Cell cycle uncoupling, elimination, and functional modification of centrioles during *C. elegans* development

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ABSTRACT

Centrosome duplication is coupled with cell division to ensure that centrosome is duplicated only once per cell cycle. This coupling, however, can be altered in specific developmental contexts although how this uncoupling occurs remains generally unclear. In *C. elegans*, the larval intestinal and the hypodermal cells will endocycles, while germ line stem cells eventually exit mitosis and enter meiosis. We use these models to better understand how the centrosome is intimately coupled to the cell cycle and the mechanisms though which the duplication of the centrioles can be uncoupled from cell division during the course of development.

By monitoring the levels of SPD-2, a protein that is critical for centriole duplication in *C. elegans*, we found that the centriole duplicates normally at the intestinal cell nuclear division, but does not re-duplicate during the first endocycle, Subsequently SPD-2 becomes diffuse within the nucleus before it is subsequently eliminated. These dynamic changes seem to be actively regulated since they are not observed in situations of un-quantized DNA re-replication.

To test whether cell cycle regulators might regulate centrosome/cell cycle uncoupling and elimination, we generated phosphomimetic and non-phosphorylable variants of SPD-2. We found that altering the highly conserved CDK-phosphorylation site of Serine 545 uncouples the centriole duplication/cell cycle coupling, whereas mimicking PLK-mediated phosphorylation or reducing the activity of ubiquitylation pathway by RNAi leads to nuclear accumulation of SPD-2 potentially by stabilizing SPD-2 without affecting centrosome duplication and uncoupling. Overall our study reveals that phosphorylation of SPD-2 by key cell cycle kinases may regulate centrosome/cell cycle uncoupling and elimination during in *C.elegans* development.

Secondly, we studied the role of RNF-1, a RING-domain protein that interacts with Cip/Kip family member CKI-2 in *C. elegans*. We found that RNF-1 mediates the ubiquitylation of CKI-2, which consequently results in its proteasome-dependent degradation. Consistent with this, RNF-1 reduces the embryonic lethality caused by

misexpression of CKI-2. We also found that RNF-1 is localized at nuclear periphery, although the significance of this localization still requires further characterization.

Finally, we analyzed the localization and the function of γ-tubulin during germ cell progression. We found that γ-tubulin undergoes a re-distribution from its association with the centriole to germ cell membrane at the onset of meiosis. This re-distribution causes the centriole to lose its microtubule nucleating capacity and appears to be triggered by signals that occur during the mitosis-meiosis transition. We are continuing a characterization of the significance and the mechanism of this re-distribution.

RÉSUMÉ

La duplication des centrosomes est couplé à la division cellulaire afin qu'elle n'ait lieu qu'une seule fois par cycle cellulaire. Cependant, lors de certains contextes développementaux, ce couplage n'a pas lieu et ceci reste mal compris à ce jour. Chez *C. elegans*, alors que les cellules hypodermales et intestinales font de l'endo-replication, les cellules souches germinales sortent de la mitose pour entrer en méiose. L'utilisation de ces différents modèles cellulaires, nous permet de mieux comprendre comment la duplication des centrosomes est dans la plupart des cas intimement couplée au cycle cellulaire, et d'étudier les mécanismes où la duplication des centrioles est indépendante à la division cellulaire au cours de contextes développementaux particuliers.

SPD-2 est une protéine essentielle à la duplication des centrioles chez *C.elegans*. En observant ses niveaux d'expression, nous avons pu montré qu'alors que les centrioles sont correctement dupliqués lors de la division des cellules intestinales, ils ne se re-dupliquent pas au cours du 1er-cycle d'endo-replication. En effet, SPD-2 diffuse dans le noyau, avant d'être éliminé. Cette dynamique semble être activement régulée, car elle n'est pas observée dans des situations où l'ADN est très anormalement répliqué. Afin d'étudier l'importance des régulateurs du cycle cellulaire dans ce découplage centrosome/cycle cellulaire, nous avons généré des variants SPD-2, phosphomimétiques ou non-phosphorylables. De manière très intéressante, la modification de la serine 545, site très conservé pour la phosphorylation par CDK, entraine le découplage de la duplication du centriole par rapport au cycle cellulaire. Au contraire, en mimant la phosphorylation par PLK ou en diminuant l'activité de la voie de l'ubiquitylation, par ARNi, la protéine SPD-2 s'accumule dans le noyau. Cette accumulation est probablement due à la stabilisation de la protéine, mais elle n'affecte pas la duplication du centrosome. Notre étude révèle donc l'importance des phosphorylations de SPD-2 par différentes kinases clés du cycle cellulaire dans la régulation son activité. En effet, celles ci pourraient réguler le découplage de la duplication des centrosomes du cycle cellulaire et affecter leur élimination au cours du développement chez *C. elegans*.

Parallèlement, nous nous sommes aussi intéressé au rôle de RNF-1, une protéine contenant des domaines RING qui interagit avec CKI-2, un membre de la famille Cip/Kip. Nous avons démontré que RNF-1 joue un rôle crucial dans l'ubiquitylation de CKI-2, qui est alors dégradé par la voie du protéasome. Ainsi, l'expression de RNF-1 réduit la létalité embryonnaire causée lors d'une mauvais expression de CKI-2. RNF-1 se localise à la périphérie du noyau, toutefois la fonction associée à cette localisation nécessite une étude plus approfondie.

Finalement, nous avons etudié le rôle de la γ-tubuline au cours de la progression des cellules germinales. Nous avons trouvé que la γ-tubuline est redistribuée depuis sa localisation centriolaire vers la membrane cytoplasmique des cellules germinales pendant la méiose. Cette redistribution inhibe les capacités du centriole à générer la nucléation des microtubules, ceci résultant probablement de signaux transmis lors de la transition entre la mitose et la méiose. Nous continuons notre travail afin de comprendre le rôle et les mécanismes impliqués dans cette redistribution.

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PREFACE

This is a manuscript-based thesis presented in accordance with the "Guidelines for thesis preparation" from the Faculty of Graduate Studies and Research. It consists of an Introduction (Chapter I), which provides a literature review as well as the rationale and objectives of my research; three research chapters (Chapter II, III and IV) and a chapter for general discussion (Chapter V). The research chapters are formatted as research manuscripts composed of the following sections: Abstract, Introduction, Materials and Methods, Results, Discussion, Figures and Tables. Each chapter also contains its own Reference sections.

I have prepared and written all the chapters of my thesis. My thesis supervisor Dr. Richard Roy has contributed to this research at all levels. He suggested ideas and edited all manuscripts. In the case of multi-authored manuscripts, the details contributions of each co-authors has been outlined in the ensuing sections. Contributors who are not authors are recognized in the corresponding Acknowledgement sections.

CONTRIBUTIONS OF AUTHORS

Chapter II

This chapter is a reformatted version of

Lu, Y. and Roy, R. (2013). Cell cycle uncoupling and centriole elimination in the endoreduplicating intestinal cells of *C. elegans.* Submitted to *Development.*

I designed the experiments with Roy, R. and performed all the experiments presented in this paper. I wrote the manuscript and prepared all the figures. My supervisor edited the manuscript.

Chapter III

This chapter details my unpublished work on the functional characterization of RNF-1 in order to better understand the regulation of CKI-2. This work will be submitted for publication published in the near future:

Lu, Y., Kim, D.Y. and Roy, R. RNF-1, a *C. elegans* RING domain protein, modulates CKI-2 through ubiquitin-dependent proteolytic pathway.

Kim, D. Y. conducted the initial yeast-two hybrid screen and contributes to the Figure 1. I designed and performed all the rest of experiment. I wrote the present version of the manuscript and prepared all the other figures and tables other than Figure 1. My supervisor edited the manuscript.

Chapter IV

This chapter demonstrates my unpublished work on the localization changes and subsequent effects on the microbutule network of γ-tubulin during the mitosis to meiosis transition. This work will be finished and published in a developmental journal as followed:

Lu, Y. and Roy, R. The analysis of the function and the re-distribution of γ-tubulin during the switch from mitosis to meiosis in the *C. elegans* germ line.

I designed with Roy, R. and performed all the experiment presented in this Chapter. I wrote the entire Chapter and made all the figures. My supervisor edited the Chapter.

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Literature Review

1.1. OVERVIEW

The development of a multi-cellular organism from a one-cell embryo requires cell proliferation to increase tissue mass. Specification gives rise to tissue and organs while differentiation allows a cell type to possess a distinct function. This complicate process is achieved by a series of mitotic cell cycle modulated by temporal and spatial control.

The mitotic cell cycle includes a gap phase G1, a synthesis phase (S), during which the entire genome is replicated, another gap phase G2, and mitosis (M phase). During mitosis the replicated sister chromatids are segregated into two genetically identical daughter cells (Murray and Hunt, 1993). The passage through the cell cycle must be tightly regulated in order to guarantee that key cellular events, including DNA replication and segregation, take place accurately and in accordance with other developmental events. This regulation is imposed by the activity of both positive and negative components of the cell cycle machinery.

1.1.1. Cyclin Dependent Kinase and its regulatory system

The basic cell cycle machinery is highly conserved throughout eukaryotes. A series of serine/threonine protein kinases referred to as Cyclin-Dependent Kinases (CDKs) drive the cell cycle through the different phases by inducing phosphorylation of their downstream substrates.

The activation of these CDKs requires their association with stage-specific regulatory Cyclins that act as cofactors while also conferring target specificity to the CDK enzyme. The budding yeast *S. cerevisiae* and the fission yeast *S. pombe* each have only one CDK gene called *cdc28* or *cdc2* respectively. The stage-specific Cyclins turn on the activity of CDC28 or CDC2 accordingly (Wittenberg et al., 1990; Forsburg and Nurse, 1991). A more complex regulatory network that consists of multiple CDKs and cyclins, however, exist in multi-cellular organisms. A single D-type cyclin is encoded in mice, *Drosopholia*, *C. elegans* and many other organisms, while three D-type cyclins, cyclin D1, D2 and D3 have been identified in humans (Assoian and Klein, 2008; Park and Krause, 1999). In multi-cellular organisms, growth factor signaling stimulates the

expression of D-type cyclins. Subsequently Cyclin D binds and activates CDK4 and CDK6 during G1, which is essential for the entry into the progression of cell cycle (Assoian and Zhu, 1997; Assoian and Klein, 2008).

One key substrate of CDK4/6 is retinoblastoma tumour suppressor (pRb). pRb binds and inhibits the transcription factor E2F; the CDK4-dependent phosphorylation of pRb causes the release of E2F, consequently activating the expression of many genes essential for G1/S transition and S phase progress, including cyclin E (Geng et al., 1996; Kato et al., 1993). Cyclin E forms a complex with CDK2 in the later stage of G1 to progress beyond the G1/S transition and to stimulate the assembly of the DNA replication complex (Converley et al., 2002; Tsai et al., 1993).

During S phase, the binding of CDK2 and Cyclin A activates DNA synthesis with the assembled replication complex while concomitantly inhibiting new complex assembly to ensure that DNA replication only occurs once per cycle (Converly et al., 2002; Copeland et al., 2010; Walker and Maller, 1991). In addition to binding with CDK2, Cyclin A also complexes with CDK1 during G2 and initiates the entry into M phase (Yam et al., 2002).

B-type Cyclins are predominantly expressed during G2/M and interact with the M phase kinase CDK1. In humans, the Cyclin B1-CDK1 complex is involved in the characteristic events of M phase, such as nuclear envelope breakdown and chromosome condensation (Gavet and Pines, 2010). Cyclin B2 may function redundantly to Cyclin B1, since both of them show membrane-associated expression and cyclin B2-defective mice develop normally (Brandeis et al., 1995). On the other hand, cyclin B3 expresses specifically in the testis in humans (Nguyen et al., 2002), while the female *Drosophila* cyclin B3-defective animal is sterile (Jacobs et al, 1993), suggesting the involvement of cyclin B3 in meiosis.

Due to the essential roles of CDKs during cell cycle progression, it is not surprising that numerous regulators affect cell cycle progression by controlling the activity or stability of CDKs or CDK-Cyclin complexes. Indeed, regulation can occur at many levels, including gene expression, substrate

recognition/concentration and proteolysis. These regulatory mechanisms are mediated by many highly conserved proteins in different species.

CDK7-Cyclin H, a CDK activating kinase (CAK) phosphorylates specific threonine residues in the CDK activation loop on CDK4, CDK2 and CDK1, to cause conformational changes that enhances the association of CDKs with their appropriate Cyclin partners (Harper and Elledge, 1998). In contrast, Wee1 and Myt1 family kinases catalyze an inhibitory phosphorylation on tyrosine-15 and threonine-14 of CDK1 respectively and thus prevent M phase entry. Dephosphorylation of these two amino acids by the phosphatase CDC25 is thus necessary to restore the activity of CDK1 (Lew and Kornbluth, 1996).

In addition to the negative regulatory phosphorylation on CDKs, CDK inhibitor proteins (CKI) inhibit or block cell cycle progression by counteracting CDK activity through their association with CDKs. Two families of CKIs have been identified, namely the INK4 (inhibitor of CDK4) family and the Cip/Kip family. The INK4 family includes $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$ and specifically targets G1 phase CDKs, i.e. CDK4 and CDK6. INK4 binds to its CDK substrate prior to cyclin binding, thereby preventing it from complexing with Cyclin D (Roussel, 1999). The enhanced expression of INK4 family members induces cell cycle arrest at G1 (Sherr and Roberts, 1995).

The Cip/Kip family includes $p21^{Cip1}$, $p27^{Cip2}$ and $p57^{Kip1}$. Various numbers of Cip/Kip family members have been identified in diverse model organisms. For example, a single Cip/Kip CKI called Dacapo is encoded in *Drosophila*, whereas four Cip/Kip CKIs have been identified in *Xenopus* (Daniels et al., 2004; Lane et al., 1996; Shou and Dunphy, 1996). Cip/Kip CKIs play broader roles than the INK4 family, affecting G1/S, S, and G/M through their capabilities to alter the activities of Cyclin D-, E-, A- and B-dependent kinases (Hengst and Reed, 1998; Sherr and Roberts, 1999). In fact, Cip/Kip CKIs are able to bind both CDK and Cyclin. For instance, $p27^{Cip2}$ interacts with the CDK2/Cyclin A complex and inhibits substrate binding at the ATP binding site of the catalytic subunit (Russo et al., 1996), potentially disrupting the function of CDK2/Cyclin A complex. Interestingly, this capacity of binding both CDK and Cyclin enables Cip/Kip

family members to promote the assembly and activation of CDK4/CyclinD (LeBaer et al., 1997), suggesting Cip/Kip family members also have a positive role in cell cycle activity.

Overall, a regulatory complex exists in eukaryotes to ensure that cell cycle progression occurs in a timely manner. CDKs/Cyclins function as the core of the regulatory complex and various positive and negative regulators balance the activity of CDKs through many mechanisms. Misregulation of any essential factors in this regulatory complex may result in diseases that arise due to abnormalities in cell cycle progression. For instance, low expression of p27 occurs frequently in many cancers (Sherr and Robert, 1999).

1.1.2. Ubiquitylation system

In addition to CDK activity, cell cycle progression is controlled by successive cycles of directed protein degradation. For instance, the degradation of Wee1 at the beginning of M phase is required for entry into mitosis, whereas the mitotic Cyclins need to be eliminated in order to exit mitosis (Li and Yang, 2007; Watanabe et al., 2004). Protein destruction plays other important roles, such as removing damaged proteins in the cell. Commonly, the degradation of protein is accomplished by the ubiquitin-proteosome system in two successive steps. Target substrates are first marked by the covalent attachment of multiple ubiquitin molecules and secondly the poly-ubiquitinated proteins are degraded by the 26S proteosome complex (Thrower et al., 2000).

Ubiquitin is a highly conserved 76-amino acid polypeptide. The covalent attachment of ubiquitin to substrates requires three enzyme activities. Ubiquitin is firstly activated by E1 activating enzyme, and then activated ubiquitin is transferred to E2 ubiquitin-conjugating enzymes. E3 ligases responsible for recognizing target proteins, catalyze the final step by ligating a ubiquitin chain onto key lysine residues. A poly-ubiquitinated chain is generated by the sequential addition of activated ubiquitins to either the lysine 63 residue or lysine 48 residue of the previous ubiquitin (Hochstrasser, 2009). Lysine 63-linked ubiquitin chains play a role in the regulation of substrate localization, while lysine 48-linked poly-

ubiquitinated proteins are eventually degraded by the 26S proteosome. The 26S proteosome, present in both cytoplasm and nucleus, consists of a proteolytic 20S core and two 19S regulatory complexes, the latter of which are responsible for recognizing, deubiquitinating and unfolding substrates (Pickart and Cohan, 2004; Wojcik and DeMartino, 2003). In the end, the poly-ubiquitin chain is recycled for other reactions, whereas the unfolded substrates are degraded by the 20S core.

Consistent with the roles of E3s in determining the specificity of substrates, hundreds or even thousands of E3 enzymes exist in most eukaryotes, whose genomes meanwhile encode only a single E1 and a few E2 enzymes (Hershko and Ciechanover, 1998). The functions of E3s have therefore been a subject of intense investigation. Indeed, defects in E3 enzymes have been found responsible for several human diseases. For example, defective human ITCH E3 ligase causes autoimmune diseases, while dysfunction of Parkin, another E3 ligase, is involved in Parkinson's disease (Dawson and Dawson, 2003; Lohr et al., 2010).

Based on the presence of conserved motifs, E3 enzymes can be further categorized into 4 classes: HECT, U-box, PHD-finger, and RING (Really Interesting New Gene)-finger class (Nakayama and Nakayama, 2006). RING-finger class E3s, the largest group of E3s, can function in either a monomeric form, or in multisubunit complexes that contain a Cullin or a Cullin homolog protein. In fact, two complexes, Skp1/Cullin/F-box (SCF) ubiquitin ligase and anaphase promoting complex/cyclosome (APC/C) have been well characterized and impinge on cell cycle progression via timely degradation of several key cell cycle regulators (Nakayama and Nakayama, 2006).

The SCF complex is composed of four subunits. Scaffold protein Skp1, a Cullin (Cul1) and RING-finger component (Rbx1) are invariable elements and an interchangeable adaptor protein such as an F-box family member is required for substrate recognition (Zheng et al., 2002). The SCF complex is implicated in the degradation of a variety of positive and negative cell cycle regulators, including Cyclin A, Cyclin D1, and Cyclin E, as well as Cip/Kip family CKIs (Abbas and Dutta, 2009; Lin et al., 2006; van Drogen et al., 2006). Not surprisingly, misregulating of the SCF complex components such as F-box proteins often

causes inappropriate stability of cell cycle regulators and consequently results in abnormal cell cycle progression (Kipreos et al., 2000; Nakayama and Nakayama, 2006).

Larger than SCF yet with some structural similarities, the APC/C complex consists of 11-13 subunits (Penas et al., 2011), including a RING finger protein APC11 and the Cullin-related scaffold protein APC2. APC/C is implicated in the promotion of sister chromatid segregation because it degrades securin, the inhibitor of anaphase and chromatid segregation (Peters, 2006). Also, APC/C is also responsible for the degradation of Cyclin B (Izawa and Pines, 2011).

1.1.3. Endocycle regulation

During development, although most tissues grow by increasing overall mass through cell proliferation (Wilson, 1925), some cell types deviate from regular cell division to instead execute an alternative endocycle, or endoreduplication. The endocycle is distinct from the canonical G1-S-G2-M cell cycle due to the absence of cell division. One typical form of endocycle thus features alternating S and G phases. However, DNA replication occurs only once during one endo-S phase, which is distinct from the un-quantized DNA re-replication that is stimulated by a stabilized DNA replication complex (Zhong et al., 2003). As a result, endocycling cells periodically double their DNA content (Lilly and Duronio, 2005).

Endocycles exist in many organisms, from plants to humans (Lee et al., 2009). It has been documented in leaves and root hairs in plants (Kodonrosi, 2000), nurse cells and follicle cells in *Drosophila* (Lilly and Spradling, 1996), in the intestinal cells in *Drosophila* and *C. elegans*, and some epidermal cells in *C. elegans* (Hedgecock and White, 1985; Smith and Orr-Weaver, 1991). In mammals, megakaryoctes and trophoblast cells in the placenta undergo endoreduplication and the latter of which can achieve greater than 1000C of DNA content (Pang et al., 2005; Zybina and Zybina, 1996). The elevated DNA content of endocycling cells is associated with an increase in mRNA as well as protein synthesis, and enlarged cell volume/mass. Endocycling cells are often implicated in supporting

the growth of other cells or tissues by providing nutrients and biological molecules.

In the egg chamber of *Drosophila* 16 germ line cells are present, and yet only one of these will adopt an oocyte fate. The remaining 15 cells become supporting nurse cells. These 16 germ line cells remain interconnected and share a common cytoplasm. The nurse cells undergo endoreduplication and generate a high degree of polyploidy. They synthesize large quantities of maternal mRNA and proteins that are transferred to the oocyte (Bastock and St Johnston, 2008). These maternal deposits contributed during oogenesis later play important roles for early embryogenesis when zygotic transcription is still silent (Bastock and St Johnston, 2008; French et al., 2003). Failure to execute endocycles in nurse cells results in sterility (Lilly and Spradling, 1996). In mammal, endocycles are also important for fertility. For example, rodent placental trophoblast cells nourish the developing embryo (Cross, 2005).

The endocycle is also important in other specialized differentiated somatic tissues. In *C. elegans*, 20 intestinal cells envelop the monolayered intestinal lumen. The total size of intestine increases significantly during larval development. However, *C. elegans* takes advantage of endocycles to enlarge the volume and metabolic output of individual cells, instead of increasing the cell number (Kipreos, 2005). This probably circumvents the disruption of intestinal epithelia integrity and corresponding physiological function of intestine that would result from performing cell division.

Like the canonical cell cycle, the endocycle is highly regulated. In fact, the progression of the endocycle is controlled by a subset of the factors that program mitosis. For example, the S phase of mitotic cell cycle is driven by CDK2 and its associated Cyclin, such as Cyclin E, whose activity is also required for DNA replication in the S phase of endocycle in mammals and *Drosophila* (Aleem et al., 2005; Lane et al., 2000). Disrupting the functions of CDK2-associated Cyclin E can halt the endocycle (Geng et al., 2003; Lilly and Spradling, 1996), comfirming that CDK2/Cyclin E is a major regulator of the endocycle.

Conversely, a constitutively active CDK2 blocks DNA replication during the endocycle (Lilly and Spradling, 1996). It appears that the periodic increase of DNA during endocycles requires the oscillation of CDK2 activity, since low CDK activity is absolutely necessary to permit the DNA pre-replicative complex (pre-RC) to assemble prior to the upcoming S phase, while elevated CDK activity leads to the DNA replication and dismantles the pre-RC (Labib and Gambus, 2007; Weiss et al., 1998).

The major difference between the endo-S phase and the mitotic cell cycle S phase is the absence of control mechanism that ensures the completion of DNA replication, also referred to as the DNA replication checkpoint, is missing in endo-S phase. As a result the replication of late-replicating sequences, such as satellite DNAs, remains incomplete (Lilly and Spradling, 1996).

