55)*. Por el contrario, en estos gusanos se observaron más células en mitosis luego del tratamiento con HU (Figura 28). Esto significa que las células germinales deficientes para *par-5* son capaces de entrar en mitosis bajo condiciones de estrés replicativo y por tanto eluden el *checkpoint* de fase S. Esta función de *par-5* en el control de la entrada en mitosis también se hizo evidente en gusanos *par-5(it55)* y *par-5(RNAi)* no tratados, los cuales también muestran un aumento del porcentaje de células en mitosis con respecto a lo observado en la cepa salvaje (Figura 29).

8.3.5 PAR-5 se acumula en el núcleo luego de la activación del checkpoint

Por medio de la construcción de una cepa transgénica que contenía la secuencia genómica de *par-5* fusionada a *gfp* se pudo observar que GFP::PAR-5 se expresaba en varios tejidos somáticos, principalmente intestino y neuronas (Figura 30). Por otro lado, mediante la utilización de un anticuerpo específico se observó que PAR-5 se expresa en todas las células de la línea germinal, aunque su nivel de expresión es menor en la esperma comparada con el resto de la gónada (Figura 32). A nivel sub-celular, PAR-5 se encuentra localizada principalmente en el citoplasma de las células germinales. Sin embargo, después del tratamiento con HU, se observó un cambio en la distribución de la proteína la cual se encontró acumulada en el núcleo de las células arrestadas (Figura 33). Ya que no se observaron cambios en los niveles de expresión de la proteína en respuesta a HU, se infiere que el cambio en la localización de la proteína puede ser importante para su función en la respuesta al daño genómico (Figura 33).

8.3.6 La inhibición por fosforilación de CDK-1 depende de la función de *par-5*

La entrada en mitosis está regulada por la actividad de CDK-1. Luego de la inducción de daño genómico, CDK-1 es fosforilada en un residuo inhibitorio (Tyr15) como parte de la respuesta del *checkpoint*, y de esta manera se evita la entrada en mitosis. Por lo tanto, se examinó si la inactivación de *par-5* afectaba la fosforilación de CDK-1. De igual forma que la HU, la Camptotecina (CPT) induce la respuesta al daño genómico en la línea germinal. El

tratamiento con CPT promueve la fosforilación de CDK-1 y el consecuente arresto en las células de la región proliferativa (Figura 34). Sin embrago, la inactivación de *par-5* reduce de manera drástica la fosforilación de CDK-1. El mismo efecto fue observado en una cepa mutante de *atl-1* (homologo de ATR), lo cual sugiere que la falta de CDK-1 fosforilada es una consecuencia de una activación deficiente del *checkpoint*. Estos resultados indican que la función de *par-5* es necesaria para la fosforilación de CDK-1 en respuesta al daño genómico.

8.3.7 *par-5* actúa de la misma manera que *wee-1.3* y de forma contraria a *cdc-25.1* en la regulación de CDK-1

Para profundizar en la relación entre la función de *par-5* y la fosforilación de CDK-1, se examinó la interacción funcional entre *par-5* y la fosfatasa *cdc-25.1*. En levadura y en mamíferos, las fosfatasas Cdc25 remueven la fosforilación de Cdk1 con el objetivo de promover la entrada en mitosis. De acuerdo con la conservación de esta función en *C. elegans,* se observó que la inhibición de *cdc-25.1* incrementa la fosforilación de CDK-1 y el arresto celular en respuesta al tratamiento con CPT (Figura 34). Esto se evidenció en el mayor tamaño de los núcleos de las células arrestadas y una señal más fuerte de la fosforilación de CDK-1. Este fenotipo es el opuesto al observado luego de la inhibición de *par-5,* lo cual implica un antagonismo funcional entre estos dos genes. De acuerdo a lo reportado anteriormente, se observó que el RNAi de *cdc-25.1* produce un arresto del ciclo celular en la región proliferativa. Este arresto es similar al observado en respuesta al tratamiento con HU o IR. Notablemente, el arresto inducido por la inactivación de *cdc-25.1* es rescatado en el trasfondo genético del mutante de *par-5*, reforzando la idea de una función opuesta de ambos genes en la regulación de la progresión del ciclo celular (Figura 35).