Since M phase is absent from the endocycle, it is not surprising that CDK1 activity, essential for mitotic entry, is generally downregulated in the endocycling cells (Lilly and Duronio, 2005). In *Drosophila*, B-type Cyclins are absent in endocycling cells, while the constitutively active form of CDK1 and its associated Cyclin A force cells to resume mitotic cycles instead of carrying out endocycles (Hayashi, 1996; Edgar and Lehner, 1996; Lehner and O'Farrell, 1990; Weigmann et al., 1997). In humans, Cyclin B undergoes a more rapid degradation in endocycling megakaryocytes than in mitotic megakaryoctes (Zhang et al., 1998). The degradation of mitotic cell cycle regulators is fulfilled by APC/C-mediated ubiquitin-dependent degradation (Narbonne-Reveau et al., 2008; Schaeffer et al., 2004; Zhang et al., 1998).

Endocycling cells perform a variety of important developmental and physiological functions in many organisms. The mechanisms that drive the initiation of the endocycle program act to reduce the activity or expression level of M phase cell cycle regulators, and oscillation of S phase cell cycle regulators result in increased DNA content during cycling.

1.1.4. Cell cycle regulation in *C. elegans*

In *C. elegans*, many somatic cells use a typical mitotic cycle, consisting of G1-S-G2-M phases. Cell cycle regulation resembles that of mammalian cells and most key proteins of the cell cycle machinery are conserved. *cdk-4* and the D type cyclin homolog *cyd-1* play key roles in G1 during late embryonic and larval development. Depleting either of them gives rise to cell cycle arrest at G1. The interaction between CDK-4 and CYD-1 has also been demonstrated *in vitro* (Park and Krause, 1999). Surprisingly, homozygous *cdk-4* mutants can still develop through embryogenesis and arrest only at late L2. This may be due to the absence of G1 phase during early embryonic development and maternal deposits of *cdk-4* could support development through embryogenesis and the early larval stages. *lin-35*, the homolog of the tumor suppressor pRB has also been identified in *C. elegans* and it functions downstream of *cdk-4* and *cyd-1*. Inactivating *lin-35* rescues the *cdk-4*- or *cyd-1*-associated larval cell cycle arrest (Boxem and van den Heuvel, 2001; Lu and Horvitz, 1998). Considering the importance of pRb in other organisms, it is surprising that the loss of function of *lin-35* alone does not give rise to ubiquitous defects in cell proliferation in *C. elegans*. In fact, only the intestinal cell lineage has so far been shown to exhibit cell cycle abnormalities in *lin-35* alleles (Boxem and van den Heuvel, 2001; Ouellet and Roy, 2007). Nevertheless, transcription is likely misregulated for many genes in *lin-35* mutants, confirming that LIN-35 plays key roles in regulating gene expression (Kirienko and Fay, 2007). Consistent with the conserved models, both *cdk-2* and the cyclin E orthologue *cye-1* are among the downstream targets of LIN-35, and their transcriptional levels are elevated in *lin-35* mutants (Ouellet and Roy, 2007). As expected, CDK-2 and CYE-1 also play essential roles in S phase progression. RNAi against *cdk-2* or *cye-1* notably causes cell cycle arrest at G1/S, which may be the basis for several developmental defects (Fay and Han, 2000).

The defects in the mitotic kinases CDK-1 in *C. elegans* blocks typical M phase events such as chromosome condensation and nuclear envelope breakdown, and consequently prevents the progression of M phase (Boxem et al., 1999). Interestingly, CDK-1 is also involved in meiotic M phase. Loss of CDK-1

function results in failure to segregate homologous chromosomes (Ellefson et al., 2011).

C. elegans encodes one A-type cyclin (*cya-1*) and four cyclin B family members, including three typical B-type cyclins *cyb-1*, *cyb-2.1*, and *cyb-2.2* as well as one B-3 subclass member *cyb-3*. Inhibition of *cyb-1* gives rise to a defect in chromosome congression and consequently causes aneuploidy, whereas *cyb-3* (*RNAi*) animals fail to initiate sister chromatid separation (van der Voet et al., 2009). However, only collective RNAi on *cyb-1*, *cyb-3* and *cyb-2.1*/*2.2* fully resembles the phenotype of *cdk-1* (*RNAi*) animals, suggesting that the B-type cyclins function redundantly (van der Voet et al., 2009).

The homologs of proteins that regulate CDK activity, including Wee1/Myt1, Cdk7, Cdc25, Plk1 and Aurora kinase are also present and functional in *C. elegans*. They play key roles in cell cycle progression so misregulation of these factors cause developmental abnormalities (Aschcroft and Golden, 2002; Clucas et al., 2002; Kipreos et al., 2000; Kostic and Roy, 2002; Wallenfang and Seydoux, 2002). INK4 family CKIs are missing from the *C. elegans* genome, yet two Cip/Kip-family CKIs, *cki-1* and *cki-2* have been identified (Feng et al., 1999; Hong et al., 1998). CKI-1 demonstrates a general G1/S inhibition and loss of CKI-1 function gives rise to precocious entry into S phase as well as excessive embryonic and larval cell division (Boxem and van den Heuvel, 2001; Fukuyama et al., 2003; Hong et al., 1998). Moreover, in absence of CKI-1 extra germ line precursor cells result from aberrant somatic gonadal precursor cell divisions, suggesting that CKI-1 also play a role in the timing of cell fate acquisition (Kostic et al., 2003). CKI-2 also seems to mediate cell cycle quiescence, as *cki-2* knockout animals undergo extra vulva cell divisions (Buck et al., 2009). More interestingly, reducing CKI-2 may trigger a CDK-2-dependent stabilization of centrioles that would normally be eliminated during oogenesis, which ultimately results in the formation of a multipolar spindle in the one-cell embryo following fertilization (Kim and Roy, 2006).

In summary, many somatic cells undergo the standard mitotic cell division during *C. elegans* development. The progression of these divisions is coordinated by the

CDK-cyclin complexes and a regulatory network that is highly conserved and comparable to that observed in most eukaryotes.

1.1.5. Ubiquitylation system in *C. elegans*

Conserved in many eukaryotes, the ubiquitylation-proteosome system is also found in *C. elegans*. The *C. elegans* genome sequence predicts that two ubiquitin genes are present, *ubq-1* and *ubq-2*. *ubq-1* is a polyubiquitin locus and encodes 11 tandem ubiquitin sequences, whereas *ubq-2* includes one intact canonical ubiquitin fused to the L40 ribosomal large subunit protein (Graham et al., 1989; Jones and Candido, 1993). Protein cleavage occurs to the gene products of both *ubq-1* and *ubq-2*, giving rise to individual ubiquitin molecules. RNAi of either gene results in arrest at the one-cell stage (Gonczy et al., 2000; Tijsterman et al., 2002).

Only one E1 ubiquitin-activating enzyme *uba-1* and less than 30 E2 conjugating enzymes are predicted by the *C. elegans* genome annotation (Jones et al., 2002; Kukarni et al., 2008). More than one hundred E3s, including HECT-domain E3s, U-box E3s as well as RING-finger E3s, have been identified. The RING-finger E3 family proteins account for more than 85% of the predicted *C. elegans* E3s and have been found to function either in a monomeric form or in a multisubunit complex (Kipreos, 2005). RING-finger E3s play a variety of essential roles during the development of *C. elegans*. For instance, RFP-1, a monomeric RING-finger E3, interacts with E2 UBC-1 *in vitro* and *rfp-1* (*RNAi*) animals demonstrate an L1 stage arrest (Crowe and Candido, 2004). RNF-5, another monomeric RING-finger E3, induces the ubiqtuitination of UNC-95, the function of which is important for the organization of body wall muscle dense bodies (Broday et al., 2004).

The important multisubunit E3 enzymes that contain RING-finger proteins, such as SCF and APC/C, are also conserved in *C. elegans*. *lin-23* encodes a F-box protein in the SCF complex and loss of LIN-23 results in hyperplasia in many cell lineages, as the mutated animals fail to terminate cell division at the appropriate time (Kipreos et al., 2000). On the other hand, APC/C is involved in regulating

vital events during mitosis. For example, downregulationg of APC/C components affects sister chromatid separation at anaphase (Bezler and Gonczy, 2010).

1.1.6. Alternative cell cycles during *C. elegans* development

The early embryonic cell cycle in many metazoans, such as *Drosophila* and *Xenopus*, progresses fast and only consists only of S and M phases without apparent gap phases (Foe and Alberts, 1983; Heasman, 2006). In *C. elegans*, the early embryonic cell cycle similarly features rapid DNA synthesis and divisions in the absence of obvious G phases (Edgar and McGhee, 1988). These cell cycles do not require the typical G1 cell cycle regulators CYD-1 and CDK-4 (Boxem and van den Heuvel, 2001; Park and Krause, 1999; Yanowitz and Fire, 2005).

Two *C. elegans* cell lineages undergo endocycling during larval stages. Prior to the end of the first larval stage 1 (L1), the intestinal cells undergo an incomplete mitosis-like cycle during which the genome replicates and the intestinal nuclei divide without executing cytokinesis, thereby generating bi-nucleate intestinal cells. Following this nuclear division, four endocycles occur at the end of each larval stage, each of which precedes the molt, eventually giving rise to adults with two polyploid intestinal nuclei (32C each). The transition from mitotic cell cycle to endocycle is mediated in part by LIN-35, the loss-of-function of which causes supernumerary nuclear divisions in the intestine prior to the eventual transition to the endocycle program. *lin-35* animals can possess up to ~50 intestinal nuclei instead of the 32 found in the wild type animals. One LIN-35 downstream target is *cye-1*, the transcription of which is significantly elevated in *lin-35* mutants. Interestingly, *cye-1* (*RNAi*) partially rescues the supernumerary nuclear division phenotype in *lin-35* mutants, suggesting that CYE-1 levels may be important for the proper initiation of endocycle (Ouellet and Roy, 2007). Endocycles also occur in the hypodermal V cell lineage. During L1 stage, an anterior daughter cell that undergoes endocycles is generated and fuses with the hyp7 syncytium, while the posterior seam cell daughter divides once mitotically. After an equational division at the L1-L2 transition, the V cell lineage repeats its L1 pattern of cell division in each subsequent larval stage (Hedgecock and White, 1985; Kipreos, 2005).

1.2. THE CENTRIOLE AND ITS BIOLOGICAL FUNCTIONS

The growth and duplication of organelles are also under cell cycle-dependent regulations. For instance, rapid Golgi growth occurs coincidently with M phase during cell cycle (Garcia-Herdugo et al., 1998). Moreover, the partition of Golgi requires PLK activity in several organisms, including humans (de Graffenried et al., 2008; Lowe et al., 1998), suggesting organelle biogenesis is under cell cycle control.

Similarly, another organelle, the centriole is under strict cell cycle regulation and in turn contributes to normal cell cycle progression. The centriole is a cylindrical microtubule-based organelle that is found in almost all eukaryotic species with the exception of higher plants and yeasts (Marshal, 2009). A single centriole is usually composed of nine microtubule triplets. A single triplet consists of a complete tubule, or an "A tubule", and two incomplete tubules ("B" and "C tubule") that share common microtubule molecules with neighboring tubules (Marshall, 2009; Nigg and Raff, 2009). Though largely conserved, structural deviations from the triplet are also observed in *Drosophila* and *C. elegans* embryos with nine doublets or nine singlets, respectively (Delattre and Gonczy, 2004; Leidel et al., 2005). Despite these variations, the alternative centrioles with doublets and singlets appear to be functionally regulated in a similar manner to those composed of triplets. The lengths and diameters of centrioles vary between organisms and even within the same species, depending on cell type and developmental context (Mashall, 2009). However, the typical centriole is 100-400 nm in length and 100-250 nm in diameter (Pelletier et al. 2006; Riparbelli et al., 2009).

The centriole plays diverse essential cellular and developmental roles through its function in the centrosome and/or the undulipodium, the latter of which includes two antenna-like organelles: the cilium and the flagellum (Kobayashi and Dynlacht, 2011; Marshall, 2009).

1.2.1. The centrosome: Primary MTOC and other functions

One typical centrosome consists of a pair of orthogonally juxtaposed centrioles embedded in an amorphous network of proteins collectively referred to as pericentriolar material (PCM) (Nigg and Raff, 2009). The centrosome has been actively implicated in a variety of cellular events from guiding cell division to regulating cell cycle progression. Many important functions of the centrosome rely on its microtubule nucleating capacity. In fact, the centrosome serves as the primary microtubule-organizing center (MTOC) in most animal cells, which enables them to define the microtubule geometry within the cell while consequently influencing cell shape, form and microtubule-associated transport (Brinkley, 1985). Furthermore, functioning as the major MTOC makes centrosomes of utmost importance in dividing cells, as it is a pair of centrosomes that establish the bipolar mitotic spindle during mitosis in order to accurately segregate genomic material. The centrosome is absolutely critical for normal mitotic progress in many organisms. Absence or dysfunction of the centrosome causes the failure of cell division in early embryos of *Xenopus*, *Drosophila*, and *C. elegans* (Basto et al., 2006; Gergely et al., 2000; Klotz et al., 1990; Krikham *et al*., 2003; Leidel and Gonczy, 2003). Conversely, extra centrosomes may give rise to multipolar spindles in early embryos, resulting in cell cycle arrest or abnormal chromosome segregation (Ganem et al., 2009; Kim and Roy, 2006).

In addition to facilitating the faithful division of the genome, centrosomes can affect daughter cell fates by regulating asymmetric cell division. This function also depends on microtubule nucleating activity. Centrosomes generate astral microtubules that align the spindle relative to asymmetrically distributed cortical cell fate determinants, consequently ensuring the accurate segregation of these determinants between two daughter cells (Nigg and Raff, 2009). For example, in the *C. elegans* early embryo, cytoplasmic microtubules constrain the centrosome near the cortex, which facilitates the establishment of embryo polarization (Bienkowska and Cowan, 2012; Cowan and Hyman, 2004; Tsai and Ahringer, 2007). Ablating the centrosomes by laser beam inhibits embryonic symmetry-breaking in the *C. elegans* one-cell embryo (Cowan and Hyman, 2004). Furthermore, during the asymmetric division of either *Drosophila* male germline

stem cells or neuroblast stem cells, one centrosome nucleates a more robust microtubule array than the other (Yamashita et al., 2007, Rebollo et al., 2007), which ultimately distinguishes the fates of the two resulting daughter cells (Januschke and Gonzalez, 2010). Disrupting this process leads to cells that undergo symmetrical division. For example, a significant percentage of asymmetric neuroblast division becomes symmetric in centrosome-less *Dsas-4* homozygous animals in *Drosophila* (Basto et al., 2006).

The microtubule nucleating function of the centrosome is largely dependent on the γ-tubulin Ring complex (γTuRC), a ring-shaped pericentriolar complex that consists of a well-conserved γ-tubulin and at least six other interacting proteins, including γ-tubulin complex protein-2 (GCP2), GCP3, GCP4, GCP5, GCP6 and GCP-WD (previously named NEDD1) (Kollman et al., 2011; Zheng, et al., 1995). γ-tubulin directly associates with GCP2 and GCP3 to form the γ-tubulin Small complex (γTuSC), which constitutes the structural core of γTuRC (Kollman et al., 2011). GCP4, GCP5 and GCP6 may form a scaffolding cup to organize multiple γTuSC and stabilize the γTuRC (Xiong and Oakley, 2009). GCP-WD does not directly affect the stability or assembly of γTuRC, but mediates the centrosomal targeting for γTuRC (Lüders et al., 2005). Exactly how γTuRC nucleates microtubules remains poorly understood, though a dominant model has suggested that γTuRC functions as a template, providing a ring of γ-tubulins. Each γ-tubulin can contact α-tubulin longitudinally and mediate the outgrowth of α-/β-tubulin dimers perpendicular to the γ-tubulin ring (Mortiz et al., 2000).

In addition to being an MTOC, the centrosome also functions as a regulatory platform to influence cell cycle progression. In fact, a number of positive or negative cell cycle regulators, such as Cyclin B, PLK1, Checkpoint kinase 1 or Aurora A kinase are present on the centrosome during the cell cycle (Jackman et al., 2003; Kramer et al, 2004). The centrosomal localization of these regulators plays a key role in their respective activities. For instance, at the onset of mitosis in mammalian cells the mitotic Cyclin B1 accumulates at the centrosome, where Cyclin B1 is phosphorylated by PLK-1 and consequently activates CDK1 (Jackman et al., 2003; Kramer et al., 2004).

Additionally, some centrosomal components contribute to the regulation of centrosome function. Cep57 is involved in maintaining centrosome integrity (Wu et al., 2012), while SPD-5, a pericentriolar protein in *C. elegans,* regulates centriole formation by targeting a key centriole-duplicating protein to the centrosome (Kemp et al., 2004; Wu et al., 2012).

1.2.2. Cilia and flagella

During the process of terminal differentiation centrioles migrate to the cell surface where they root to form a basal body and then nucleate the microtubule outgrowth of an axoneme, a nine-doublet structure (Ishikawa and Marshall, 2011; Kobayashi and Dynlacht, 2011). The axoneme is surrounded by membrane constituents continuous with the surrounding cellular membrane, and together with the basal body it serves as the core building block for both cilia and flagella, the antenna-shaped protrusions that emanate from the cell surface (Haimo and Rosenbaum, 1981; Ishikawa and Marshall, 2011).

Cilia are present in almost every cell type in mammals, although their existence is restricted to neural systems in invertebrates such as *C. elegans* and *Drosophila* (Ishikawa and Marshall, 2011). Cilia can be categorized into two groups: primary cilia and motile cilia. Primary cilia are immotile yet they play important roles in signal transduction and sensory function (Kobayashi and Dynlacht, 2011). Many essential signaling pathways, such as the Hedgehog (Hh), β-catenin and Wnt signaling pathways are mediated by primary cilia (Kim et al., 2011; Kumamoto et al., 2012). It is not surprising that dysfunctions in primary cilia have been found to be associated with many human diseases (Brueckner, 2007; Wagner, 2008).

Motile cilia, on the other hand, are present on some epithelial cell surfaces, where they beat in a rhythmic manner often to create fluid movement. For example, the motile cilia of trachea epithelial cells generate flow in order to remove particles in the airway (Kobayashi and Dynlacht, 2011). The beating and bending abilities of motile cilia are enabled by two extra microtubule pairs at the center of their axoneme as well as radial spokes and dynein arms that are attached to the 9-microtubule doublets (Ishikawa and Marshall, 2011).

Flagella are present in some prokaryotes, one-cell eukaryotes and sperm (Ishikawa and Marshall, 2011). The major function of flagella is to drive cellular locomotion. A typical example is that of the single flagellum that propels the sperm cell using a whipping action (Ishijima et al., 1986). In eukaryotic cells, the structures of flagella and motile cilia are identical, although the average length of flagella is generally longer than cilia (Ishikawa and Marshall, 2011).

1.2.3. Conclusion

Overall, the centriole is implicated in faithful genome segregation, asymmetric cell division, signal sensing as well as locomotion through its function in the formation of undulipodium. The proper execution of these functions contributes to normal cellular and developmental homeostasis. It is therefore not surprising that defects in centriole-based structures are associated with many human diseases referred to collectively as "ciliopathies" (Nigg and Raff, 2009). In line with the importance of the centriole, its dynamics and functions have been under active investigation for many decades. The remaining chapters of this thesis will be dedicated to reviewing the current understanding of regulatory mechanisms in centriole biology.

1.3. CENTRIOLE DUPLICATION AND MATURATION

1.3.1. Centriole duplication licensing and duplication pathway

In cycling cells, bipolar spindles are usually organized by two centrosomes (two pairs of centrioles) that are generated from the duplication of one pair of pre-existing centrioles during interphase. Analogous to DNA replication, this canonical centriole duplication must ensure that one and only one daughter centriole shall be generated next to each parental centriole per cell division. Failure to correctly duplicate centrioles can either give rise to mono- or poly-polar spindles.

Centriole duplication is orchestrated by a centriole-intrinsic licensing mechanism and the execution of the daughter centriole duplication pathway. The licensing mechanism depends on the maintenance of a tight orthogonal configuration between a pair of centrioles (one mother and one daughter), which inhibits centriole duplication. Therefore, disengagement (losing this configuration) must occur prior to the subsequent round of centriole duplication (Nigg and Raff, 2009; Tsou and Stearns, 2006). Understanding of the various steps and molecules involved in duplication was greatly enhanced by genetic screenings that identified genes essential for centriole duplication in *C. elegans*. Four coiled-coil proteins have been identified, namely Spindle-defective protein 2 (SPD-2), Spindle-assembly abnormal protein 4 (SAS-4), SAS-5 and SAS-6, as well as a kinase called Zygotic lethal-1 (ZYG-1) (Dammermann et al., 2008; Delattre et al., 2004; Kirkham et al., 2003, O'Connell et al., 2000; O'Connell et al., 2001). Intriguingly, a molecular epistasis study suggested that centriole duplication requires the strict sequential recruitment of these five proteins to the centriole (Delattre et al., 2006; Pelletier, 2006).

The centriolar localization of SPD-2 is indispensable for the subsequent function of all downstream centriole duplication proteins in *C. elegans* and the ZYG-1 is loaded to the centriole immediately after SPD-2 (Delattre et al., 2006; Kemp et al., 2004). In *C. elegans*, loss-of-function of *zyg-1* completely blocks centriole duplication (Delattre et al., 2006; O'Connell et al., 2001). SAS-6, the target of the kinase activity of ZYG-1, is directly phosphorylated by ZYG-1 on Serine 123 *in*

vitro. The same phosphorylation also occurs in a ZYG-1-dependent manner *in vivo*. Intriguingly, the phosphorylated Serine 123 helps maintain the centriolar localization of SAS-6 (Kitagawa et al., 2009). Moreover, the phosphatase PP2A dephosphorylates SAS-5, a protein that interacts with SAS-6 in *C. elegans.* This dephosphorylation further enhances SAS-5/SAS-6 centriolar localization (Delattre et al., 2004; Kitagawa et al., 2011).

Although the ortholog of SPD-2 in *Drosophila* plays important roles in centrosome maturation but is not indispensable for centriole duplication, the human ortholog of SPD-2, CEP192 functions as an essential PCM component and is required for centriole duplication (Dix and Raff, 2007; Giancenti et al., 2008; Zhu et al., 2008). However, the centriolar recruitment of PLK4 (SAK in *Drosophila*), the functional equivalent of ZYG-1, relies on another coiled-coil protein CEP152 (asterless in *Drosophila*) that plays an analogous role to *C. elegans* SPD-2 as a beacon to initiate centriole duplication. Both CEP152^{asl} and PLK4/SAK are essential for centriole duplication, as depleting either of them compromises centriole duplication. Moreover, overexpressing PLK4/SAK alone is sufficient to drive centriole amplification (Bettencourt-Dias et al., 2005; Cizmecioglu et al., 2010; Habedanck et al., 2005). Although it is still unknown whether PLK4 mediates the phosphorylation of SAS-6 in *Drosophila* and mammals, SAS-6 is indeed located at the centriole in a PLK4-dependent manner in these species, suggesting that the general mechanisms of recruiting SAS-6 are likely conserved (Strnad et al., 2009).

In both mammalian cells and *C. elegans*, the centriolar presence of SAS-6 directly triggers the building process of the procentriole, the immature daughter centriole at its early stage. The procentriole assembles at the proximal base of the mother centriole (Guichard et al 2010). The centriole is formed according to a cartwheel-like structure that consists of a central hub from which nine spokes radiate outward and link to nine microtubule blades (Strnad and Gonczy, 2008). SAS-6 is localized at the central hub and its self-assembly through its conserved N-terminus is a direct contributing factor in the structural organization of the centriolar wheel-like core (van Breugel et al., 2011). Interestingly, another protein

that bears the same N-terminus, Bld12p in *C. reinhardtii* shows similar self-organizing capacity, confirming that the N-terminus of SAS-6 is intrinsically capable of constructing the framework for centriole formation (Kitagawa, et al., 2011). Lack of SAS-6 results in abnormal centriole structures in many organisms (Jerka-Dziadosz et al., 2010; Nakazawa et al., 2007; Rodrigues-Martins et al., 2007). Ironically, the centrioles of *C. elegans* do not show this cartwheel structure. Instead, the first detectible procentriolar structure is a 60nm central tube. The oligomerization of SAS-6 through its N-N interaction contributes to central tube formation (Pelletier et al., 2006; Kitagawa, et al., 2011). During the formation of the central tube another coiled-coil protein SAS-5 is required for the centriolar localization of SAS-6 and these two proteins are recruited to the centriole together (Pelletier et al. 2006).

The next steps involve the attachment of centriolar microtubules to the cartwheel or the central tube followed by subsequent growth of these microtubules. This attachment and growth is mediated in many organisms by a highly conserved coiled-coil protein called SAS-4 (CPAP in humans) (Leidel et al., 2003; Basto et al., 2006). SAS-4 accumulates at the centriole during S phase and directly interacts with γ-tubulin. γ-tubulin further stabilizes the centriolar localization of SAS-4, and more importantly mediates the attachment or growth of centriolar microtubles (Dammermann et al., 2008; Kohnmaier et al., 2009; Pelletier et al., 2006). Consistent with its established role, SAS-4 downregulation results in the failure of centriolar microtubule attachment, while overexpression of CPAP generates an abnormally elongated centriole (Dammermann et al., 2008; Pelletier et al., 2006; Schmidt et al., 2009).

The elongation of centriole microtubules is limited by a negative regulator called CP110. CP110 is localized at the distal end of centrioles, which suggests a capping function for CP110 during centriole formation (Kleylein-Sohn et al., 2007; Schmidt et al., 2009). Indeed, depleting CP110 gives rise to a similar phenotype as that observed by overexpressing CPAP, confirming the role of CP110 in counteracting CPAP (Schmidt et al., 2009).

Overall, the centriole duplication licensing mechanisms and the centriole duplication pathway coordinate to regulate centriole duplication. The duplication pathway involves sequential protein recruitment to the centriole. Although the proteins involved in recruitment vary among different organisms, functional equivalents perform the critical steps required for faithful duplication (Bettencourt-Diaz et al., 2005; Habedanck et al., 2005; Leidel et al., 2005; Zhu et al., 2008).

1.3.2. Cell cycle regulators control centriole duplication by regulating the licensing and duplication pathway

The licensing of centriole duplication and the generation of a new centriole from its mother are highly regulated in cycling cells. In fact, many vital cell cycle regulators are also implicated in regulation of centriole duplication; every essential step of the duplication cycle is coupled with the progression of the cell cycle (Brito et al., 2012).

Although centriole formation *per se* does not initiate until G1/S, a pair of centrioles needs to disengage as early as G2/M of the preceding cell cycle (Hinchcliff and Sluder, 2002; Tsou and Stearns 2006; Tsou et al., 2009). This disengagement licenses the subsequent round of centriole duplication that will take place in the following cell cycle (Tsou and Stearns, 2006).

An important M phase kinase, Polo-like kinase 1 (Plk1), promotes centriole disengagement during G2/early M phase. Reducing Plk1 gives rise to incomplete or compromised disengagement, thereby blocking subsequent centriole duplication (Tsou and Stearns, 2006). Separase, a protease that is activated by APC/C that cleaves the cohesin complex at anaphase to allow for sister chromatid separation, acts in parallel with Plk1 to independently promote centriole disengagement (Peters, 2006; Tsou and Stearns, 2006). Depleting both Plk1 and separase completely blocks centriole disengagement (Tsou et al, 2009).

After the completion of cell division, the disengaged centrioles are eventually inherited by daughter cells and remain loosely tethered to each other by cohesion fibers (Bahe et al, 2005).

Centriole duplication initiates during S phase, and like DNA replication, this process requires the activities of the G1/S phase cell cycle regulators CDK-2 and its associated Cyclins in many organisms, including *Xenopus* and *C. elegans* and in mammalian cells (Hinchcliffe and Sluder, 2002). Although the direct interaction between S phase-specific CDK activity and centriole biogenesis remains undetermined, SPD-2 intriguingly possesses several putative CDK phosphorylation sites *in C. elegans*. Depleting *cdk-2* or *cye-1* affects centrosomal accumulation of SPD-2 in one-cell embryos, suggesting that SPD-2 may be a target of CDK-2 activity (Cowan and Hyman, 2006; Pelletier et al., 2004).

Following S phase, the newly formed daughter centriole remains engaged with the mother centriole, thereby intrinsically blocking any re-duplication. Meanwhile, the daughter centrioles elongate to full length until late G2/M (Tsou and Stearns, 2006).

Overall, different cell cycle regulators, e.g. CDK or Plk, impinge on centriole duplication by directly or indirectly regulating licensing and the centriole duplication pathway. By doing so, all essential steps during centriole duplication are strictly coupled to cell cycle progression and as a result, the correct number of centrioles is faithfully passed on to the next generation.

1.3.3. Centriole maturation

Following cell division, each daughter cell inherits a pair of centrioles, consisting of one mother and one newly formed daughter centriole. Although the daughter centriole reaches the length of the mother centriole after one cell cycle, only the mother centriole bears the distal or sub-distal appendages that enable microtubules anchoring. The assembly of these appendages onto the daughter centriole is termed centriole maturation (Azimzadeh and Marshall, 2010; Piel et al., 2000). In mammals, OFD1 and ODF2 have been identified as required for centriole maturation, and depleting either of them results in failure to assemble the appendages (Ishikawa et al., 2005; Singla et al., 2010). Although the regulation of centriole maturation remains poorly understood, this process is coupled to cell cycle progress as with centriole duplication. Following the

completion of centriole duplication, it takes another half cell cycles for the daughter centriole to mature. Within that half cycle, M phase must be completed and the next cell cycle must be initiated, as noted by the lack of centriole maturation in prolonged M phase condition (Guarguaglini et al., 2005).

1.4. CENTROSOME MATURATION AND SEPARATION

1.4.1. Centrosome maturation

In order to establish a functional bipolar spindle, both centriole duplication and the MTOC capacities of the centrosome must be under strict control. The microtubule-organizing capacity relies mainly on rapid recruitment of γTuRC and other key PCM components to the centrosome during maturation. As a result, the physical size of the centrosome as well as its microtubule-organizing capacity increase significantly to peak during metaphase (Gomez-Ferreria and Sharp, 2008; Palazzo et al., 2000). Centrosome maturation functionally enables the centrosome to properly establish the mitotic spindle (Lee and Rhee, 2011).

Not surprisingly, like centriole duplication, centrosome maturation also progresses in synchrony with the cell cycle. The rapid recruitment of essential PCM components occurs at the onset of M phase, which is regulated by key cell cycle regulators (Gomez-Ferreria and Sharp, 2008; Palazzo et al., 2000). In mammalian cells, Plk1 is localized at the centrosome and phosphorylates a PCM protein called Pericentrin at the onset of mitosis. The phosphorylated Pericentrin subsequently triggers the centrosomal targeting of other PCM components, including CEP192 (Lee and Rhee, 2011). Interestingly, the ortholog of CEP192 in *C. elegans*, SPD-2, interacts with PLK-1 and enhances its centrosomal localization, since the disruption of specific amino acids on SPD-2 diminishes the appropriate PLK localization at the centrosome and reduces centrosome size (Decker et al., 2011). CEP192 further contributes to loading GCP-WD onto the centrosome (Gomez-Ferreria et al., 2007; Luders et al., 2006; Zhu et al., 2008). GCP-WD is the centrosome-targeting subunit of γTuRC (Luders et al., 2006). The indispensable role of PLK-1 during centrosome maturation is also conserved in *Drosophila*, in which a genome-wide RNAi screen confirmed that Polo kinase, together with another centrosomal protein Centrosomin, are absolutely required for centrosome maturation. Depleting either of them completely blocks maturation and centrosomes fail to nucleate bipolar spindles (Dobbelaere et al., 2008). Taken together, these findings indicate that mitotic kinase Plk1 triggers a sequential recruitment of essential PCM components, including γTuRC, to the

maturing centrosome during M phase. The recruitment of γTuRC increases the MTOC capacity of centrosome.

In addition to the enrichment of microtubule nucleating capacity, centrosome maturation involves the attachment of microtubule stabilizing factors to the centrosome, a process that is also mediated by essential cell cycle regulators. For instance, TACC is a protein family conserved from *C. elegans* to humans (Peset and Vernos, 2008). In *Drosophila*, TACC is phosphorylated *in vitro* by M phase kinase, Aurora A kinase, and localizes at the centrosome in an Aurora A-dependent manner *in vivo*. TACC brings Minispindle (Msps)/XMAP215 protein to the centrosome to form a TACC-Msps complex, where Msps functions as a stabilizer for the centrosome-associated MTs, a key function in establishing the spindle (Barros et al., 2005). A similar interaction between TACC and Msps/XMAP215 is also conserved in other species (Peset and Vernos, 2008). However, TACC is not essential for the initial centrosomal recruitment of γ-tubulin, since loss of function of TACC homolog in *C. elegans* or *Drosophila* has no effect on the subcellular distribution of γ-tubulin (Bellanger and Gonczy, 2003; Gergely et al., 2000).

Overall, centrosome maturation enhances the MTOC capacity of the centrosome. This maturation involves the centrosomal recruitment of microtubule nucleating and stabilizing factors at the onset of M phase. M phase cell cycle regulators, such as PLK-1 and Aurora A, cooperate to regulate centrosome maturation and couple this process with the initiation of M phase.

1.4.2. Centrosome separation

As previously stated, despite being disengaged in the preceding G2/M, the two mother centrioles remain loosely connected by cohesion fibers that consist of Rootletin, C-Nap1 and β-Catenin (Bahe et al., 2005; Bahmanyar et al., 2008; Yang et al., 2006). For the organisms in which centrioles are indispensable for the formation of spindles, such as somatic cells of mammals, chicks and *C. elegans*, the successful separation of the centrosome is required to properly establish the spindle. In mammals, an M-phase kinase Nek2, which belongs to the Never in

mitosis A (NimA) related kinase family, phosphorylates components of the cohesion fibers and consequently disrupts their interactions. As a result, two mother centrioles disassociate from each other (Bahe et al., 2005; Bahmanyar et al., 2008; Moniz et al., 2011). Following disjunction, the further separation of two centrosomes relies on Eg5, a member of the Kinesin-5 subclass of kinesins. Eg5 is a target of two mitotic kinases: Plk1 and CDK1. Plk1 plays an important role in the centrosomal recruitment of Eg-5, whereas CDK1-dependent phosphorylation of Eg5 enhances its microtubule attachment capacity. The cooperative function of both kinases finally triggers the full separation of the centrosomes (Kapitein et al., 2005; Smith et al., 2011). Consistent with this model, loss of function of Plk1, CDK1 or Eg5 causes defects in centrosome separation, which give rise to an abnormal monopolar spindle (Valentine et al., 2006).

1.5. ALTERNATIVE CENTRIOLE BIOGENESIS

In cycling cells, one daughter centriole is generated per mother centriole per cell cycle. This semi-conservative centriole duplication is driven, at least in part, by S phase-specific enzymatic cell cycle activities. However, studies have revealed that alternative centriole biogenesis pathways also exist and are developmentally programmed in some non-cycling cells in order to generate centrioles, or centriole-like structures in the absence of S phase-specific enzymatic activities (Hagiwara et al., 2004; Vladar and Stearns, 2007). Typically, in non-proliferating multiciliated cells, such as tracheal epithelial cells, hundreds of centrioles/basal bodies are present. These centrioles are generated through two ways: Either multiple centrioles are simultaneously produced in proximity to a single existing centriole, or they are assembled without the presence of mother centriole, the latter of which is also referred to as *de novo* centriole assembly (acentriolar pathway) (Vladar and Stearns, 2007).

de novo centriole assembly also occurs at the blastocyst (64 cells) stage during early embryonic development in the mouse. Rodents eliminate all detectable centriole-like structures in both the oocyte and the sperm (Manandhar et al., 1999). Following fertilization, mitotic spindles are only organized by periodic aggregates of PCM in the absence of any centriole structure (Calarco-Gillam et al., 1983). The typical centriole structures are restored through *de novo* assembly and eventually become visible in electron micrographs (Coutois et al., 2012; Gueth-Hallonet et al., 1993).

Alternative centriole assembly begins with the formation of amorphous aggregates containing essential centrosomal proteins, such as centrin, pericentrin, γ-tubulin and SAS-6 (La Terra et al., 2005; Kim et al., 2005; Vladar and Stearns, 2007), which suggests that the alternative pathway deploys the same proteins that are essential for typical centriole duplication. Consistent with this hypothesis, the expression of centrosomal proteins is upregulated in the cells undergoing cilia formation through alternate assembly (Vladar and Stearns, 2007). Furthermore, depleting SAS-6 significantly blocks multiple centriole formation in the multi-ciliated cells (Vladar and Stearns, 2007), whereas overexpressing canonical

centriole-duplicating factors, such as PLK4, results in abnormal centriole amplification (Holland et al., 2012; Rodrigues-Martins et al., 2007; Vladar and Stearns, 2007).

These aggregates of centrosomal proteins subsequently consolidate into an intermediate structure called a deuterosome (Sorokin, 1968). Deuterosomes further organize the growth of various numbers of procentrioles, an immature yet morphologically recognizable centriole structure (La Terra et al., 2005; Sorokin, 1968). The centriolar microtubules eventually elongate and mature following release from deuterosomes (Sorokin, 1968).

de novo centriole biogenesis can be also triggered by experimentally removing the centriole via laser ablation or needle microsurgery in cycling cells (La Terra et al., 2005; Uetake et al., 2007). Removing the centriole during G1 phase can induce cell cycle arrest in cycling cells (Hinchcliff et al., 2001; Uetake et al., 2007). However, transformed HeLa cells and human mammary epithelial cells are able to restore the removed centriole by *de novo* centriole assembly, which consequently allows them to resume their regular cell cycles.

Similar to *de novo* centriole assembly during normal development, this induced assembly begins with aggregates containing centrosomal proteins (La Terra et al., 2005; Uetake et al., 2007). During induced *de novo* centriole assembly in cycling cells, random numbers of centrioles can form (La Terra et al., 2005; Song et al., 2008). However, the formation of just one centriole is sufficient to aggregate PCM (Song et al., 2008) and block further *de novo* centriole assembly probably by destabilizing other aggregates (La Terra et al., 2005; Song et al., 2008).

Interestingly, alternative centriole biogenesis, especially the *de novo* centriole assembly pathway, strongly challenges the centriole duplication-based hyothesis, that mother centrioles function as obligatory templates to guide daughter centriole duplication. In fact, the spontaneous *de novo* assembly takes more time than centriole biogenesis that occurs via existing mother centriole templates, suggesting that mother centrioles may instead serve as a catalytic platform to concentrate centriolar proteins and consequently facilitate centriole duplication (Rodrigues-Martins et al., 2007).

1.6. CENTROSOME REDUCTION

In the gametes of a variety of organisms and some differentiated cells that carry out specialized functions, centrosomes lose their typical PCM components and consequently their MTOC capacity, while in certain contexts complete elimination of the centriole may occur. These processes are collectively considered mechanisms of centrosomal reduction (Mahowald et al., 1979; Manandhar et al., 2005; Wilson, 1925). Despite having been described for many decades, the precise mechanism that drives centrosome reduction has remained poorly understood compared to centriole biogenesis.

1.6.1. Centrosome reduction in endocycling cells

During development, some cell types deviate from the mitotic cell cycle and instead undergo endocycles. This alternative cell cycle takes place in the germ line-derived nurse cells and the somatic follicle cells of *Drosophila* during oogenesis.

Prior to the onset of the endocycle, the centrioles in endocycling nurse cells begin to lose their initial juxtanuclear localization, gradually migrating away toward the oocyte rather than duplicating in response to the endo-S phase (Mahowald and Strassheim, 1970; Mahowald et al., 1979). They eventually localize between the oocyte nucleus and the follicle cell border to aggregate into a cluster with intensified MTOC activity, presumably guiding transport of nutrients and mRNA along microtubules from the nurse cells into the oocyte (Bolivar et al., 2001; Mahowald and Strassheim, 1970). Subsequently, the clustered centrioles gradually lose their PCM, reducing the microtubule-nucleating activity, and complete removal of these centrioles may eventually take place (Mahowald and Strassheim, 1970).

An analogous centrosome reduction is observed in the endocycling somatic follicle cells. By the time initiation of the endocycle occurs, centrioles are disassociated from nuclei. Next, the pair of centrioles migrates away from each other, losing their perpendicular orientation, suggesting the structural integrity of

centrioles is affected. Centrioles translocate to the basal surface of the follicle cell plasma memberane before their complete disappearance (Mahowald et al., 1979).

1.6.2. Centrosome reduction during gametogenesis

One challenge that fertilization poses for organisms is that the diploid zygote would possess two centrosomes if each haploid gamete maintained its centrosomes. This could result in the formation of polypolar spindles and cell cycle arrest following zygotic S phase when disengagement and duplication are triggered (Kim and Roy, 2006). It has been conventionally believed that in order to maintain numeral integrity following fertilization, animals maintain their centrioles and degenerate most of the PCM in the sperm, whereas oocytes discard their centrioles yet retain PCM (Sun and Schatten, 2007). This reciprocal and complementary centrosome reduction process ensures that the zygote restores centrosome numeral integrity in cells after fertilization. The latest data suggests that although this model is very typical, other patterns of centrosome reduction/elimination during male or female gametogenesis may exist (Manandhar et al. 2005).

During spermatogenesis, centrosomes usually function as a MTOC until the completion of two rounds of meiosis. Subsequently, centrosome reduction takes place, during which centrosomes start sequentially losing their microtubule nucleating function and selectively discard pericentriolar material, including γ-tubulin (Manandhar et al., 2005). In some rodents, a more thorough centrosome reduction may occur, involving the complete disappearance of centrioles. Paternal centriolar structures therefore remain in sperm (Manandhar et al., 1999).

Maternal centriolar structures, however, are usually eliminated during oogenesis even prior to meiosis. As a result, unlike in sperm, the meiotic MTOC and the spindle in oocytes are organized in an acentriolar manner (Szollosi et al., 1972). This lack of a centriole during maternal meiosis is also observed in many vertebrates, including mouse, rabbits, *Xenopus* and human (Calarco-Gillam et al., 1983; Sutovsky et al., 1999), and in invertebrates, such as *Drosophila* and *C. elegans* (Kim and Roy, 2006; Mahowald and Strassheim, 1970).

Although the absence of centrioles in the ooctyes of many species seems relatively typical, the essential pericentriolar components are still present (Sutovsky et al., 1999). For example, γ-tubulin is present in mouse, *Xenopus* and *Drosophila* oocytes (Hughes et al., 2011; Palacios et al., 1993; Zheng et al., 1995). This γ-tubulin deposited in the oocyte plays an essential role in nucleating microtubules to establish an acentrosomal meiotic spindle (Endow and Hallen, 2011; Hughes et al., 2011). Moreover, the maternal reserve of γ-tubulin restores the microtubule-nucleating function of paternal centrioles contributed following fertilization during early embryonic development, when zygotic gene expression is still silent.

Overall, despite being less accurate in some species, especially rodents, the general model of centrosome reduction in gametes is that sperm retain their centrioles, whereas oocytes degenerate their centrioles, while maintaining pericentriolar proteins (Sutovsky et al., 1999). By doing this, early embryos ensure that they inherit the correct number of centrosomes with proper function.

1.6.3. The regulation of centriole elimination in oocytes

Since proper centrosome reduction during gametogenesis contributes to successful early embryonic cell division, we were interested in understanding how this reduction takes place. However, although centriole elimination from oocytes has been described for almost a century, little is known regarding the regulation of this event. This is largely due to the difficulty of collecting and analyzing mammalian ooctyes.

Recent findings in *C. elegans* suggest that a negative cell cycle regulator, CKI-2, plays a key role in centriole elimination. Reducing CKI-2 results in failure to eliminate centrioles in mature oocytes. Although these oocytes can still be fertilized normally by sperm, the extra maternal centrioles duplicate and organize polypolar spindles together with their paternal counterparts, resulting in genetic aneuploidies and chromosomal abnormalities (Kim and Roy, 2006).

CKI-2 probably affects maternal centriole elimination by antagonizing the activity of CDK-2 and simply downregulating CDK-2 can significantly ameliorate the

CKI-2 associated defect in centriole elimination. These findings suggest that CDK-2 stabilizes centrioles during oogenesis, although the canonical role of CDK-2 is usually to trigger duplication of centrioles during S phase (Kim and Roy, 2006).

In addition to CKI-2, another *C. elegans* factor that is implicated in the centriole elimination is CGH-1. CGH-1, an RNA helicase, is involved in various aspects throughout gametogenesis, including ooctye fertilization, sperm function and inhibition of apoptosis in the germline (Navarro et al., 2001). CGH-1-reduced or depleted animals show significantly delayed centriole elimination, which is supposed to occur during diplotene (Mikeladaze-Dvali et al., 2012).

In addition to genes responsible for centriole elimination during oogenesis, the germ cell karyotype seems to be another contributing factor for timely centriole elimination. More XO diakinesis oocytes harbor centrioles than XX diakinesis oocytes. However, the exact relationship between centriole elimination and germ cell karyotype remains to be elucidated (Mikeladaze-Dvali et al., 2012).

Overall, genetic and cell biological studies using *C. elegans* have shed some light on the mechanisms of centriole elimination during oogenesis. Some of the genes and other developmental factors responsible for this process have been revealed, although parallel studies in other systems are still lacking. More thorough investigation will be required to better understand how centrosome reduction occurs during oogenesis in other organisms.