En levadura, la fosforilación de Cdk1 depende del balance entre las actividades de la quinasa Wee1 y la fosfatasa Cdc25 (Raleigh and O'Connell, 2000). Por consiguiente se probó si *par-5* podría estar actuando en el misma vía que *wee-1* en la regulación del ciclo celular. Usando doble inactivación mediada por RNAi, se observó que el RNAi de *wee-1.3* suprime de manera parcial el arresto inducido por el RNAi de *cdc-25.1* (Figura 36). Si bien, bajo el efecto del RNAi de *cdc-25.1* el número de células en mitosis es casi nulo, la co-inhibición con *wee-1.*3 causó un aumento evidente de figuras mitóticas en las células germinales. Este resultado indica que *wee-1.3*, igual que *par-5*, actúa contrariamente a *cdc-25.1* en la regulación de la

entrada en mitosis. De acuerdo con esta hipótesis, se observó que la supresión de *wee-1.3* suprime la fosforilación de CDK-1 en respuesta al daño genómico (Figura 34).
Adicionalmente, se observó que la inhibición de *wee-1.3,* suprime el arresto celular inducido por estrés replicativo, promoviendo mitosis aberrantes y fragmentación de los núcleos (Figura 37). No obstante, la inhibición de *wee-1.3,* en contraste con la de *par-5,* no afectó la proliferación de la línea germinal en ausencia de estrés replicativo (HU). Por lo tanto las funciones de *par-5* en la línea germinal no van siempre acopladas a la función de *wee-1.3.*

Estos resultados sugieren que luego de la inducción de daño genómico, *par-5* controla la entrada en mitosis de la misma manera que lo hace *wee-1.3*, promoviendo la fosforilación e inactivación de CDK-1 y contrarrestando la función de *cdc-25.1*.

8.4 DISCUSIÓN

La disminución del número de células germinales en los animales deficientes para *par-5* puede ser explicada, al menos en parte, por una entrada prematura en la fase M, lo cual resulta en defectos mitóticos. De acuerdo con esta hipótesis, luego de la inhibición de *par-5* se detectaron núcleos con cromatina hipercondensada y fragmentada. Estos núcleos probablemente han sufrido una catástrofe mitótica al entrar en mitosis con ADN que no estaba correctamente replicado o con algunos daños no reparados y por lo tanto, no son viables para continuar dividiéndose, lo cual contribuiría a la fuerte reducción de células germinales en los gusanos *par-5(RNAi)*. Este fenotipo fue rara vez observado en los mutantes *par-5(it55)*, en los cuales, a pesar de que la proliferación se ve afectada, los niveles de *par-5* son suficientes para mantener las divisiones celulares sin entrar en catástrofe mitótica (Figura 16). Además, la fragmentación de los núcleos observada en los gusanos *par-5(RNAi)* se vio acompañada por la acumulación de focos de RAD-51 en la región proliferativa de la línea germinal (Figura 21). Ambos fenotipos están asociados con defectos en el mantenimiento de la estabilidad del ADN durante la replicación, y han sido observados luego de la inhibición de genes clave del *checkpoint* como *atl-1* y *chk-1* (Kalogeropoulos et al., 2004; Garcia-Muse and Boulton, 2005; Lee et al., 2010).

Por otro lado, existen otras vías de señalización que podrían ser moduladas por *par-5* y que podrían estar implicadas en la proliferación de la línea germinal. Por ejemplo, *par-5* has sido relacionado con *daf-16*. Este gen codifica un factor de transcripción que se mueve al núcleo para activar la transcripción de genes relacionados con estrés y longevidad y su localización parece estar regulada por *par-5* y *ftt-2* (Berdichevsky and Guarente, 2006). Se ha demostrado que *daf-16* inhibe la proliferación de la línea germinal en los estadios de larva, en condiciones en las que la vía de la insulina esta suprimida (Michaelson et al., 2010). Por tanto, una posibilidad es que *par-5*, sea necesario para la inhibición de *daf-16* como efector de la vía de la insulina durante la proliferación de la línea germinal. Esta función podría contribuir a la reducción de la línea germinal observada desde L3 en los animales deficientes para *par-5* (Figura 16B).