1.7. *C. elegans* SYSTEM

1.7.1. General biological advantages of *C. elegans* system

The nematode *Caenorhabditis elegans* has been used successfully as a model to study many developmental and cell biological phenomena. *C. elegans* uses the same highly conserved cell cycle machinery to regulate cell progression, suggesting that the knowledge gathered from *C. elegans* studies may contribute to our general understanding of the corresponding processes in other animals. Furthermore, compared to mammalian cell culture or yeasts, examining cell division control in *C. elegans* allows us to better understand how cells respond to environmental cues and communicate among themselves to control cell division in a dynamic, organismal context. This advantage is even more obvious considering that *C. elegans* invariable cell lineage has been fully documented, (Sulston and Horvitz, 1977; Sulston et al., 1983), facilitating the identification of cell division defects in different genetic backgrounds. Meanwhile, the transparency of the *C. elegans* egg shell and cuticle facilitates the direct observation of cells or fluorescent markers under the light microscope. Because of these features, *C. elegans* is currently broadly used as a model to address many fascinating questions in modern biology.

1.7.2. RNAi and tissue-specific RNAi

The discovery of RNA-mediated interference (RNAi) in *C. elegans* provides yet another powerful tool to study gene function (Fire et al., 1998). Simply introducing synthesized double stranded RNAs into *C. elegans* can selectively and robustly eliminate the products of specific genes of interest. The dsRNAs can be introduced into animals by various means, such as feeding, microinjecting or soaking. So far, RNAi libraries that cover more than 80% of the predicted *C. elegans* genes are available, facilitating the use of systematic genome-wide surveys using RNAi (Fraser et al, 2000; Kamath et al, 2003). Furthermore, a tissue-specific RNAi can be achieved to address the function of essential genes in a specific lineage after embryogenesis (Chotard et al., 2010; Quadota et al., 2007).

1.8. RATIONALE AND OBJECTIVES OF MY PH.D. WORK

The primary objective of my research project is to better understand the developmental mechanisms that couple/uncouple centriole duplication and cell division, in addition to gaining further insight as to how centriole elimination may occur in a given cell type.

1.8.1. Developmental uncoupling of centriole duplication

In cycling cells, cell cycle regulators ensure strict coupling between centriole duplication and DNA replication. However, such coupling can be altered in specific developmental contexts. In sperm, one round of centriole duplication occurs between meiosis I and meiosis II, although DNA replication is absent. Consequently each haploid sperm possesses one pair of centrioles (Albertson and Thomson, 1993; L'Hernault, 2006). Moreover, in the respiratory epithelium, hundreds of centrioles/basal bodies are generated in an almost spontaneous manner, without any DNA replication (Nigg and Raff, 2009; Vladar and Stearns, 2007). These examples suggest that centriole biogenesis can occur independently of the regular requirements for cell cycle-dependent enzyme activities in some situations. The converse is also true; in the follicle cells or the nurse cells of the *Drosophila* egg chamber, instead of duplicating, the centriole migrates away from the endoreplicating cell, which eventually results in its elimination (Bolivar et al., 2001; Mahowald et al., 1979).

In each of the developmental situations described above, centriole duplication is uncoupled from S-phase, yet how this uncoupling occurs remains poorly understood. By examining these mechanisms where cells deviate from regular mitosis, I hope to shed more light on about how centrioles duplication can be uncoupled from the cell division cycle, and perhaps more importantly, how these two key cellular processes are coupled.

In *C. elegans*, two cell lineages, the intestinal and the lateral hypodermal cells, undergo endocycles (Hedgecock and White, 1985; Kipreos, 2005). It is unknown how the centriole responds in this context as cells begin to undergo successive rounds of DNA replication typical of the periodic waves of endo S-phase CDK

activity. I therefore sought to first describe the fate of the centrosome in these situations and to identify the various gene activities that govern the developmentally-regulated coupling or uncoupling of the cell cycle with the centrosome cycle.

Five proteins involved in centriole duplication and sequentially recruited to the centriole have been well characterized in *C. elegans*. Among these five proteins, the coiled-coil protein SPD-2 is the first factor to be recruited to the disengaged centriole, thereafter triggering centriole duplication (Delattre et al., 2006; Pelletier et al., 2004). SPD-2 is also a pericentriolar protein that plays an additional role in centrosome maturation (Kemp et al., 2004). SPD-2 may therefore be a prime target of regulation via upstream developmental signals. We decided to examine SPD-2 as a marker for centrioles as they undergo the changes associated with centrosome/cell cycles uncoupling and potential dynamics (clustering, elimination) during the endocycle.

1.8.2. The regulation of CKI-2

In. *C. elegans*, CKI-2 acts as an important negative cell cycle regulator and regulates cell cycle quiescence in vulva precursor cells. More importantly, CKI-2 also contributes to centriole elimination during oogenesis (Buck et al., 2009; Kim and Roy, 2006). However, CKI-2 regulation remains poorly understood.

As a part of our attempts to reveal the regulatory mechanisms that affect CKI-2 activity, we previously carried out a yeast-two hybrid screen in order to identify CKI-2 interacting partners, from which we identified a RING-finger protein RNF-1 and the SUMO orthologue SMO-1.

RING finger motif proteins have been found to typically function as E3 ligases for ubiquitylation and SUMOylation, although the RING domains in ubi-E3 and SUMO E3 are slightly different (Gareau and Lima et al., 2010). The RING-domain in RNF-1 belongs to the ubi-E3 RING, suggesting that RNF-1 may be involved in the ubiquitylation of CKI-2. Ubiquitylation plays an important role in regulating the level of cell cycle regulators, including the CIP/KIP family CDK inhibitors. For instance, SIC1, a CIP/KIP orthologue in yeast, is degraded through

ubiquitin pathway (Nash *et al*., 2001). In *C. elegans*, CKI-1 is also regulated by ubiquitylation (Feng et al., 1999).

Considering the importance of the ubiquitylation pathway on cell cycle progression and homeostasis, I became interested in verifying whether RNF-1 functions as an E3 ligase for CKI-2. Because ubiquitylation can affect multiple aspects of protein function, I also monitored localization and stability of RNF-1 as both may contribute to its regulation of CKI-2.

1.8.3. γ-tubulin dispersal from centriole during oogenesis

γ-tubulin is conserved across eukaryotes and functions as the major microtubule nucleating protein (Kollman et al., 2011). In *C. elegans*, the orthologue of γ-tubulin, *tbg-1*, has been identified and TBG-1 appears to exhibit similar functional properties as the major microtubule-nucleating factor. Similar to its orthologues in other organisms, TBG-1 demonstrates centrosomal accumulation during M phase in mitotic cells (Strome et al., 2000). γ-tubulin has also been found to stabilize the centrosome localization of SAS-4, a protein essential for centriole duplication (Dammermann et al., 2008). Moreover, depleting γ-tubulin by RNAi results in abnormal centriole structures (O'Toole et al., 2011), suggesting that γ-tubulin plays several roles in centrosome and centriole biology.

During *C. elegans* meiosis, γ-tubulin is reduced to an undetectable level at centriolar foci following the transition from mitosis to meiosis (Bobinnec et al., 2000). The remaining centrioles no longer organize the germ cell microtubule network, which coincides with a change in the positioning of ZYG-12, a gene product required for appropriate centrosome attachment during mitosis, to the nuclear membrane to distinct patches around the envelope (Malone et al., 2003). The germ cell membrane replaces the centrosome as the major microtubule nucleating site and ultimately mediates germ cell nuclear positioning, alone with homologue pairing through ZYG-12 (Zhou et al., 2009). How γ-tubulin re-distributes to the germ cell membrane from the centrioles and how this re-localization during meiosis affects germ cell function or meiosis *per se* still remains elusive. I am interested in characterizing the mechanisms responsible for

γ-tubulin re-distribution during mitosis-meiosis transition and hope to better understand the biological significance of this re-distribution.

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Chapter II

Cell cycle uncoupling and centriole elimination in the endoreduplicating intestinal cells of *C. elegans*

2.1. ABSTRACT

The centrosome cycle is often coupled with cell division to ensure that the centriole is duplicated only once per cell cycle. This coupling can be altered in specific developmental contexts, although how this uncoupling occurs remains misunderstood. In *C. elegans*, the larval intestinal cells undergo one nuclear division followed by four endocycles, characterized by successive rounds of S-phases without intervening mitoses. By monitoring the levels of SPD-2, a protein critical for centriole duplication in *C. elegans*, we show that the centrioles lose their pericentriolar material after the nuclear division and no longer respond to the endocycle-associated S-phase activities that normally drive centriole duplication. Subsequently, SPD-2 translocates to the nucleus, which precedes its elimination. Cell division/centrosome cycle uncoupling relies on the transcriptional regulation of centriole duplication genes, SPD-2 phosphorylation, and ubiquitin-dependent protein degradation. Our study highlights the role of key cell cycle kinases in both uncoupling the centrosome cycle from cell division and in regulating centriole stability in these cells.

2.2. INTODUCTION

In many animal cells, the centrosome acts as the major microtubule organization center (MTOC), thus playing key roles in defining microtubule geometry and ultimately, cellular morphology (reviewed in Nigg and Stearns, 2011). This MTOC function is of special importance in proliferating cells where the two centrosomes are responsible for accurately establishing the bipolar spindle typical of mitotic cell division, in order to facilitate timely and faithful DNA segregation. Although recent studies suggest that some cells may utilize alternative strategies to bypass the challenges resulting from numeral defects of centrosomes (Basto et al., 2006; Kwon et al., 2008), centrosome function and its numeral integrity are still largely believed to be essential for many organisms. Altering their appropriate regulation often leads to genomic instability and/or tumorigenesis (Basto et al., 2008; Ganem et al., 2009). The numbers of centrosomes must therefore be strictly regulated whereby the centrosome is duplicated once, and only once, each cell cycle.

The centrosome consists of a pair of barrel-shaped centrioles, surrounded by pericentriolar material (PCM) (reviewed in Nigg and Raff, 2009). During the centrosome cycle, the centrioles must disengage, duplicate, and separate; each event taking place at an appropriate stage of the cell cycle under the control of stage-specific enzymes, including protein kinases (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; reviewed in Hinchcliffe and Sluder, 2002; Lee and Rhee, 2011; Tsou et al., 2006; Tsou et al., 2009; Warnke et al., 2004)**.** During G2/M, the tethered parental centrioles separate, which is regulated by Cyclin-dependent kinase-1 (CDK-1) and Polo-like kinase-1 (PLK-1) (Bahe et al., 2005; Crasta et al., 2006; Smith et al., 2011). Coincidently, two centrosomes undergo maturation by rapidly recruiting PCM and consequently increasing the microtubule organizing capacity of the centrosome. This process is regulated by M phase kinases, such as PLK-1 and Aurora A kinases (Hannak et al., 2001; Lee and Rhee, 2011).

Centriole assembly is rate limiting and therefore of pivotal importance in the generation and duplication of the centrosome. The proteins that drive this process have been intensively studied in various cell culture systems and in model

organisms. In *C. elegans*, centriole assembly involves the sequential recruitment of a series of proteins that exert various centriolar biogenesis functions (Delattre et al., 2006; Pelletier et al., 2006), many of which are also conserved from metazoans to humans (Giansanti et al., 2008; Rodrigues-Martins et al., 2007; Zhu et al., 2008). SPD-2 is a coiled-coil protein that is the first factor to be localized to the mother centriole (Pelletier et al., 2004; Pelletier et al., 2006). It is thereafter joined ZYG-1, a protein kinase that is presumed to function in a manner analogous to PLK-4 (Bettencourt-Dias et al., 2005; O'Connell et al., 2001). SAS-6, another coiled-coil protein that is a likely ZYG-1 target (Dammermann et al., 2004; Kitagawa et al., 2009; Leidel et al., 2005), associates with SAS-5 to form the "central tube" stucture of the new centriole (Delattre et al., 2004; Pelletier et al., 2006). Eventually SAS-4 will reside and mediate the attachment of the *de novo* generated centriolar tubule onto the central tube (Delattre et al., 2006; Kirkham et al., 2004; Pelletier et al., 2006).

Despite the conserved coupling between centriole duplication and cell division, such coupling can be altered in specific developmental contexts. The basal bodies, another form of centrioles that exist in cells with cilia or flagella, are critical for early ciliogenesis (reviewed in Carvalho-Santos et al., 2011). In some cells, such as the respiratory epithelium, hundreds of centrioles/basal bodies are generated in an almost spontaneous manner, without any requirement for DNA replication (reviewed in Nigg and Raff, 2009; Vladar and Stearns, 2007), suggesting that at least in some situations, centriole biogenesis can occur independently of the regular requirements for cell cycle-dependent enzyme activities. The converse is also true; in the follicle cells of the *Drosophila melanogaster* egg chamber, the centrosome migrates away from the endoreduplicating DNA and is eventually eliminated (Bolivar et al., 2001; Mahowald et al., 1979). Initially it was assumed that loss of centriole might have been a mechanistic switch to drive the onset of the follicle cell endocycles (Mahowald et al., 1979), although this did not seem to be the case. In each of the developmental duplicating situations described above, the centriole duplication must be uncoupled from S-phase during these novel cell cycles, yet how this uncoupling occurs remains poorly understood. By examining

these mechanisms in cells that deviate from conventional mitotic divisions, we may learn how centrioles can uncouple with the cell division cycle, and perhaps more importantly how these two key cellular processes are coupled.

In *C. elegans*, two cell lineages, the intestine and the lateral hypodermal cells, undergo endocycles; where cells duplicate their genomes for successive cycles without intervening mitoses thus giving rise to polyploid cells. The intestinal nuclei undergo karyokinesis at the end of the first larval stage 1 (L1 stage) followed by endocycles at the end of each larval stage, giving rise to adults with two polyploid intestinal nuclei (32C each). In the hypodermal V cell lineage, an anterior daughter cell that undergoes endocycles is generated and fuses with the hyp7 syncytium, while the posterior seam cell daughter will divide once mitotically during the L1 stage. After an equational division at the L1-L2 transition the V cell lineage repeats its L1 pattern of cell division in each subsequent larval stage (Hedgecock and White, 1985; Kipreos, 2005). Although these unique cell cycles undergo reiterative rounds of DNA replication, it is unclear how the centrosome would respond to these successive waves of S-phase CDK activity. We therefore sought to better understand the various mechanisms that may govern the developmentally-regulated coupling or uncoupling of the cell cycle with the centrosome cycle.

Using the postembryonic intestinal cell to monitor centrosome dynamics, we noticed that centrosomes lose their PCM component following the mitotic nuclear division in the first larval stage. Centriole duplication is subsequently uncoupled from the DNA replication steps of the ensuing endocycle before the centrioles are gradually eliminated through a mechanism that requires the activities of CDK and PLK as the animal progresses through the L2 stage. The uncoupling and the final elimination of centrioles also rely on the proper initiation of the endocycle program, during which centriole-duplicating genes are transcriptionally downregulated.

2.3. MATERIALS AND METHODS

2.3.1. Nematode strains

C. elegans Bristol strain N2 was used as the standard wild-type strain. Nematode culture was performed as described previously (Brenner, 1974). All the strains carrying temperature-sensitive *spd-2* (*oj29*) allele were shifted to 25˚C 6 hours before the corresponding experiments were performed. The alleles used in this study are *spd-2* (*oj29*), *lin-35* (*n745*) and *cul-4* (*gk434*). MR1657 (*unc-13* [*e1091*] *spd-2*[*oj29*] I; (*rrIS1495* [*spd-2*::SPD-2::GFP; *unc-119*(+)]II; *unc-119*(*ed3*)III), MR1672 (*unc-13*[*e1091*] *spd-2*[*oj29*] I; (*rrIS1488* [*spd-2*::SPD-2(S545A)::GFP; *unc-119*(+)]II; *unc-119*(*ed3*)III), MR1667 (*unc-13*[*e1091*] *spd-2*[*oj29*] I; (*rrIS1514* [*spd-2*::SPD-2(S545E)::GFP; *unc-119*(+)]II; *unc-119*(*ed3*)III), MR1652 (*unc-13*[*e1091*] *spd-2*[*oj29*] I; (*rrIS1587* [*spd-2*::SPD-2(S357E)::GFP; *unc-119*(+)]II; *unc-119*(*ed3*)III), MR1792 (*rrEX1560* [*elt-2*::RDE-1; *inx-6::*GFP]; *rde-1*(*ne219*)V), MR1778 (*rrIS1778* [*elt-2*::FLAG::PAB-1; *unc-119*(+)]II; *unc-119*(*ed3*)III) and MR1779 (*lin-35*(*n745*)I; *rrIS1778* [*elt-2*::FLAG::PAB-1; *unc-119*(+)]II; *unc-119*(*ed3*)III).

2.3.2. DNA constructs, site-directed mutagenesis, and RNAi

For the transgenes encoding either wild type or variant GFP-tagged SPD-2, a 2433bp sequence upstream to and the genomic DNA encoding full length *spd-2* lacking its natural stop codon was first amplified from N2 and clonedin frame with GFP at C-terminus with SalI and XmaI into pPD95.79 (a gift from the Fire Lab) to create pMR812. Subsequently a fragment containing *spd-2*::SPD-2::GFP::*unc-54* 3'UTR was removed from pMR812 and then cloned into pCFJ151 with SpeI to yield pMR831. PCR for site-directed mutagenesis was performed using Gene-Tailor site-directed mutagenesis PCR kit (Invitrogen) on pMR831 in order to generate different variants including pMR832 (S545A), pRM833 (S545E) and pMR857 (S357E). For the intestinal mRNA enrichment experiment, the *elt-2* promoter was cloned from N2 and FLAG::PAB-1 was cloned from pPBSK9 (Roy et al., 2002), both flanked with sequences compatible

for Gateway cloning (Invitrogen) . Then the *elt-2::*FLAG::PAB-1 was cloned into pCFJ150 to generate pMR869. For the tissue-specific RNAi experiment, *rde-1* was cloned into pPD49.26 with BamHI and NheI and then *elt-2p* was cloned with BamHI to generate pMR542.

For RNAi experiments, staged L1 animals were allowed to recover for two hours on regular culture plates (Brenner 1974) before being transferred to plates for feeding RNAi (Timmons and Fire, 1998).

2.3.3. Antibodies, immunological methods and microscopy

The following primary antibodies and dilutions were used: 1:100 anti-SPD-2 rabbit polyclonal (Kemp et al., 2004. a gift from Dr. K. O'Connell, National Institutes of Health, Behesada, MD), 1:100 anti-SAS-4 rabbit polyclonal (Santa Cruz Biotech), 1:50 anti-γ-tubulin (Sigma-Aldrich, T1450) and 1:100 mouse monoclonal anti-GFP (Abcam, ab1218). 1:250 secondary antibodies were anti-mouse or anti-rabbit AlexaFluor488 (Molecular Probe). DAPI (Sigma-Aldrich) was used to reveal DNA. L1, L2 or L3 larvae were fixed and stained as described previously (Finney and Ruvkun, 1990). With the exception of Figure 2.S2, other indirect microscopy was performed using 60X oil-immersion objective lens in a LSM510 META Confocal microscope (Zeiss) 1-4 μm optical sections were acquired with a 109.6 μm pinhole and 1024X1024 pixels resolution. Planes containing centrioles were projected using LSM510 Version 3.2 SP2. Images were processed using Adobe Photoshop, preserving relative image intensities within a series. The microscopic work of Supplemental Figure 2 was performed using 100X oil-immersion objective lens in a DeltaVision Image Restoration System (Applied Precision). Data were collected as a series of 13-27 optical sections in increments of 0.2 μm under standard parameters with the softWoRx 3.0software (Applied Precision). All microscopy was performed at 20°C.

2.3.4. Intestinal mRNA enrichment and RT-PCR

The intestinal mRNA tagging protocol was modified from Roy et al., 2002. The following modifications were made. For each sample, we collected 2.5 mL

packed worms. Then the worms were processed in original recipe of homogenization buffer containing one protease inhibitor cocktail tablet (Roche) resulting in a 2x concentration compared to Roy et al., 2002, We also found 60 passes/sample through a Wheaten homogenizer yield more abundant and better quality intestinal RNA during the lysis.

RT-PCR was performed with ProtoScript® M-MuLV *Taq* RT-PCR Kit (NEB, E6400S) and gene-specific primers. The primers were designed and analyzed with on-line tool (www.basic.northwestern.edu/biotools/oligocalc.html). Two pairs of primers were designed for each tested gene except one pair for each *elt-2* and *htp-3*. Primers: *spd-2* (5'primer 5'- GTG GTG GAG AGA ATG CCA TTG AAG-3' and 3' primer 5'- CAG GAG TGA TGC TTG TCT TTA GGC-3'; 5'primer 5'- GAA CAA GAG ACG GCT TTG CGA TG-3' and 3' primer 5'- GAA GAA TAC GTC GCT GGA ATG AGG-3'). *zyg-1*(5'primer 5'- GGA GAG AAG GTG GCG ATT AAG AGG-3', 3' primer 5'- CCT CCT TCG CAC AAC TCC ATG AC-3'; 5' primer 5'- CTA ATA TGG TTG GCA GTT CGC CG-3'; 3' primer 5'- CCC TTC TCT GAA CAT TAG CCG ATG-3'), *sas-4* (5'primer 5'- GTC GAA TGC TCT CCC GAA CTC TG-3', 3' primer 5'- GCA ACG GAA GGT GCG GGA TTT AG-3'; 5' primer 5'- CAC AGA AAC GAT AGC CAC TCT CCG-3', 3' primer 5'- GTG CTC TTT AGC CGG TTT CCG TC-3'), *sas-5* (5'primer 5'- CCA AAC TGT CGA GGG AAC ATC TCG-3', 3'primer 5'- GGG CCG TTT CTT CTT GAA TCT CTG-3'; 5' primer 5'- GAT AAT CGT GCT CCT GAC TCA TAC CG-3' 3' primer 5'- CGC TTG CTC GTG ATA GTT CTG TC-3'); *sas-6* (5' primer 5'- GGA GCT AAT TTG AAC TCG CGC ACC-3', 3' primer 5'- CGT CAC ACT TGA ACC AGT AGT CTC G-3'; 3' primer 5'- CGT CAC ACT TGA ACC AGT AGT CTC G; 5' primer 5'- CCA TCA TTC AAG CCT GTT CTT GGA CC-3', 3' primer 5'- CGT CGG CGA TTA GTT GAC TGT TG-3'); *glo-1* (5' primer, 5'- CGA CAA TCG GTG TTG ACT TTG CTC TC-3', 3'primer 5'- CAG TCT TCC AGC GTA AAG CAC CTT C-3'; 5' primer 5'- GAA GGT GCT TTA CGC TGG AAG ACT G-3', 3' primer 5'- GTT CAG TGG AGA TCA CCG TAT TTG CC-3'); *dlg-1* (5' primer 5'- GGA GGT ATG GAC CAA CCA ACA GAA GAC GG-3'; 3'primer 5'- CAA GTT GCC GGC GCT

CAC TGA TG-3'; 5' primer 5'- CAG TAG CTG CGT TCC ACA CAC TTC C-3', 3' primer 5'- GGC ATT GCT TTG AAG TCG TCG GAT AG-3'); *elt-2* (5'primer 5'- CCA TAT ACG ACA AAC CCT CTC TGT ACG ACC-3', 3' primer 5'- GTG ACG TTG ATG GTG TGG AAG AGT CTC C-3'); *htp-3* (5'primer 5'- CCA AGA GGC CTC ATT CCA TCG A-3', 3' primer 5'- GGA GCT CTC GTT GAA CGT TTC-3').

2.4. RESULTS

2.4.1. Centriole duplication is uncoupled from DNA replication prior to elimination in endocycling cells

During post-embryonic development in *C. elegans* cells in both the hypodermal V cell and the intestinal E lineage will abandon mitotic division and alternatively execute endocycles to generate polyploid cells in the adult (Hedgecock and White. 1985; Kipreos 2005). The successive cycles of DNA replication that are characteristic of the endocycle are driven by canonical S-phase regulators, many of which have previously been shown to trigger centriole duplication during the mitotic cell cycle (reviewed in Hinchcliffe and Sluder. 2002; Hemerly et al., 2009; reviewed in Lee et al., 2009). Because of the repeated oscillations of the S-phase cyclin/CDK activity during endoreduplication we questioned how centrioles might react in endocycling cells. If centrosome duplication remains coupled with the cell cycle, the centrioles could duplicate at each S-phase, resulting in an accumulation of centrioles in the polyploid adult cells. Alternatively, the centrioles might uncouple from the cell cycle, and become refractory to S-phase activities. This would manifest as two centrioles being present in the 64C adult intestinal cells. By determining centriole numbers in the polyploid cells of the *C. elegans* larva we should be able to distinguish between each of these possible scenarios.

SPD-2, the *C. elegans* orthologue of mammalian CEP192, is the first protein to be recruited for the initiation of centriole duplication in *C. elegans* (Delattre et al., 2006; Joukov et al, 2010; Kemp et al., 2004; Pelletier et al., 2004), therefore we monitored SPD-2 levels as a marker for the centrioles to gain a better understanding of centriole dynamics in the polyploid cells of *C. elegans* intestine. A wild type SPD-2 genomic DNA fragment including its endogenous promoter region was fused to GFP (*spd-2*::SPD-2::GFP) to visualize SPD-2 expression, localization and stability (Frøkjær-Jensen et al., 2008; Kemp et al., 2004). Although GFP expression was observed throughout the germ line, most prominently in the distal region (Figure 2.1A, a and a'), it was notably absent from the adult intestinal cells, suggesting that SPD-2 was either not expressed in

the intestinal lineage, or SPD-2 was eliminated by the adult stage (Figure 2.1A, b and b').

We therefore examined the temporal profile of SPD-2 expression in the intestine during post-embryonic development. The intestinal nuclear division was used as a developmental landmark to discern between the L1 and L2 stage in our study, since it represents the end of mitotic cell cycles. Wild type larvae were staged and stained at the L1, the L1/L2 transition (~3 hours post intestinal nuclear division), L2, and L3 stages. In the L1 stage, as well as at the L1 nuclear division, SPD-2 was apparent on the majority of intestinal nuclei (Figure 2.1B; 2.1C). However, the SPD-2 signal gradually became undetectable on the intestinal nuclei as the L2 stage progressed, whereby only half of the intestinal nuclei possessed SPD-2 foci 6-8 hours after the nuclear division (Figure 2.1B; 2.1C). This progressive loss of SPD-2 eventually results in the complete elimination of SPD-2 signal by the L3 stage (Figure 2.1B; 2.1C). Similar dynamics were also observed for another centriolar protein, SAS-4 (Kirkham et al., 2004) (Figure 2.S1), confirming that the loss of SPD-2 most probably reflects the fate of *bona fide* centrioles in these cells. Taken together, our observations of SPD-2 reveal that centrioles are still present until the completion of the post-embryonic nuclear division in the L1, but are progressively eliminated in the later stages post-embryonic development in the intestinal lineage.

The intestinal nuclear division is therefore a critical landmark that is associated, not only with a transition from one type of cell cycle to another, but also with a change in sensitivity to S-phase CDK activity and centriolar stability. Prior to the intestinal nuclear division, centrioles duplicate normally in a cell cycle-dependent manner since two SPD-2 positive foci are detectable (Figure 2.1B), consistent with findings in mitotic cells (Kemp et al., 2004). However, after the nuclear division, the centrioles do not re-duplicate despite S-phase entry during the first endocycle, since only a single SPD-2 focus is detectable in the majority of the nuclei (Figure 2.1B), suggesting that centriole duplication becomes uncoupled from S-phase at the transition from the mitotic cell cycle to the onset of the first endocycle. This may be a general feature of endoreduplicating cells, since similar

uncoupling occurs in the anterior daughter cells of V cell lineage. The endoreduplication in the anterior daughter results in a 4C nucleus, which is visibly larger than its posterior sister. We noticed that a SPD-2 singlet was present in the anterior cells, whereas a doublet is present in the posterior seam cell nuclei that do not undergo endocycles (Figure 2.1D). Interestingly, the majority of the SPD-2 signal becomes transiently diffuse throughout the nuclei beginning at the L2, before the signal becomes completely undetectable later during the L2 stage (Figure 2.1B). In the germ line, where centrioles are eliminated at the onset of oogenesis, we noticed that SPD-2 also becomes diffuse prior to the complete loss of centriolar markers (Figure 2. S2). This transient change in SPD-2 localization appears to precede the destabilization or elimination of the centrioles and may reflect specific modifications of SPD-2 that converge upon a common developmentally-regulated mechanism that determines centriole stability in both the intestine and in the germ line.

2.4.2. Intestinal nuclear division is followed by a loss of PCM.

One of the major functions of the centrosome is to organize the mitotic spindle during mitosis, which, to a great extent, is fulfilled by components that make up the PCM, including the γ-tubulin complex. Previous studies indicated that γ-tubulin recruitment is subject to cell-cycle dependent regulation in mitotic cells (Bobinnec et al., 2000). During maturation γ-tubulin accumulates around the centrioles, resulting in substantially enlarged γ-tubulin foci, the intensity of which returns to baseline levels at the onset of the next interphase (Bobinnec et al., 2000). We observed a similar basal level of γ-tubulin expression during the L1 stage (Figure 2.2A). However the size of the γ-tubulin focus greatly increases and peaks when the metaphase chromosomes become detectable (Figure 2.2A). Following chromosome segregation during anaphase, most γ-tubulin disperses prior to onset of the following S-phase and its levels around the centrioles never recover. (Figure 2.2A). This anaphase dispersal does not occur in mitotically proliferating somatic cells (Figure 2.2B left), or in the germ cells that undergo mitotic divisions around the same time (Figure 2.2B right). Our observation

suggests that at the L1/L2 transition, just subsequent to the nuclear division, the centriole loses its MTOC function, which is associated with a dispersal of γ-tubulin.

2.4.3. Centriole duplication can be uncoupled during unscheduled DNA synthesis, but elimination is under developmental control.

The centrioles in endocycling cells uncouple from the successive cycles of DNA replication and eventually are eliminated before the L3 stage. The uncoupling may be a means to limit centriole numbers in cells that become polyploid and thus may be under developmental/genetic control. To determine whether centriole/cell cycle uncoupling is unique to the onset of endocycles we determined whether a similar uncoupling step might still occur in cells that undergo non-developmentally regulated, un-quantized DNA replication. We monitored SPD-2 levels in a *cul-4* loss of function mutant wherein a critical ubiquitin E3 ligase that specifically targets the key DNA replication licensing factor CDT-1 has been compromised. DNA replication licensing is abnormal in these animals resulting in un-quantized DNA synthesis and cells with oversized nuclei containing 100C DNA content in some lateral hypodermal cells (Kim and Kipreos, 2007; Zhong et al. 2003). Interestingly, in *cul-4* (*gk434*) homozygous animals, SPD-2 is neither overduplicated nor eliminated in cells that contain nuclei that have undergone massive DNA re-replication (Figure 2.1E), suggesting that centriole duplication becomes uncoupled from unscheduled/un-quantized DNA re-replication, but that their elimination is not linked to the repetitive cycles of DNA replication. Centriole elimination is therefore not an obligate downstream consequence of uncoupling, but rather it must be a developmentally-regulated event associated with cells that undergo these unique cell cycles that do not undergo mitosis/cytokinesis prior to initiating a subsequent phase of DNA synthesis.

Although the centrioles persist in the *cul-4* hypodermal cells, it is not apparent whether they maintain their MTOC capacity. In order to test this, we measured the levels of γ-tubulin in *cul-4* mutants and found that the levels remain restricted to centriolar foci but no longer fluctuate and are maintained at a low basal

intensity level typical of interphase cells (Figure 2.2C). Our data suggest that the centrioles uncouple from the cell cycle in *cul-4* mutant cells and no longer respond to S-phase CDK activities that would normally promote duplication. However, their uncoupling in this circumstance is not associated with their eventual elimination as is observed in the cells undergoing the successive rounds of DNA replication typical of the endocycles.

Because the uncoupling and eventual elimination of the centrioles occur invariably at or around the L1/L2 transition in the intestinal cells, we wondered whether elimination was under temporal control, or whether it depended on the appropriate execution of the endocycle program. To distinguish between these possibilities, we used a *lin-35* (*n745*) mutant that is disrupted for the *C. elegans* orthologue of mammalian Rb (Lu and Horvitz, 1998). *lin-35* (*n745*) does not disrupt developmental timing globally, but *lin-35* mutants do repeat the nuclear divisions typical of the L1 stage due to transcriptional misregulation of cell cycle effectors, thus giving rise to supernumerary intestinal nuclei prior to their eventual switch to the endocycle program later in L2 stage (Ouellet and Roy, 2007). Interestingly, in contrast to the wild type, two SPD-2 foci per nuclei were visualized in L2 stage *lin-35* mutants (Figure 2.3A; 2.3B), while some animals occasionally possessed even more than two foci, if they were fixed and stained one hour later. (Figure 2.3A; 2.3B). This supernumerary centriole phenotype is likely due to the failure to uncouple centriole duplication from DNA replication after the first nuclear division since centriole numbers were unaffected in *lin-35* (*n745*) animals prior to this point in the L1 stage (data not shown). Because we see numeral defects in *lin-35* mutants, which do not appropriately initiate the endocycle program, our data suggest that centriole uncoupling from DNA replication and elimination is contingent on the appropriate onset of the endocycle program.

2.4.4. Centrosome elimination requires transcriptional attenuation of genes that drive duplication.

lin-35 acts as a transcriptional repressor, therefore *lin-35* mutants exhibit a plethora of defects that arise due to the misexpression of numerous genes that would normally be silenced (Kirienko and Fay, 2007; Lu and Horvitz, 1998; Ouellet and Roy, 2007). Because we observed aberrant centriole duplication at the L1 nuclear division in the *lin-35* mutants we reasoned that some of the misregulated gene targets in these animals might include genes involved in centriole duplication. In order to precisely analyze the expression levels of these gene products exclusively in the intestine we performed mRNA tagging to enrich for intestinal-specific transcripts following immunoprecipitation (Figure 2.3C) (Roy et al, 2002). Taking advantage of this enriched fraction of intestine-specific mRNA, we first compared the levels of transcripts that correspond to genes that were previously determined to play a role in centriole duplication, for their expression levels during the pre-nuclear division L1 stage, and then after the L1/L2 transition. In wild type larvae, all the known genes that affect centriole duplication were reduced in the intestinal mRNA fraction following the L1/L2 transition, while their expression levels were maintained at similar levels in the whole worm (Figure 2.3D). We then compared the wild type transcriptional levels of these centriole duplication genes with those in *lin-35* (*n745*) mutants. Our data suggest that the genes involved in centriole duplication are not appropriately attenuated following the first intestinal nuclear division when compared to wild type (Figure 2.3E). However, despite their inability to downregulate these genes early in the L2, their expression eventually drops to near wild type levels 6-10 hours after the final nuclear divisions take place in the *lin-35* mutants (Figure 2.3F), resulting in a significant delay in the appropriate modulation of these gene activities, similar to what is observed with the cyclin genes prior to the onset of the endocycle program (Ouellet and Roy, 2007). These transcriptional analyses are further corroborated by our immunostaining results and suggest that many of the key genes that regulate centriole duplication are transcriptionally attenuated at, or around, the time that uncoupling takes place as the intestinal cells exit the mitotic cell cycle and commence endoreduplication.

2.4.5. CDK and PLK: SPD-2 phosphorylation and centriole dynamics

Because of the intimate links between cell division and the centrosome cycle, it is not surprising that cell cycle regulators, including many essential protein kinases and phosphatases, also control centriole duplication and various important aspects of normal centrosome function. (Reviewed in Nigg and Raff, 2009). Recent biochemical and genetic analysis have further revealed that cell cycle regulators affect centriole dynamics directly, by altering the phosphorylation status of specific proteins critical for centriole duplication and/or maturation (Decker et al., 2011; Kitagawa et al., 2011; Song et al., 2011).

Using bioinformatic analysis we identified a number of predicted phosphorylation sites on SPD-2 (Xue et al., 2008; Zhou et al., 2004) (Figure 2.4A), while systematic mass spectrometric studies have experimentally confirmed that Serine 357 and Serine 545, are indeed phosphorylated (Bodenmiller et al, 2008; Zielinska et al., 2009). Intriguingly, these amino acids correspond to consensus CDK and PLK phosphorylation sites (Xue et al., 2008; Zhou et al., 2004), both of which have been implicated in various aspects of centrosome biology (Decker et al., 2011; Harrison et al., 2011) (Figure 2.4A). In order to experimentally test whether these amino acids affect centriole dynamics in the endocycling cells of the gut, the potential CDK phospho-acceptor residue S545 was mutated to either Alanine (SPD-2^{S545A}) or Glutamic Acid (SPD-2^{S545E}), to convert the wild type SPD-2 sites into non-phosphorylable or phosphomimetic variants, respectively. The variants, together with the wild type SPD-2, were integrated into the genome and the resulting transgenic animals were crossed into the *spd-2* mutant background.

We found that all the *spd-2*-associated cell division and centriolar defects were rescued by the wild type transgene (SPD-2^{WT}) (Kemp et al., 2004) (Figure 2.4B). However, in SPD-2^{S545A} transgenic animals, we noticed that the intestinal nuclei frequently failed to divide at the L1/L2 transition, compared to SPD-2^{WT} animals (data not shown). Similar phenotypes have been reported for centriole duplication mutants in *C. elegans* suggesting that the nuclear division defect that we observed

may be due to a failure in centriole duplication (Kemp et al., 2004). We therefore monitored centriole numbers in the SPD-2^{S545A} variants at the nuclear division and found that only one SPD-2 focus was detectable adjacent to the non-divided nuclei at the L2 stage, despite that two cycles of DNA replication would have already occurred by that point of time (Figure 2.4B), consistent with a problem in centriole duplication. This occurred in approximately 25% of the SPD-2^{S545A} intestinal cells that we examined, while only 4% intestinal cells in SPD-2^{WT} animals showed the same defect (Figure 2.4C). Our data suggest that the disruption of S545 phosphorylation on SPD-2 compromises centriole duplication and renders the intestinal nuclei incapable of dividing.

Conversely, centriole duplication was seemingly unaffected during the L1 nuclear division in the phosphomimetic SPD-2^{S545E} strain (data not shown), but 35% of the SPD-2^{S545E} animals possessed supernumerary SPD-2 foci in the divided intestinal nuclei during the L2 stage (Figure 2.4B; 2.4D). Distinct from the *lin-35* mutant however, the intestinal nuclei do not undergo additional divisions, indicating that SPD-2^{S545E} specifically affects the numeral control of centriole regulation without impinging on cell cycle progress *per se*.

2.4.6. The ubiquitin-mediated degradation pathway acts downstream of SPD-2^{S357} phosphorylation to eliminate centrioles in the endocycling cells.

In addition to the potential CDK-mediated phosphorylation that may occur on S545 to affect centriole duplication, bioinformatic and Mass Spectrometry analyses have revealed a conserved consensus PLK phosphorylation site at Serine S357. Consistent with a functional role for this site, the phosphomimetic modification of Serine S357 (SPD-2^{S357E}) resulted in a distinct centriole stability phenotype. SPD-2^{S357E} is associated with the nuclear accumulation of SPD-2 during the L1 stage (Figure 2.5A), prior to the nuclear division and up until the late L2 stage (Figure 2.5A). This increase in SPD-2 accumulation has no apparent effect on the normal centriolar functions of SPD-2, since the centriole-associated SPD-2 appears to form normally on the opposing poles of the condensing nuclei

prior to nuclear division (Figure 2.5A). The SPD-2^{S357E}-associated accumulation is akin to the nuclear localization of the SPD-2 that precedes its elimination during the L2 stage (Figure 2.5A), and is usually accompanied by a loss of centrosomal SPD-2 (Figure 2.1B; 2.5B). However, SPD-2^{S357E} animals show strong SPD-2 foci coincident with its nuclear localization (Figure 2.5A), while SPD-2^{S357E} is also more stable than wild type since the variant SPD-2 signal is still detectable in a significant portion of intestinal cells even after the L2 molt, long after when the SPD-2^{WT} disappears (Figure 2.5C). This suggests that PLK-mediated phosphorylation of S357 can stabilize SPD-2 and allow it to escape its normal elimination, while allowing it to accumulate abnormally in the affected nuclei. We did not however observe any related phenotype in the non-phosphorylable SPD-2^{S357A} variant animal.

The phosphorylation of specific residues on a critical protein target can act as a molecular switch to enhance either stability or degradation. Since ubiquitylation often precedes proteasome-mediated degradation (Weissman et al., 2011), we examined SPD-2 dynamics in the absence of essential ubiquitylation/proteasome components to assess the role of protein destabilization in the elimination of the intestinal centrioles. The postembryonic roles of essential genes, like those involved in the ubiquitin-proteasome machinery, are not easily discerned through conventional RNAi. Therefore, we generated a tissue-specific RNAi-sensitive strain by introducing a rescuing *rde-1* transgene into a RNAi-insensitive *rde-1* mutant strain driven under the control of promoter that is exclusively expressed in the gut (Chotard et al., 2010; McGhee et al., 2009; Tabara et al., 1999). In this transgenic strain, the RNAi effect is restricted to the intestine, allowing animals to survive even when adult animals are fed dsRNA that corresponds to an essential gene. *proteasome b-subunit* 3 (*pbs-3*) is essential for the ubiquitylation-proteasome functions (Kamath et al., 2003) and is lethal when subjected to RNAi through conventional feeding protocols. However, using our intestine-specific RNAi strain, animals survive, allowing us to observe the premature nuclear localization of SPD-2 in the L1 stage, comparable to that observed in the SPD-2^{S357E} phosphomimetic strain (Figure 2.6A; 2.6B). Moreover, in *pbs-3* (*RNAi*)

animals, SPD-2 protein can still be detected during L3, considerably later than in control animals (Figure 2.6A; 2.6C). A similar phenotype is also observed in *proteasome a-subunit 5* (*RNAi*) animals (data not shown). Overall, our data suggest that mimicking the phosphorylation on S357 in the transgenic SPD-2^{S357E} variant enhances SPD-2 nuclear localization and may interfere with normal protein degradation cues to ultimately stabilize SPD-2.

2.5. DISCUSSION

The intimate links between the cell division cycle and the centrosome cycle ensure that the duplication and maturation of the centrosome occur in synchrony with the formation of the central spindle during mitosis. Consistent with this, many of the key enzymatic activities that drive specific events required at each stage of the mitotic cell cycle, moonlight by simultaneously affecting aspects of the centrosome cycle (reviewed in Doxsey et al., 2005).

During the development of many organisms however, the mitotic cell cycle is replaced by endocycles: an alternative means to provide tissue mass or to increase nuclear numbers (reviewed in Lee et al., 2009). Under these circumstances the existing centrioles within these cells must become refractory to the same cell cycle stage-specific enzyme activities required to drive them through G1 and S-phase. In the present study we focused on when and how this uncoupling might occur, while addressing the ultimate fate of the centrioles present in the differentiated cells in the gut following the uncoupling event.

The uncoupling occurs shortly after the nuclear division that occurs in the intestinal cells at the end of the L1 stage. Following this division the centrioles are no longer duplicated and are eliminated gradually throughout the L2 stage. Cell cycle uncoupling is not governed by chronological time but is instead contingent on the transition from the mitotic cell division program that occurs during embryogenesis and early larval development to an endocycle program that occurs in the gut from the L2 through adulthood. Furthermore, the uncoupling is not obligatorily linked to the elimination of the centrioles since other situations of centriole/cell cycle uncoupling, such as following unquantized DNA re-replication, is not succeeded by centriole elimination.

In several tissues growth can occur through the duplication of the genome without undergoing subsequent segregation of the newly formed DNA. Although the reason for this has not been firmly established, it presumably allows tissues to increase metabolic capacity and/or increase in size (grow) without the need to de-differentiate, ie…to compromise an essential physiological function in order to

divide, and/or physically separate the chromosomes into different cellular compartments.

Switching from a conventional mitotic cell division program to the endocycle requires a modification of the transcriptional repertoire that affects not only cyclins and other core cell cycle components, but also includes changes to the key gene products involved in centriole duplication.

By blocking *de novo* production of the proteins required for centriolar biosynthesis the centriole could slowly be eliminated as protein components undergo progressive degradation without replenishment. Although this may be the case, the kinetics of centriole elimination are not consistent with a passive attrition, but more likely reflect an active mechanism to degrade the centrioles beginning immediately following the uncoupling phase and then throughout the L2 stage.

In *C. elegans* the first protein required to initiate centrosome biosynthesis is a conserved coiled-coil protein called SPD-2 that acts as a scaffold to recruit other polypeptides to direct the formation of the centriole (Delattre et al., 2006; Pelletier et al, 2006; Kemp et al. 2004). Since centriole duplication is strictly regulated, SPD-2 would be a logical target to mediate this event. Our data and that of others indicate that SPD-2 is phosphorylated *in vivo*, although at present we cannot distinguish if this modification occurs in a cell cycle-dependent manner. Furthermore, others have shown that SPD-2 is phosphorylated by PLK-1 *ex vivo* and this phosphorylation is critical for aspects of SPD-2 function (Decker et al., 2011), hinting that the phosphorylation state of SPD-2 might play an important role during the regulation of cell cycle-associated changes that occur during the centriole cycle.

The modification of two Serine residues, S545 and S357 present on SPD-2 to their phosphomimetic or non-phosphorylable counterparts significantly affects centriole uncoupling (S545) and SPD-2 stability (S357), respectively. The effects of these changes appear to be restricted to the centriole dynamics in the gut cells since they do not have a visible effect on the morphology or function of the centriole/centrosomes in other lineages.

Although both of these protein kinases are important for cell cycle progression, not all kinases involved in cell division and/or centrosome dynamics necessarily impinge on the uncoupling or the elimination process that we describe here. For instance, AIR-1, an aurora-like kinase in *C. elegans*, has no obvious role in these events in the larval gut, although it does play a critical role in maturation and other aspects of centrosome biology (Kemp et al., 2004). On the other hand, other conserved cell cycle regulators such as the ubiquitin-dependent proteolytic machinery, namely F-box/Slimb have been shown to affect the elimination of the centrioles both in mammalian and *Drosophila* (Cunha-Ferreira et al., 2009; Rogers et al., 2009) and here we highlight their role in the timely elimination of centrioles following the onset of the intestinal endocycles.