La reducción de células en paquitene y de ovocitos en los gusanos *par-5(RNAi),* también indica una función de *par-5* en la progresión a través de meiosis (Figura 17). Existen varios factores que podrían contribuir a este fenotipo en los gusanos deficientes para *par-5.*

En primer lugar, el defecto en la proliferación que se presenta en los gusanos *par-5(RNAi)* reduce ostensiblemente la elongación de la gónada. Debido a que la entrada en meiosis es inhibida por la señalización proveniente de la región distal de la gónada, la elongación de la gónada permite que las células más proximales se alejen de esta señalización y entren en meiosis (Killian and Hubbard, 2005). Por consiguiente, la falta de elongación en los gusanos *par-5(RNAi)* dificultaría la entrada en meiosis de las células germinales. De acuerdo con esta hipótesis, se observó un retraso en la gametogénesis de los gusanos *par-5(RNAi)*. El 60% de los gusanos de la cepa salvaje han empezado la espermatogénesis en estadio L4 temprano, mientras que los gusanos *par-5(RNAi)* en el mismo estadio, no mostraban esperma.

En segundo lugar, la acumulación de daño genómico (focos de RAD-51 y HUS1), observada en las células germinales *par-5(RNAi)* se vio asociada con la activación constitutiva de la quinasa CHK-1 (Figura 22). Probablemente, la activación de esta quinasa es la consecuencia del daño genómico elevado en la células meióticas, como se ha reportado previamente para esta modificación (Bhalla, 2010; Jaramillo-Lambert et al., 2010). Por lo tanto, es posible que en estas condiciones las células con daños en el genoma no puedan progresar correctamente a través de meiosis. Además, la maduración de los ovocitos requiere del arresto del ciclo celular en la profase I de meiosis. Al igual que el arresto de la entrada en mitosis que ocurre en células proliferativas, este arresto depende de la inhibición de CDK-1 (Boxem et al., 1999; Burrows et al., 2006). Por consiguiente, como *par-5* regula la fosforilación de CDK-1 en la

transición G2/M en las células germinales proliferativas, es posible que la desregulación de CDK-1 también contribuya a los defectos en los ovocitos observados en los gusanos *par-5(RNAi).*

Otro de los resultados importantes de este estudio es la regulación de la progresión del ciclo celular en la primera división del embrión por parte de *par-5*. Específicamente, se observó que los embriones deficientes para *par-5* presentaban una fase S más corta y una fase M más larga (Figura 26). Comparando con los datos reportados previamente para los embriones deficientes para los otros genes *par*, se vio que este patrón de ciclo celular es específico de la inhibición de *par-5* (Figura 27). Adicionalmente, se observó que *par-5* también era requerido para el retraso del ciclo celular inducido por el *checkpoint* en respuesta al estrés replicativo (Figura 26). Por tanto, teniendo en cuenta el papel que juega *par-5* en la distribución de las otras proteínas PAR para la división asimétrica, es tentador pensar que *par-5* podría ser el link entre la maquinaria de la familia PAR y el control del ciclo celular, lo cual es requerido para la determinación de los destinos celulares en las células del embrión.