Centrosome/cell cycle uncoupling is not unique to endocycling cells as this occurs in other developmental contexts as well. During spermatogenesis in several different organisms the haploid sperm fertilizes the oocyte with a pair of centrioles, indicating that centriole duplication had taken place during meiosis II in the absence of DNA replication (reviewed in Schatten, 1994; Slunder et al., 1989). Similarly, in multiciliated cells such as the tracheal cells, many centrioles begin to appear independent of the cell cycle (Vladar and Stearns, 2007). How the centriole is sensitized to the various enzymatic activities that are associated with cell cycle progression, or even better, how it can duplicate independently of these influences remains a mystery.

It is tempting to generalize a role for these modifications of SPD-2 in all developmental contexts that might include uncoupling and/or elimination. In the polyploid hypodermal V cell lineage SPD-2 undergoes similar changes in cellular distribution prior to the elimination of the centrioles. Similarly, in germ cells that initiate oogenesis, SPD-2 also changes its cellular distribution prior to the elimination of the centrioles in leptotene (Figure S2.2). However, in the germ line, modification of these residues on SPD-2 appears to have little or no consequence, suggesting that mechanisms that do not include phosphorylation on S357 and S545 underlie the disappearance of the centrioles in the germ line. Moreover, gene products that are required for elimination in the germ line do not affect

uncoupling and elimination in the gut (data not shown). Despite the differences between the two tissues however, the pathways may converge on a common component, SPD-2, where both modes of regulation independently impinge on the cellular SPD-2 distribution to ultimately affect its stability.

Although centriole elimination during oogenesis is common in many organisms it also occurs in various other contexts, namely after the cell commits to a terminally differentiated state. In most mammalian cells, the centriole pair that is present following the last mitotic division preceding terminal differentiation, will migrate to the cell membrane where it will undergo specific modifications to generate a basal body from which will form the primary cilium (Dawe et al., 2007). In *Drosophila* and *C. elegans* only a small collection of cells are ciliated and the centrioles in these non-ciliated cells are not retained following differentiation. Instead they disappear by an as of yet uncharacterized mechanism. It is not clear why it would be advantageous to remove the centrioles from a differentiated cell; perhaps its presence could sensitize the cell to regenerate the mitotic spindle through some aberrant acquisition of MTOC capacity; potentially a critical step toward unscheduled/unequivalent divisions and potentially hyperplasia. In this light, in addition to its co-opted role in signaling, perhaps the role of the primary cilium may be to sequester the centriole for future entry into the mitotic cell cycle, following wound healing for example; something that rarely occurs in the *C. elegans* adult under normal conditions. Nonetheless, our interrogation of how such organelles are removed in a developmentally regulated manner will be informative to identify genes that are generally required to control this process in a coordinated manner in diverse cell types.

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2.8. FIGURES

Figure 2.1. Centrioles are eliminated in the endocycling cells of *C. elegans*. **(A)** SPD-2::GFP signal is undetectable in many adult somatic cells, (a) but can be seen throughout the germline until the onset of oogenesis, while in (b) it is undetectable in the intestinal cells of the young adult hermaphrodite. a' and b' are high magnification images of the field identified by two white rectangles designated as a and b. **(B)** SPD-2 foci are detectable up to the nuclear division in the L2. The signal is no longer detectable at the L3 stage. Animals were stained with DAPI (red) and anti-SPD-2 (green). Arrowheads indicate the centrioles, while the asterisks indicate intestinal nuclei. The insets represent magnified SPD-2 signal of the respective highlighted regions (white rectangles). **(C)** Quantification of centriole elimination described in **(B)** based on SPD-2 detection ($n \geq 56$ for each stage). **(D)** Centrioles duplication is uncoupled from the lateral hypodermal V cells in the late L1 stage. The centriole appears to be uncoupled in the anterior daughter cell, but persists in the posterior daughter, which will continue to divide. The square brackets indicate the anterior and posterior daughters of a common V cells, while arrowheads point to the centrosomes. The inset is a magnified view of the region delineated by the white rectangle. A, anterior; P, posterior. hyp7, the nucleus of hyp7 cell. **(E)** Centriole duplication becomes uncoupled from the cell cycle, but centrioles are not eliminated in the context of un-quantized DNA re-replication. Homozygous *cul-4* (*gk434*) mutant was stained with DAPI (red) and SPD-2 (green). The inset shows the SPD-2 signal in the region delineated by the white rectangle. Arrowheads point to centrioles. Scale bar, 5 μm.

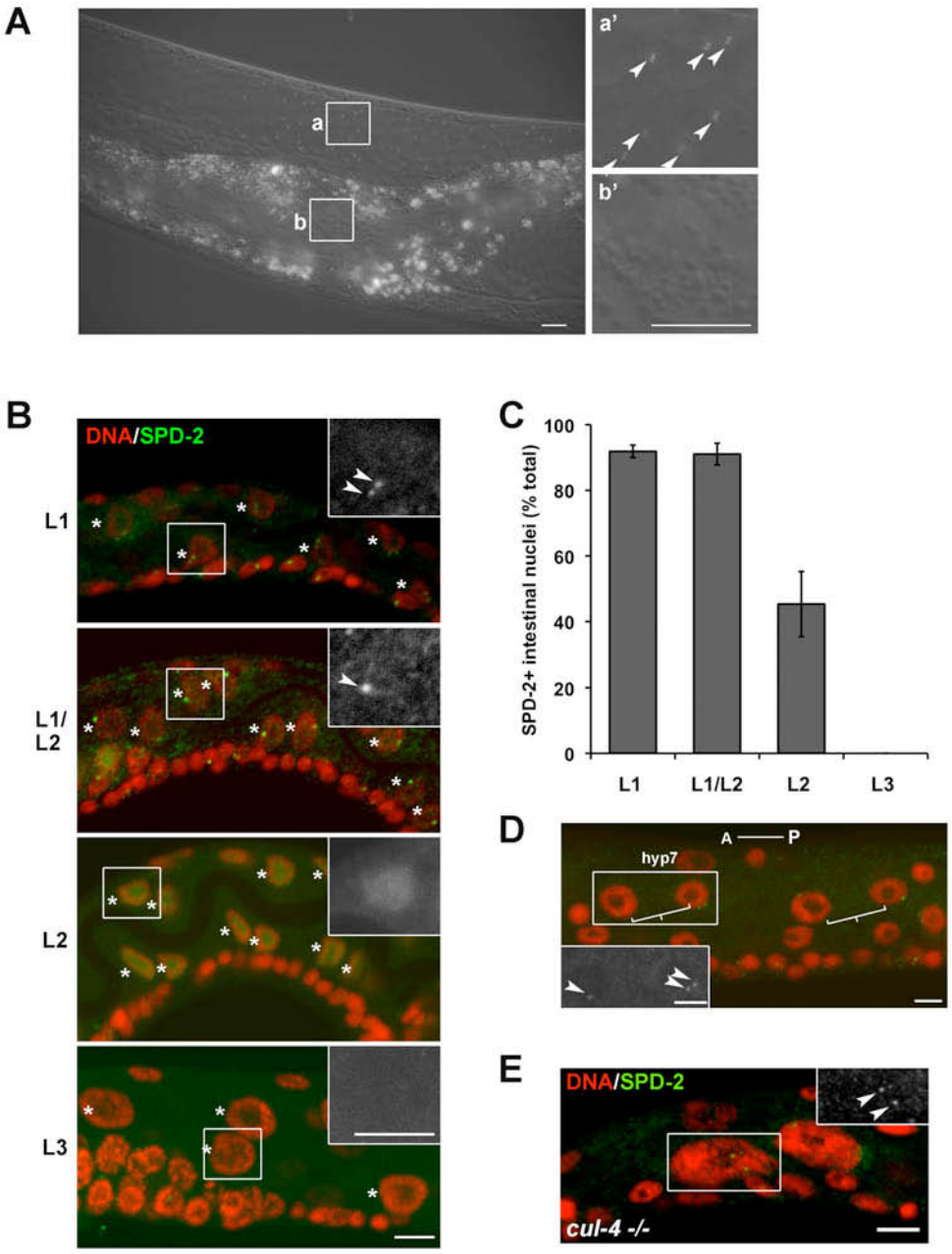

A
a
b
a'
b'
B
DNA/SPD-2
L1
L1/
L2
L2
L3
C
SPD-2+ intestinal nuclei (% total)
100
80
60
40
20
0
L1
L1/L2
L2
L3
D
A
P
hyp7
E
DNA/SPD-2
cul-4 -/-

Figure 2.2. Centrioles lose their capacity to recruit γ-tubulin following the intestinal nuclear division that precedes the onset of endoreduplication. (A) Wild type worms were stained for the PCM marker γ-tubulin before and during the intestinal nuclear division. The asterisks indicate the intestinal nuclei and the square bracket highlights a pair of sister intestinal nuclei. **(B)** High resolution micrograph of cells following division during the L1 stage. Note that γ-tubulin re-associates or remains associated with the centrioles in the nascent sister cells. Left, ventral somatic cell; right, germ line precursor cells. Square brackets highlight sister cells. **(C)** Similar staining was performed in *cul-4* (*gk434*) homozygous animals to show that centrioles lose their capacity to recruit γ-tubulin following uncoupling. All cells were stained with DAPI (red) and anti-γ-tubulin (green). Scale bar, 5 μm.

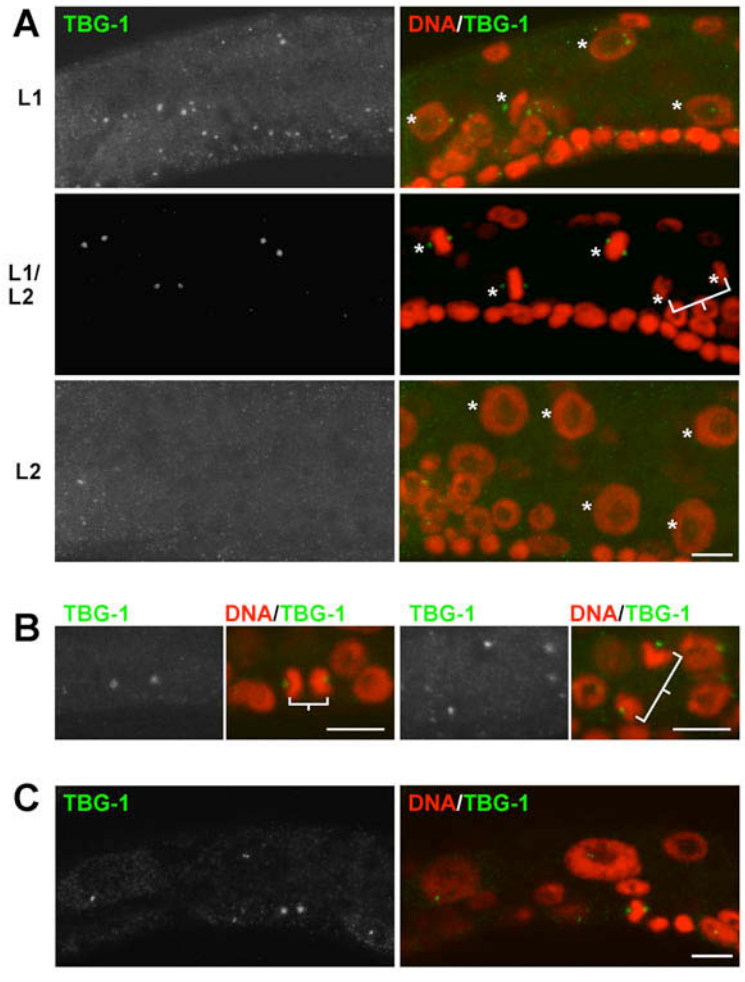

A
TBG-1
DNA/TBG-1
L1
L1/
L2
L2
B
TBG-1
DNA/TBG-1
TBG-1
DNA/TBG-1
C
TBG-1
DNA/TBG-1

Figure 2.3. *lin-35*/Rb mutants undergo additional rounds of centriole duplication. (A) *lin-35*/Rb mutant L1 larvae were stained with DAPI (red) and anti-SPD-2 (green) to monitor centriole dynamics at the nuclear division. The asterisks indicate the intestinal nuclei and the arrowheads point to the SPD-2 foci. The insets represent magnified views of the respective regions highlighted by the white rectangles. Scale bar, 5 μm. **(B)** Quantification of the number of SPD-2 foci per intestinal nucleus in both wild type and *lin-35*/Rb mutants (n=75). **(C)** RT-PCR analysis of cell-specific transcripts from N2 or *lin-35 (n745)* following expression and enrichment. *elt-2* is intestinal specific, while *htp-3* is expressed exclusively in the germ line. **(D)** *spd-2*, *zyg-1*, *sas-4*, *-5*, *-6* and *dlg-1* (control), (McMahon et al., 2001) transcript levels were quantified using RT-PCR from total or intestine-enriched mRNA from wild type (N2) or from *lin-35 (n745)* L2 stage larvae. int., intestinal. bp, base pair. **(E)** The relative levels of *spd-2*, *zyg-1*, *sas-4*, *-5*, and *-6* in *lin-35* (*n745)/*N2. The Y-axis indicates the log-fold levels of change in the expression of the various genes analyzed. **(F)** *spd-2*, *zyg-1*, *sas-4*, *-5*, *-6* and *dlg-1* transcript levels were quantified using RT-PCR from total or intestine-enriched mRNA from *lin-35* (*n745*) 6-10 hours after the last intestinal nuclear division . int., intestinal. bp, base pair.

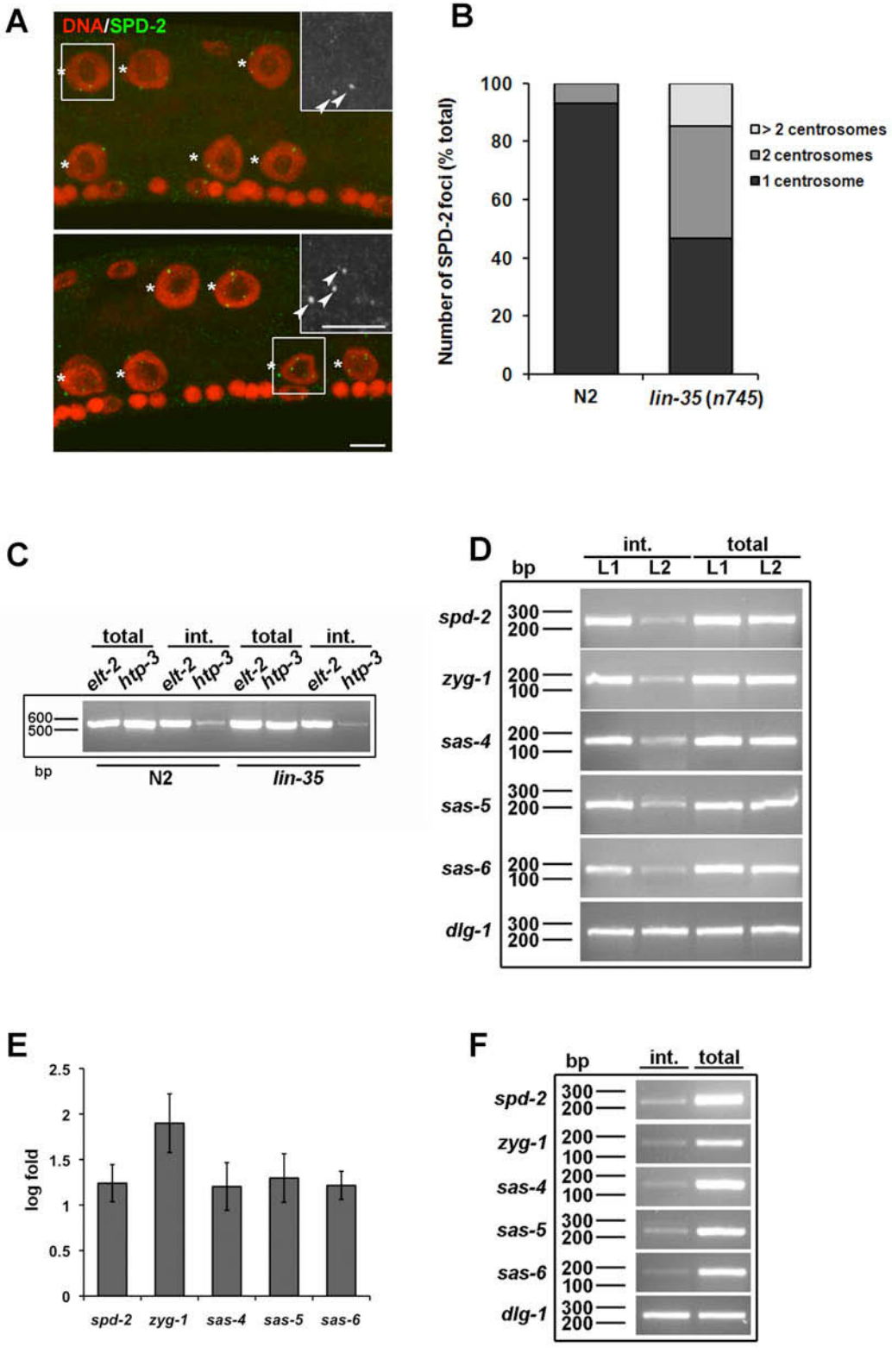

A
DNA/SPD-2
B
Number of SPD-2 foci (% total)
100
80
60
40
20
0
N2
lin-35 (n745)
> 2 centrosomes
2 centrosomes
1 centrosome
C
total
int.
total
int.
elt-2
htp-3
elt-2
htp-3
elt-2
htp-3
elt-2
htp-3
600
500
bp
N2
lin-35
D
bp
int.
total
L1
L2
L1
L2
spd-2
300
200
zyg-1
200
100
sas-4
200
100
sas-5
300
200
sas-6
200
100
dlg-1
300
200
E
log fold
2.5
2
1.5
1
0.5
0
spd-2
zyg-1
sas-4
sas-5
sas-6
F
bp
int.
total
spd-2
300
200
zyg-1
200
100
sas-4
200
100
sas-5
300
200
sas-6
200
100
dlg-1
300
200

Figure 2.4. Regulation of centriolar dynamics in the postembryonic intestinal cells may be mediated through CDK and PLK phosphorylation of SPD-2. (A) The schematic structure of SPD-2 and the potential phosphorylated amino acids. Numbers represent the positions of the amino acids. S, Serine. T, Threonine. Orange: predicted consensus PLK site. Blue: predicted consensus CDK site. Blue or Orange S indicates an experimentally-confirmed phosphorylated Serine according to previous studies (Bodenmiller et al, 2008; Xue et al., 2008; Zielinska et al., 2009; Zhou et al., 2004). **(B)** From top to bottom, animals carrying WT or phospho-variant SPD-2 transgenes after the intestinal nuclear division. DAPI (red) and SPD-2 (green). Asterisks indicate the intestinal nuclei and arrowheads point to SPD-2 foci. The insets show high magnification of the regions within the white rectangles. Scale bar, 5 μm. **(C)** The frequency of centriole duplication failure is represented by quantifying intestinal cells with undivided nuclei that possess a single SPD-2 focus after nuclear division. **(D)** The frequency of supernumerary centriole duplication/formation is indicated by the number of intestinal nuclei with two or more SPD-2 foci after the nuclear division. $n \geq 50$. $P < 0.05$.

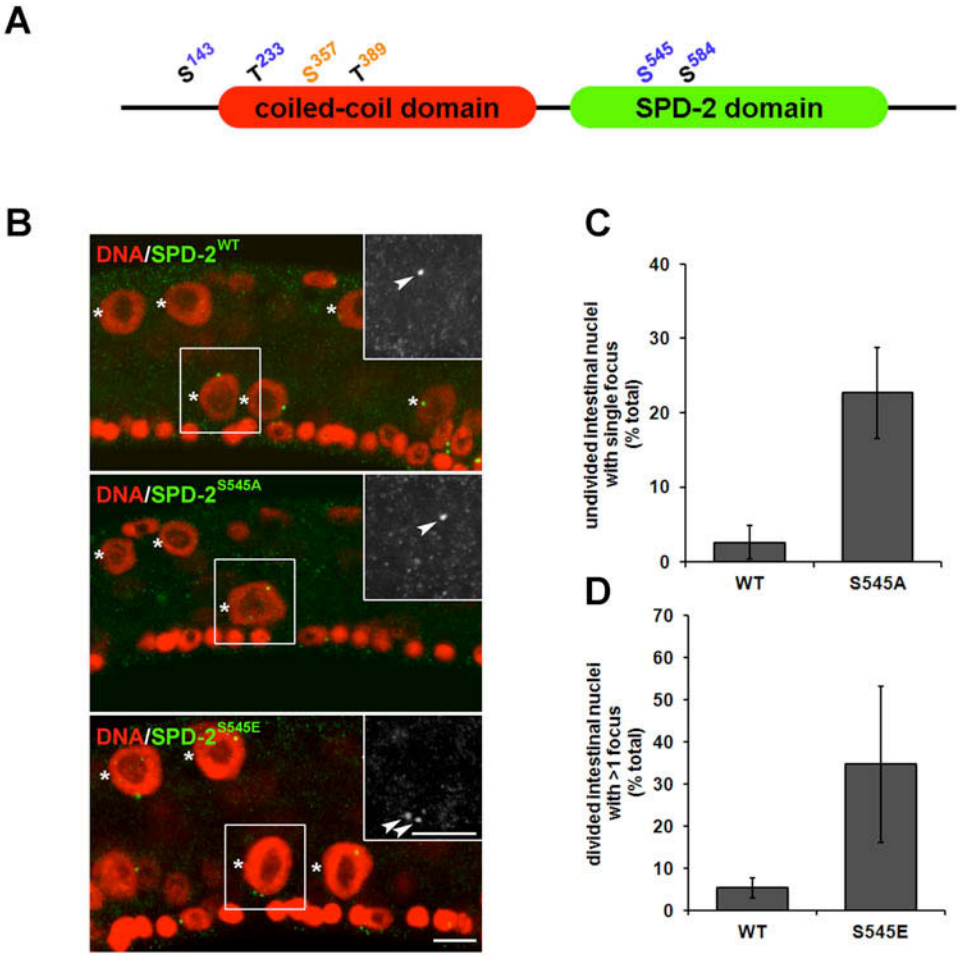

A
S143
T233
S357
T389
S545
S584
coiled-coil domain
SPD-2 domain
B
DNA/SPD-2WT
DNA/SPD-2S545A
DNA/SPD-2S545E
C
undivided intestinal nuclei with single focus (% total)
40
30
20
10
0
WT
S545A
D
divided intestinal nuclei with >1 focus (% total)
70
60
50
40
30
20
10
0
WT
S545E

Figure 2.5. Phosphorylation of S357 on SPD-2 controls appropriate localization and centriolar stability. (A) Worms carrying SPD-2^{S357E} variant were synchronized and stained with DAPI (red) and anti-SPD-2 (green) at different developmental stages. The asterisks indicate the intestinal nuclei. Scale bar, 5 μm. **(B)** SPD-2 staining was monitored in intestinal cells and the percentage of intestinal nuclei that demonstrate nuclear-localized SPD-2 at different developmental stages in strains carrying either wild type SPD-2 or SPD-2^{S357E} expressing single-copy transgenes. **(C)** The frequency of centriole persistence is quantified by counting the number of intestinal nuclei that continue to show SPD-2 signal during larval development. $n \geq 50$. $P < 0.05$.

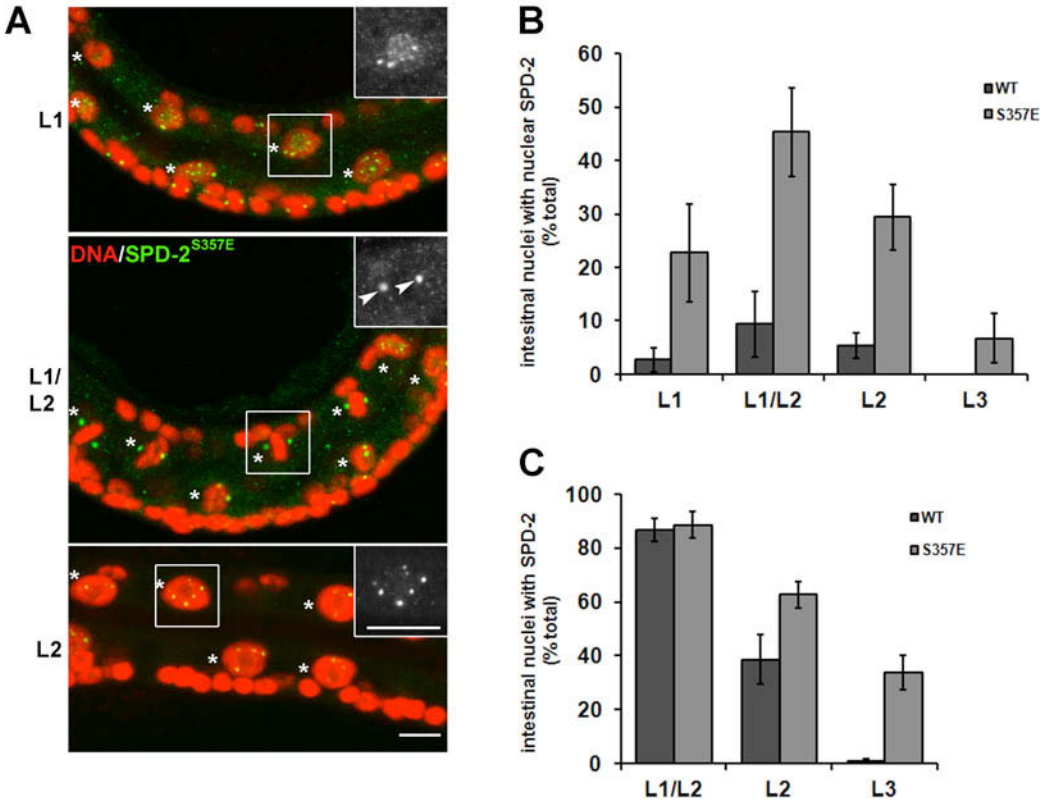
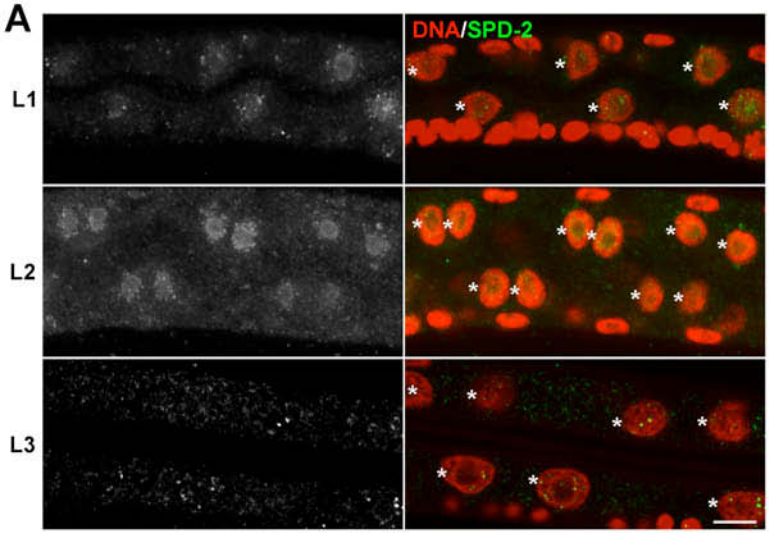
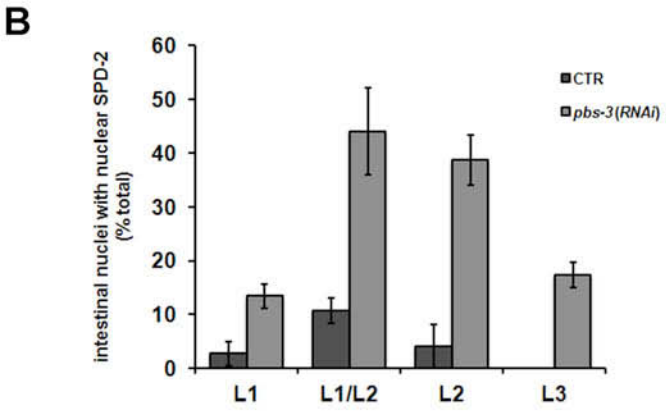

A
L1
DNA/SPD-2S357E
L1/
L2
L2
B
intesitnal nuclei with nuclear SPD-2
(% total)
60
50
40
30
20
10
0
WT
S357E
L1
L1/L2
L2
L3
C
intestinal nuclei with SPD-2
(% total)
100
80
60
40
20
0
WT
S357E
L1/L2
L2
L3

Figure 2.6. SPD-2 localization and persistence are altered by compromising proteasome activity. (A) Larvae were subjected to *pbs-3* (*RNAi*) feeding and subsequently stained with DAPI (red) and anti-SPD-2 (green) at different developmental stages. Asterisks indicate the intestinal nuclei. Scale bar, 5 μm. **(B)** SPD-2 nuclear localization was monitored and the number of intestinal nuclei that demonstrate diffuse nuclear SPD-2 staining was compared in control RNAi or *pbs-3* (*RNAi*). **(C)** The effects of *pbs-3* (*RNAi*) on centriole persistence were quantified by counting the number of intestinal nuclei that continue to express SPD-2 during larval development. $n \geq 50$. $P < 0.05$.


A
DNA/SPD-2
L1
L2
L3


B
intestinal nuclei with nuclear SPD-2 (% total)
60
50
40
30
20
10
0
CTR
pbs-3(RNAi)
L1
L1/L2
L2
L3

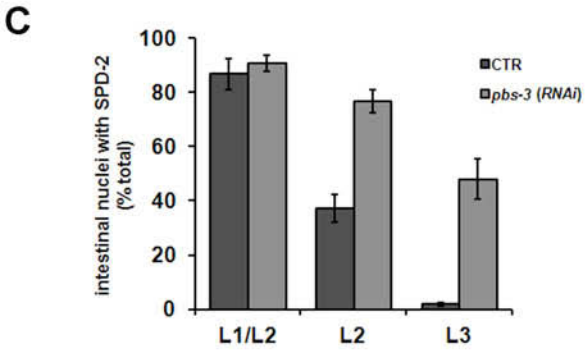

C
intestinal nuclei with SPD-2 (% total)
100
80
60
40
20
0
CTR
pbs-3 (RNAi)
L1/L2
L2
L3

Figure 2.7. A model to depict centriole dynamics in the postembryonic *C. elegans* intestine. Centrioles undergo regular mitotic-like duplication and separation during the L1 nuclear division. The centrioles then lose their PCM during anaphase and undergo cell cycle uncoupling wherein centrioles are not affected by the oscillations of successive endoreduplicative S-phases. These events precede the diffusion of SPD-2 into the intestinal nuclei followed by its eventual elimination. Substituting Serine 545 with Alanine on SPD-2 results in the failure of mitotic centriole duplication; whereas replacing Serine 545 with Glutamic Acid or, alternatively in *lin-35* mutant, centrioles overduplicate after the normal mitotic cycle. SPD-2 seems becomes stabilized if *ubq-3* is reduced or if Serine 357 of SPD-2 is replaced with Glutamic Acid.

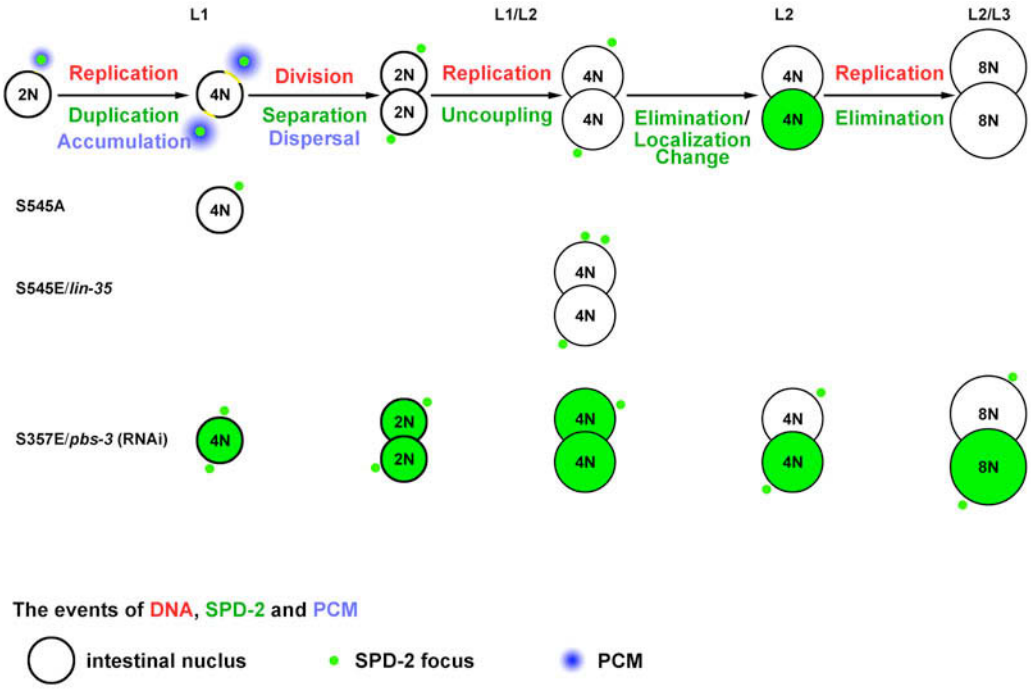

L1
L1/L2
L2
L2/L3
2N
Replication
Duplication
Accumulation
4N
Division
Separation
Dispersal
2N
2N
Replication
Uncoupling
4N
4N
Elimination/
Localization
Change
4N
4N
Replication
Elimination
8N
8N
S545A
4N
S545E/lin-35
4N
4N
S357E/pbs-3 (RNAi)
4N
2N
2N
4N
4N
4N
4N
8N
8N
The events of DNA, SPD-2 and PCM
intestinal nuclus
SPD-2 focus
PCM

Figure 2.S1. SAS-4 is eliminated in intestinal cells. N2 animals at different larval stages were stained with DAPI (red), anti-SAS-4 (green). The asterisks mark the intestinal nuclei. The inset shows the SAS-4 signal in the region delineated by the white rectangle. Arrowheads point to centrioles. Scale bar, 5 μm.

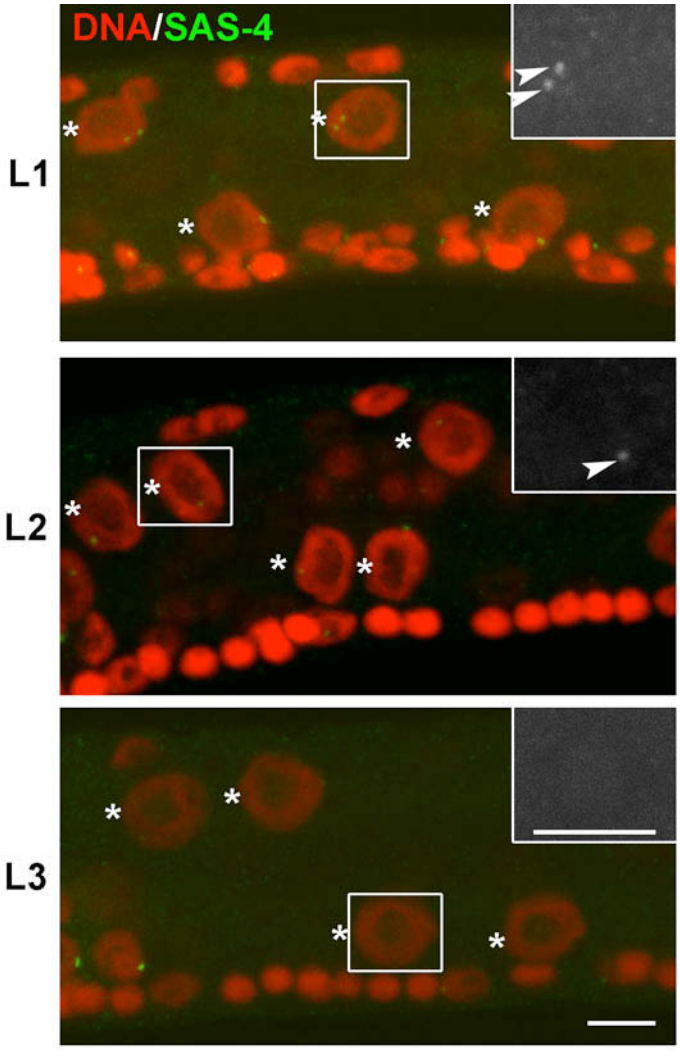
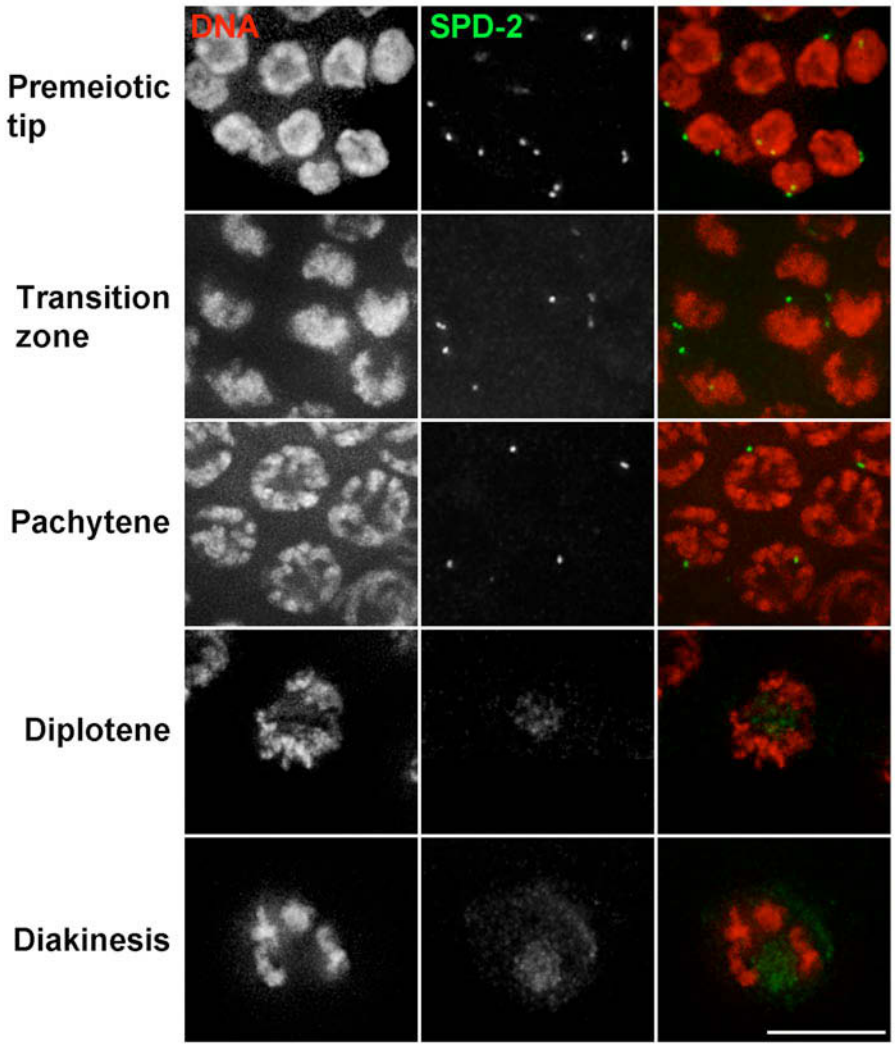

DNA/SAS-4
L1
L2
L3

Figure 2.S2. SPD-2 diffuses into nuclei before its elimination in the germline. The gonad of N2 young adult was dissected and stained with DAPI (red) and SPD-2 (green). Scale bar, 5 μm.


DNA
SPD-2
Premeiotic tip
Transition zone
Pachytene
Diplotene
Diakinesis

CONNECTING TEXT (II TO III)

In the previous chapter, I examined the function, duplication and stability of the centriole in the endocycling cells. Furthermore, I presented our candidate approach that aims to identify the potential mechanisms responsible for the uncoupling of centriole duplication and elimination in the intestinal cells. Based on my observation and candidate approach, I proposed a plausible model for the endocycling centriole uncoupling/elimination.

In the next chapter, I will present my effort in understanding the function of RNF-1, a RING-domain protein that interacts with CKI-2, which is important for cell cycle quiescence in some cell lineage as well as centriole elimination during oogenesis. By characterizing the regulation of CKI-2 through RNF-1, I hope to gain better insight into the mechanisms that control the proper level of essential cell cycle regulators.

CHAPTER III

RNF-1, a *Caenorhabditis elegans* RING domain protein, modulates the levels of CKI-2 by mediating ubiquitin-dependent proteolysis

3.1. ABSTRACT

Two CIP/KIP family CKIs are encoded in the *C. elegans* genome sequence. Post embryonic developmental cell cycle progression is regulated largely at the G1/S transition. This is controlled by the well-characterized *cki-1*, which responds to developmental or environmental signals to mediate timely cell cycle arrest. Recent studies indicate that another CIP/KIP family member, *cki-2* regulates cell cycle quiescence in parallel to *cki-1* and may contribute to timely centriole elimination. However, the regulation of this important cell cycle regulator still remains poorly understood. Here we show using a yeast two-hybrid approach that a RING domain protein (RNF-1) interacts with CKI-2. Coexpression studies suggest that RNF-1 may negatively regulate the levels of CKI-2 through the ubiquitylation pathway. Moreover, a yeast assay identified a possible role of *C. elegans* SUMO (SMO-1) to counteract the association between CKI-2 and RNF-1. Our results suggest a novel regulatory mechanism to maintain appropriate levels of CKI-2 through the interplay of a RING domain protein with SUMO, throughout the postembryonic cell cycle.

3.2. INTRODUCTION

Eukaryotic cell cycle progression is controlled mainly by the oscillation of various Cyclin-dependent kinase (CDK) activities, which are regulated by an interplay between positive regulators such as Cyclins and CDK activating kinases, or through the effects of negative regulators such as CDK inhibitors (CKIs); both of which respond to diverse developmental cues during different stages of the cell cycle.

To a great extent, proteolytic degradation of positive or negative regulatory proteins represents the major mode of regulation that controls cell cycle progression. In budding yeast, the B-type cyclin kinase inhibitor $p40^{SIC}$ is degraded at the G1/S phase transition in order to permit subsequent initiation of DNA replication, while mitotic exit takes place by inactivating the mitotic kinase (Cdk1) through the degradation of mitotic cyclins (Schwob et al., 1994; Shirayama et al., 1999).

Similarly, in multicellular organisms, the CKIs control S-phase entry and in mammalian cells CKI $p27^{Kip1}$ is eliminated at the G1-to-S phase transition, thereby allowing the onset of S phase entry (Kamura et al., 2004; Schwob et al., 1994). Many of these transitions are controlled by proteolytic degradation of key targets, which are often distinguished by ubiquitylation.

Ubiquitin (Ub) is a highly conserved small polypeptide composed of 76 amino acids that can be conjugated onto specific lysine residues of target substrates. Initially, ubiquitin is activated by ubiquitin-activating enzyme (UBA or E1) in an ATP-dependent manner. Secondly, the activated Ub is transferred to a Ub-conjugating enzyme (UBC or E2) through a thioester bond between E2 and Ub. Finally, the E3 enzyme will then recruit a given E2 to catalyze the ubiaquitylation of its target proteins (Varshavsky, 2012). Since E3s directly confer the specificity to ubiquitylation by their recognition of different target substrates, they are hence considered to be rate-limiting enzymes (Deshaies and Joazeiro, 2009).

E3 ligases can be further classified into four families by their characteristic domains: HECT domain, U-box domain, PHD domain and RING (Really Interesting New Gene)-finger domains (Bernassola et al., 2008; Coscoy and

Ganem, 2003; Deshaies and Joazeiro, 2009; Hatakeyama and Nakayama, 2003). The RING domain family represents the largest group amongest E3s. For example, they account for more than 85% of predicted E3s in the *C. elegans* genome (Kipreos, 2005).

In *C. elegans*, RING proteins can function as monomeric E3s. For instance, RNF-5 recognizes and ubiquitinates UNC-95, a protein essential for the integrity of dense body in muscle (Broday et al., 2004). Nevertheless, most RING-finger proteins act in multisubunit E3 complexes, such as in the SCF (Skp/Cullin/F-box) complex and in the APC/C (anaphase promoting complex/cyclosome).

SCF complexes are well conserved among eukaryotes and consist of three invariable components, the adaptor SKP-1, the scaffold protein Cullin (CUL-1), RING-finger protein RBX and an interchangeable F-box protein that is responsible for recognizing specific substrates (Cardozo and Pagano, 2004).

In *C. elegans*, the SCF complex is involved in critical aspects of cell cycle regulation. LIN-23 is an F-box protein and *lin-23* mutants have hyperplasia in all somatic lineages due to a failure of cycling blast cells to terminate their cell divisions at the appropriate time (Kipreos et al., 2000). Consistent with this, loss of CUL-1 function, the scaffold protein in the *C. elegans* SCF complex, shows a similar hyperplasic phenotype, together with accelerated G1/S transition and failure to trigger mitotic arrest, indicating that CUL-1 plays an indispensible role in the normal cell cycle progression during *C. elegans* development (Kipreos et al., 1996).

Like CUL-1, many of the Cullin family members affect cell cycle regulation in *C. elegans*. In fact, *cul-2* is positively involved in the G1/S phase transition and mitosis, as the *cul-2* mutant demonstrates a G1 phase cell arrest in germ cells as well as the failure to condense DNA during mitosis in the embryo (Feng et al., 1999; Liu et al., 2004; Sonneville and Gonczy, 2004). Intriguingly, the G1 arrest in the *cul-2* mutant results from elevated CKI-1; *cki-1* (*RNAi*) is capable of rescuing the cell cycle arrest (Feng et al., 1999). Considering that CUL-2 also functions as a component in the multisubunit E3 ligase that structurally resembles SCF (Wu et al., 2003), these findings strongly suggest that in *C. elegans*,

ubiquitylation on CKI-1 is critical to control decisions of cell cycle progression and arrest.