En este estudio se ha demostrado que la función del gen *par-5* es necesaria para promover el arresto celular en respuesta al daño genómico. Sin embargo, aunque sólo los gusanos *par-5(RNAi)* mostraron una acumulación de daño genómico endógeno y fragmentación en el ADN (Figura 21), tanto los gusanos *par-5(RNAi)* como los *par-5(it55)* mostraron defectos del *checkpoint* similares en la respuesta al daño inducido de manera exógena (después del tratamiento con HU o IR) (Figura 23 y 24). Por consiguiente, teniendo en cuenta que la cepa mutante expresa niveles bajos pero no nulos de la proteína PAR-5 (Morton et al., 2002), se puede inferir que una reducción moderada en los niveles de PAR-5 es suficiente para afectar la respuesta al daño extrínseco, mientras que una reducción casi completa (como la observada con el RNAi) afecta también la proliferación de las células germinales y la estabilidad genómica. La acumulación de daño genómico endógeno observada en los gusanos *par-5(RNAi)* podría explicarse por la incapacidad de las células germinales para detener el ciclo celular y reparar los daños que normalmente se presentan en el ADN. Sin embargo, no se puede descartar la posibilidad de que la inhibición de *par-5* por sí misma, genere más daño genómico a través de otras vías directas o indirectas.

De acuerdo con la función de las 14-3-3 en otros organismos, los resultados de este estudio son compatibles con la idea de que *par-5* colabora con *wee-1.3* y contrarresta la función de

cdc-25.1 en la entrada en mitosis y en respuesta al daño genómico. Este modelo colocaría a PAR-5 como una de las proteínas efectoras requeridas para el arresto del ciclo celular en respuesta al daño en el ADN (Figura 38). Es de notar que a pesar de su conservación, la función de los genes *par*-5, *wee-1*.3 y *cdc-25.1* en el *checkpoint* en *C. elegans* no habían sido descritas antes. No obstante, si bien *par-5* y *wee-1.3* parecen colaborar en el *checkpoint*, la inhibición de *wee-1.3*, a diferencia de *par-5*, no parece afectar la proliferación de la línea germinal en ausencia de daño exógeno. Por tanto, no todas las funciones de *par-5* en la línea germinal están relacionadas con *wee-1.3*.

Igualmente, en mamíferos las proteínas 14-3-3 secuestran CDK1 fosforilada en el citoplasma en respuesta a la activación del *checkpoint*. Sin embargo, en *C. elegans* la CDK-1 fosforilada se localiza en el núcleo. Por tanto, si PAR-5 regula a CDK-1, el mecanismo debe ser diferente al secuestro en citoplasma. Además, PAR-5 se vio localizada en el núcleo de las células germinales luego de la inducción de estrés replicativo (Figura 33), sugiriendo que su función debe estar relacionada con interacciones que suceden dentro del núcleo, por ejemplo con WEE-1.3 o CDK-1. En este sentido, una hipótesis sería que la unión dentro del núcleo de PAR-5 a CDK-1 fosforilada es necesaria para bloquear la interacción de CDK-1 con sus dianas y por tanto, para detener la entrada en mitosis. En este sentido, se requieren experimentos adicionales para identificar las interacciones de PAR-5 y su relevancia en la respuesta del *checkpoint* y la proliferación de la línea germinal.

Finalmente es importante considerar las implicaciones de este estudio en relación al uso de *C. elegans* como modelo para investigar la función de las proteínas 14-3-3. A pesar de que los homólogos de 14-3-3 en mamíferos han divergido estableciendo una familia de siete genes, en este estudio se confirma que las funciones básicas de las 14-3-3 están conservadas en *C. elegans*. De hecho, la catástrofe mitótica que presentan los gusanos *par-5(RNAi)* también ha sido observada en células humanas a las cuales se les inhibe una 14-3-3 en respuesta al daño genómico (específicamente la isoforma sigma) (Chan et al., 1999). Sin embargo, a diferencia de lo mamíferos, en *C. elegans* sólo existen dos 14-3-3 y sólo una de ellas se expresa en la línea germinal. Esto podría explicar el por qué *par-5* es esencial para mantener las estabilidad genómica y la proliferación de la línea germinal, mientras la inhibición de una única 14-3-3 en mamíferos no tiene tanta influencia en las células en ausencia de daño exógeno (probablemente por la redundancia funcional entre algunas isoformas de los mamíferos). Por otro lado la fragmentación de ADN en las células germinales de animales deficientes para *par-5* tratados con diferentes agentes genotóxicos (CPT, HU, IR) implica una sensibilidad

elevada de las células proliferativas a estos agentes en ausencia de *par-5*. Esta observación coincide con los múltiples estudios que muestran que la sobreexpresión de las 14-3-3 está relacionada con la resistencia a la quimioterapia en líneas celulares humanas y también los reportes que muestran que la inhibición de las 14-3-3 sensibiliza a dichas células a la apoptosis. Estos estudios han llevado a proponer las 14-3-3 como posibles dianas para el tratamiento del cáncer (Porter et al., 2006; Tzivion et al., 2006; Neal and Yu, 2010; Bergamaschi et al., 2011; Hodgkinson et al., 2012).

Aunque hay muchos estudios acerca de la función de las proteínas 14-3-3, la mayoría se han enfocado en una isoforma (14-3-3 sigma) y muy pocos han mostrado los efectos de la inhibición de las 14-3-3 en animales modelo. Por lo tanto, esta investigación abre las puertas para el uso de *C. elegans* para el estudio de las funciones y regulación de las proteínas 14-3-3, así como plataforma para realizar ensayos de RNAi a escala genómica con el objetivo de identificar otras proteínas que interactúen o supriman la función de las 14-3-3. Teniendo en cuenta la gran cantidad de interacciones putativas que se han identificado para las 14-3-3 utilizando métodos proteómicos (Jin et al., 2004; Meek et al., 2004; Pozuelo Rubio et al., 2004; Benzinger et al., 2005; Ge et al., 2010; Pozuelo-Rubio, 2010), *C. elegans* se presenta como un organismo potente para testar la relevancia funcional de estas interacciones, así como las interacciones genéticas y las vías moleculares involucradas. Además, *C. elegans* podría ser usado para testar drogas que puedan suprimir las funciones de las 14-3-3, específicamente las relacionadas con la resistencia a la quimioterapia. No obstante, la extrapolación de los resultados obtenidos en *C. elegans* debe ser analizada con cuidado debido a la divergencia de la familia 14-3-3 en mamíferos.

8.5 CONCLUSIONES

En este estudio se analizó el papel del gen *par-5* en la línea germinal de *C. elegans* mediante el uso de un RNAi que produce la depleción total de la proteína y un mutante hipomorfo que provoca una pérdida parcial de la función. Esta aproximación permitió la identificación de varias funciones de *par-5* que se resumen a continuación:

- La supresión de *par-5* afecta de manera severa la proliferación y la progresión meiótica de las células de la línea germinal. Los defectos en estos procesos se ven reflejados en la esterilidad de los animales deficientes para *par-5*.
- *par-5* es necesario para el mantenimiento del ADN, ya que su inactivación promueve fragmentación de la cromatina y acumulación de daño genómico tanto en células en proliferación como en células en meiosis de la línea germinal.
- *par-5* es esencial para el arresto del ciclo celular en la fases S y G2/M en respuesta a diversos agentes genotóxicos. Esta función no había sido descrita antes para un miembro de la familia PAR.
- En adición a su rol en la división asimétrica del embrión, *par-5* se requiere para el *checkpoint* de replicación del ADN y también para la progresión del ciclo celular que se dan en la primera división del embrión.
- *par-5* previene la entrada prematura en mitosis en respuesta a agentes genotóxicos y también durante la proliferación de las células germinales.
- PAR-5 se expresa en varios tejidos somáticos, principalmente neuronas e intestino, y en todas las células de la línea germinal. Aunque a nivel sub-celular su expresión es predominantemente citoplasmática, PAR-5 se encuentra en los núcleos de las células germinales en respuesta al estrés replicativo.
- En respuesta al daño genómico, *par-5* bloquea la entrada en mitosis mediante la regulación de la fosforilación de CDK-1, del mismo modo que *wee-1.3* y en contraposición a la función de *cdc-25.1*.
- Dada la implicación de las proteínas 14-3-3 en enfermedades humanas, este estudio presenta *C. elegans* como modelo apropiado para identificar nuevos genes o validar drogas que puedan regular la actividad de las 14-3-3.

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