Contrary to the well-characterized CKI-1, little is known about the function of CKI-2, the second CIP/KIP family CKI encoded in *C. elegans*. This is mostly due to the refractoriness of CKI-2 to RNAi (Kim and Roy, 2006). Recent evidence suggests that CKI-2 mediates cell cycle quiescence, as the putative *cki-2* null allele shows extra cell divisions (Buck et al., 2009). Moreover, depleting *cki-2*, and potentially in combination with other orthologues also results in failure to eliminate centriole in the mature oocyte (Ambros, 2009; Kim and Roy, 2006). Considering the potential importance of CKI-2 in these processes, it is important to gain thorough knowledge regarding the regulation of CKI-2, in order to better understand how its activity is integrated during development in *C. elegans*. We therefore performed a yeast two-hybrid screen to identify interacting partners of CKI-2, as our initial attempt to decipher its various functions.

Here we report that CKI-2 interacts with RING domain protein (RNF-1) as a CKI-2 interactor. Our coexpression studies reveal that RNF-1 negatively regulates the levels of CKI-2, and which seem to be mediated by the ubiquitin-dependent proteolytic pathway.

3.3. MATERIALS AND METHODS

3.3.1. Nematode Strains

The following *C. elegans* strains were used: N2 Bristol was used as the wild-type. MR251 (*unc-119* (ed3); *rrEX251* [*hs*::CKI-2::GFP; *unc-119*(+)]), MR1227 (*unc-119* (ed3); *rrEX664* [*hs*::GFP::RNF-1; *unc-119*(+)]), MR1225 (*unc-119*; (*rrEX251* [*hs*::CKI-2::GFP; *unc-119*(+)]; *rrEX664* [*hs*::GFP::RNF-1; *unc-119*(+)])), MR1781 (*unc-119* (ed3); (*rrEX25* [hs::CKI2N::GFP; unc-119(+)]; rr*EX664* [*hs*::GFP::RNF-1; *unc-119*(+)]). All *C. elegans* strains were cultured using standard techniques and maintained at 20ºC unless stated otherwise (Brenner, 1974).

3.3.2. Yeast two-hybrid screen

Saccharomyces cerevisiae strain *W303 Y100*3 (*URA3::lexAop-lacZ 8xlexA-ADE2::URA3 ura3-1 leu2-3 his3-11 trp1-1 ade2-1 con1-100*) was used and maintained according to standard procedures (Gietz et al*.,* 1997). To generate the bait constructs, pEG202-NLS (9.8 kb), which is a LexA-DBD (DNA binding domain) fusion expression plasmid, was used. Yeast was transformed with a bait construct (LexA-DBD::CKI-2C) and grown on selective media deficient in histidine. The resulting bait strains were then transformed with 60mg of cDNA library expressing the GAL4-AD (transcriptional activation domain) fused to mixed stage *C. elegans* cDNAs (a gift from A. La Volpe) and screened as described (Gietz et al*.,* 1997).

For the directional two-hybrid assay. The yeast strain containing LexA-DBD::CKI-1 were transformed with GAL4-AD::RNF-1 and then subsequently grown on the selective media (Adenine(-)) to examine the association of the GAL4 activation domain (GAL4-AD)-fusion candidates with the LexA-DBD::CKI-1 fusion bait. The $O.D_{600}$ of the yeast cells was adjusted to 5.0 followed by 10-fold dilutions.

3.3.3. Heat-shock experiments

All the heat shock-related constructs were generated using pPD49.78 (heat shock promoter (*hs*) 16-2) and pPD49.83 (heat shock promoter (*hs*) 16-41) (Fire Lab vector kit), and each promoter containing construct was co-injected to generate heat shock-inducible CKI-2 variant transgenic animals: *hs*::GFP::RNF-1, *hs*::CKI-2::GFP, and *hs*::CKI-2N::GFP. Heat shock-induced expression was performed by floating parafilm-sealed culture plates in a 33ºC water bath for 1 hour followed by a 4 hour-recovery period at 20ºC. To check the embryonic lethality after heat shock, embryos laid from gravid adults were heat shocked for up to 30 minutes and the results were presented as the percentage of the unhatched embryos in a total population.

3.3.4. Antibodies and Immunological methods

For the generation of recombinant proteins and antiserum, GST::RNF-1C (C-terminal fragment of RNF-1) was over-expressed in *E. coli XL1-Blue* and purified according to manufacturer's recommendations (Amersham Pharmacia). GST::RNF-1C was further purified by electroelution (Bio-Rad) and rabbits were immunized using a standard protocol.

For immunoblotting, Worms were picked into SDS sample buffer and were freeze/thawed four times at -80ºC and 100ºC. The supernatant was subjected to 10% SDS-PAGE and proteins were transferred to nitrocellulose membrane (Hybond-C Extra, Amersham Pharmacia) and blotted as described elsewhere (Li et al., 2010). Primary antibodies used were rabbit polyclonal anti-CKI-2, anti-RNF-1, monoclonal α-tubulin (Sigma). Secondary antibodies were HRP-conjugated anti-rabbit or mouse. Protein bands were detected using a chemifluoresence (ECL Plus, Amersham Pharmacia) and imaged with a STORM^{TM} (Amersham Pharmacia).

For immunofluorescence, the following primary antibody was used: rabbit polyclonal anti-RNF-1 and secondary antibody were anti-rabbit Alexa 488 (Invitrogen). Embryos were fixed and stained as described elsewhere (Couteau et al., 2004), and DAPI (4,6-diamidine-2-phenylindole, Sigma) was used to

counterstain slides to reveal DNA/nuclei. Indirect immunofluorescence microscopy was performed using a Leica DMR compound microscope (x60) equipped with a Hamamatsu C4742-95 digital camera, imaging ~0.5 μm-thick optical sections (z scan). Image analysis, computational deconvolution and pseudocolouring were performed using Openlab 4.0.2 software (Improvision, UK). Images were processed using Adobe Photoshop (version 8.0).

3.4. RESULTS

3.4.1. RNF-1 interacts with CKI-2

Identifying interacting protein partners can provide a more insightful description of how a protein may be regulated and the yeast two-hybrid screen has been widely used to identify such protein-protein interactions. Therefore, we performed a standard yeast two-hybrid analysis to screen for proteins that interact with CKI-2. Since the N-terminus of CKI-2 shares some similarity with CKI-1, we conducted this analysis with the more variant C-terminus of CKI-2 (CKI-2C) as bait to isolate CKI-2-specific interactors and not factors that bind to CKI-1. From these analyses we identified a RING-finger domain protein RNF-1, which binds to CKI-2 specifically with little or no affinity for CKI-1 (Figure 3.1).

3.4.2. Endougenous RNF-1 is localized at the nuclear envelop

In order to gain further knowledge regarding the role of *rnf-1* during development, we examined the expression and/or localization in tissues during the various stages of embryonic and postembryonic development. To achieve this, we raised and purified anti-RNF-1 antisera, which recognized a band that corresponded to the expected size of GFP::RNF-1 fusion in lysates obtained from animals injected with heat-shock inducible GFP::RNF-1, whereas the same band was absent in control animals (Figure 3.2A, column 1 and 2). Furthermore, this band was equally recognized with an anti-GFP antibody, confirming that the anti-RNF-1 recognizes the GFP::RNF-1 fusion protein (Figure 3.2A, column 3).

The failure to reveal any signal from the control animal, however, suggests that our antibody could not detect endogenous RNF-1 in a western (Figure 3.2A, column 2). Nevertheless, we used this anti-RNF-1 to perform indirect immunostaining to visualize the localization of endogenous RNF-1. Interestingly, our antibody revealed signal at or around the nuclear envelope in the early embryo in N2 animals (Figure 3.2B), which is not present when stained with pre-immusera control (Figure 3.2C). To further ensure that the signal was specific to RNF-1, we subjected N2 hermaphrodite at early L4 stage with *rnf-1* (*RNAi*) and subsequently stained the embryos with anti-RNF-1 antibody. We found that the

nuclear envelope signal disappeared completely following *rnf-1* (*RNAi*) treatment (Figure 3.2D, 3.2E), which is consistent with anti-RNF-1 antibody specifically recognizing RNF-1.

3.4.3. RNF-1 mediates the ubiquitylation of CKI-2

Many RING domain proteins function in multisubunit E3 Ub ligase, therefore we speculated that RNF-1 might potentially target CKI-2 for ubiquitylation. In order to test this possibility, we assayed whether CKI-2 is indeed ubiquitylated *in vivo*. Ubiquitylation of a substrate alters its mobility on a SDS-PAGE (Obin et al., 1996), and consistent with CKI-2 being subject to this modification, we noted that overtime higher molecular weight entities could be detected by the anti-CKI-2 antibody (Figure 3.3A). Interestingly, the higher molecular weight band intensified over time following heat shock (Figure 3.3A). Unfortunately, we have been not able to directly confirm if this band is indeed ubiquitylated CKI-2 due to the lack of useful anti-Ub antisera. Nevertheless, depletion of *ubc-20*, the key Ub-conjugating E2 that interacts with RNF-1 (Gudgen *et al*., 2004), reduced the intensity of the extra band dramatically (Figure 3.3A). Taken together, our data suggests that the co-expression of RNF-1 with CKI-2 results in a post-translational modification consistent with CKI-2 ubiquitylation.

Since RNF-1 interacts with CKI-2C, we therefore tested if this modification on CKI-2 requires the direct interaction with RNF-1. We generated an animal that co-expresses an N-terminal variant of CKI-2 (CKI-2N) and full length RNF-1. The extra band was not detected from the protein extract prepared from the CKI-2N and RNF-1 co-expressing animals (Figure 3.3B), confirming that the direct interaction via CKI-2 C-terminus is required for the RNF-1-mediated modification.

SUMOylation is another ubiquitin-like post-translational modification, during which a small Ub like modifier (SUMO) is covalently linked onto substrates (Mahajan et al., 1997; Matunis et al., 1996). Resembling ubiquitylation, SUMOylation requires E1-E2-E3 catalytic cascades and also results in the mobility shift of target substrates (Geiss-Friedlander and Melchior, 2007; Park-

Sarge and Sarge, 2010). Interestingly, SMO-1, the SUMO orthologue in *C. elegans*, also interacts with CKI-2 (unpublished data). In order to test if RNF-1 is involved in the SUMOylation of CKI-2, we depleted the factors required for ubiquitylation or SUMOylation respectively by RNAi in the animals that co-expressed CKI-2 and RNF-1. In lysates prepared from these backgrounds, we found that the higher molecular weight CKI-2 band disappears if *ubq-1* is compromised (Graham et al. 1989), whereas it remains unaffected in animals that are compromised for the SUMOylation conjugating enzyme *ubc-9* or *smo-1* (Figure 3.3C) (Broday et al., 2004; Leight and Kornfeld, 2002) suggesting that the RNF-1-associated modification on CKI-2 is not SUMOylation.

3.4.4. RNF-1 promotes the degradation of CKI-2

Since ubiquitylation is often associated with protein degradation, we next tested if the RNF-1-associated ubiquitylation can affect the level of CKI-2. If this is the case, coexpression of RNF-1 should suppress the phenotype associated with the misexpression of CKI-2. Overexpression of CKI-2 or CKI-2N results in embryonic lethality (Table 1 and unpublished data), while misexpression of RNF-1 has no effect on embryogenesis (Table 3.1). However, when RNF-1 was co-expressed with CKI-2, the embryonic lethality was suppressed, while co-expression of GFP has no affect on the embryonic lethality (Table 3.1), suggesting that RNF-1 plays antagonistically to CKI-2 function. In contrast, co-expression of RNF-1 with the CKI-2N, which does not bind to RNF-1, did not suppress the embryonic lethality associated with misexpression of CKI-2N (Table 3.1). Taken into together, our data suggests that the suppression of the embryonic lethality may be mediated by the direct interaction between RNF-1 and CKI-2.

To confirm that the suppression of the embryonic lethality is due to increased rate of CKI-2 degradation, we performed western blot analyses on whole *C. elegans* extracts prepared from transgenic animals expressing CKI-2 with or without RNF-1 at various time points post heat-shock (Figure 3.4). The CKI-2::GFP peaks in expression at approximately 5h post heat-shock, while when co-expressed with RNF-1 this peak shifts substantially with a maximum at 2-3h post heat-shock,

decreasing to baseline levels thereafter (Figure 3.4). However, this peak shift of CKI-2 does not occur in *pas-4* (*RNAi*), where PAS-4 is an essential component of the proteasome (Davy *et al*., 2001; Vartiainen *et al*., 2005), (Figure 3.4). Taken together, our data suggests that RNF-1 antagonizes CKI-2 and may be involved in targeting the degradation of CKI-2 through the proteasome-mediated degradation pathway.

3.5. DISCUSSION

Post-translational modifications, such as phosphorylation, SUMOylation and ubiquitylation, play crucial roles in regulating the activity, localization or stability of critical protein targets. Such modifications of cell cycle regulators ensure that the appropriate transition of cell cycle events occur in an orderly manner. Indeed, phosphoryaltion and ubiquitylation directly regulate CKI-1 in *C. elegans*; and defects in these regulatory steps result in misregulation of CKI-1 levels, thereby giving rise to several cellular and/or developmental abnormalities (Feng et al., 1999).

CKI-2 is a second CKI encoded in *C. elegans* genome and recent emerging data suggests that *cki-2* mutants have abnormal cell divisions and may contribute to the timely failed elimination of centrioles in developing oocytes (Buck et al., 2009; Kim and Roy, 2006). In our attempt to achieve a better understanding of how CKI-2 may impinge on these process, we identified a novel RING finger protein, RNF-1, from the two-hybrid analysis with the C-terminal domain of CKI-2.

Our overexpression studies indicated that RNF-1 promoted ubiquitylation of CKI-2. This modification consequently reduced CKI-2 levels and the embryonic lethality caused by CKI-2 overexpression, suggesting that RNF-1 is likely to function as or part of an E3 ubiquitin ligase, consistent with the typical role of RING-finger proteins. Recently, a new *cki-2* deletion allele (*ok2105*) has been characterised wherein the vulva precursor cells undergo extra cell divisions during the larval stage (Buck et al., 2009), which may allow us to examine how RNF-1 affect endogenous CKI-2.

RNF-1 localizes to the region around the nuclear envelope. In fact, E3 ligases in or around the nuclear envelope have been found to play important roles in degrading nuclear proteins (Lee and Kay, 2008). For example, In yeast, the E3 ligase Doa localizes to the nuclear membrane and targets the transcriptional factor Matα-2 (Deng and Hochstrasser, 2006; Swanson et al., 2001), while the nuclear pole-associated E3 complex Nup84/Slx5/Slx8 is involved in the recovery of collapsed DNA replication forks (Nagai et al., 2008). Interestingly, CKI-2 has a nuclear localization signal, therefore it would be interesting to identify where

RNF-1 triggers the CKI-2 ubiquitylation at the subcellular level and further elucidate the biological significance of this modification.

Recently, a new method using transposon-based transgeneses has allowed investigators to generate animals that carry a single-copy transgene, which largely improves the analysis and characterization of many essential genes (Frokjaer-Jensen et al., 2008). We will generate transgenic animals expressing fluorescently tagged CKI-2 and RNF-1 using this new method to better visualize CKI-2 and RNF-1 in live tissues. These experiments will help us elucidate the importance of both RNF-1 and CKI-2 localization and potentially provide substantial insight regarding function in various developmental contexts.

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3.8. FIGURES

Figure 3.1. CKI-2 interacts with RNF-1. Summary of the interaction between LexA-DBD fused CKI-2 (top) or CKI-1 (bottom) and the GAL4-AD fused RNF-1 using directional yeast two-hybrid analysis. (+) or (-) in the table (right) indicates "interaction" or "no-interaction", respectively.

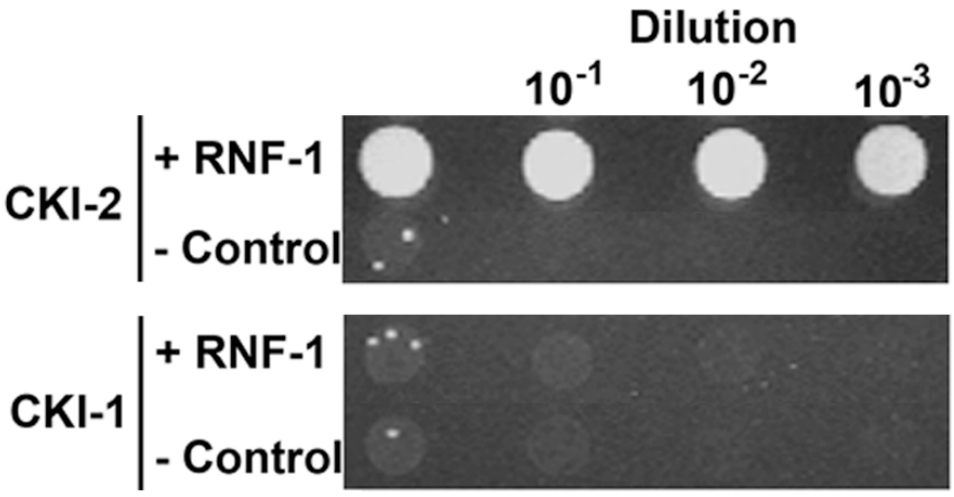
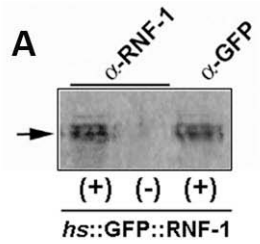

Dilution
10^{-1}
10^{-2}
10^{-3}
CKI-2
+ RNF-1
- Control
CKI-1
+ RNF-1
- Control

Figure 3.2. Immunostaining using the purified RNF-1 antiserum reveals that RNF-1 is present at or aruond the nuclear envelope. (A) The protein extract from mix-populated N2 or *hs*::GFP::RNF-1 strains were investigated with RNF-1 and GFP antiserum. **(B,C)** Embryos obtained from wide type hermaphrodites were stained with the preimmune serum **(B)** or the RNF-1 anti- serum **(C)**. **(D, E)** anti-RNF-1 specifically recognizes endogenous RNF-1. The two-cell embryo was obtained from a N2 hermaphrodite worm **(D)** or from a *rnf-1* feeding RNAi worm **(E)**. The arrow marks the polar body (anterior). The close arrowhead indicates the nuclei stained with the RNF-1 antiserum.


A
α-RNF-1
α-GFP
(+) (-) (+)
hs::GFP::RNF-1

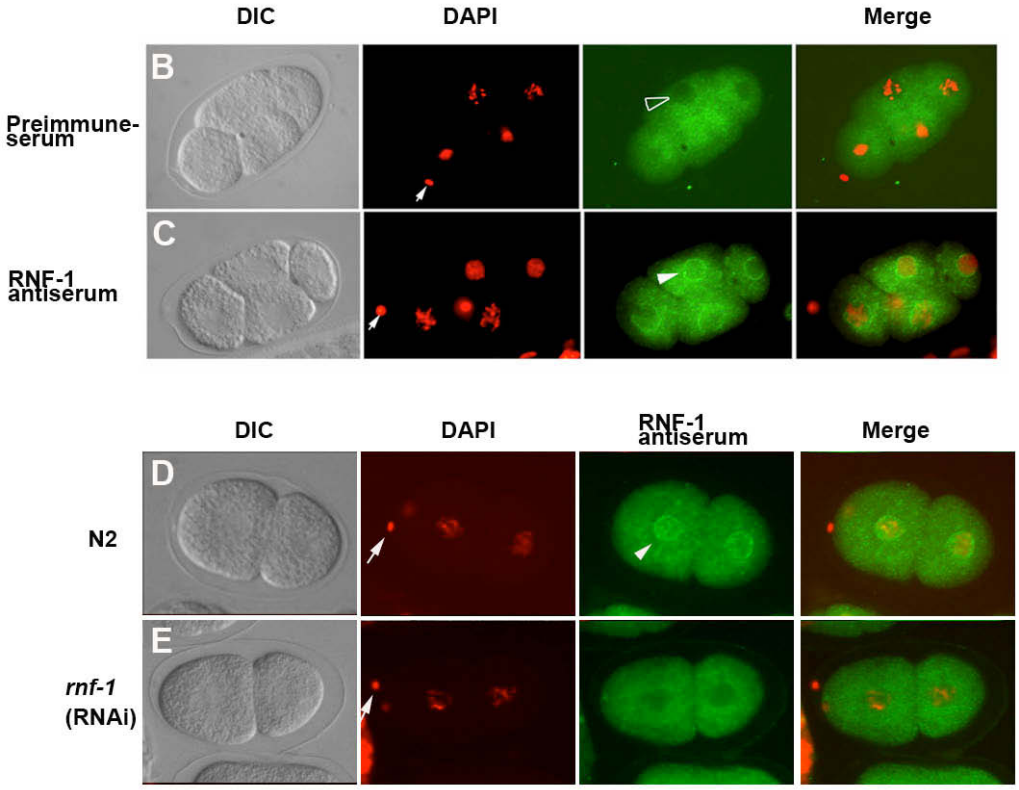

DIC
DAPI
Merge
B
Preimmune-serum
C
RNF-1 antiserum
DIC
DAPI
RNF-1 antiserum
Merge
D
N2
E
rnf-1 (RNAi)

Figure 3.3. RNF-1 regulates CKI-2 through ubiquitin-dependent modification. (A) Western blot analyses were performed using protein extracts prepared from the transgenic animals expressing CKI-2 ([*hs*::CKI-2::GFP]), or co-expressing CKI-2 and RNF-1 ([*hs*::CKI-2::GFP; *hs*::GFP::RNF-1]) at two different time points (0.5 and 1 hour) after heat shock. (-) indicates "no heat shock". In a similar manner, a western blot analysis was performed using protein extracts prepared from the *ubc-20* (*RNAi*)-treated transgenic animals co-expressing CKI-2 and RNF-1 ([*hs*::CKI-2::GFP; *hs*::GFP::RNF-1]) at 1 hour post heat shock. **(B)** Western blot analyses performed using protein extracts prepared from the transgenic animals co-expressing CKI-2 and RNF-1 ([*hs*::CKI-2::GFP; *hs*::GFP::RNF-1]), or CKI-2N and RNF-1 ([*hs*::CKI-2N::GFP; *hs*::GFP::RNF-1]) at 2 hour post heat shock. **(C)** Western blot analyses performed using protein extracts prepared from the *ubc-1* (*RNAi*), or *ubq-9* (*RNAi*), or *smo-1* (*RNAi*)-treated transgenic animals co-expressing CKI-2 and RNF-1 ([*hs*::CKI-2::GFP; *hs*::GFP::RNF-1]) at 1 hour post heat shock. The arrows indicate the position of 64 KDa or 85 KDa standard size marker, respectively.

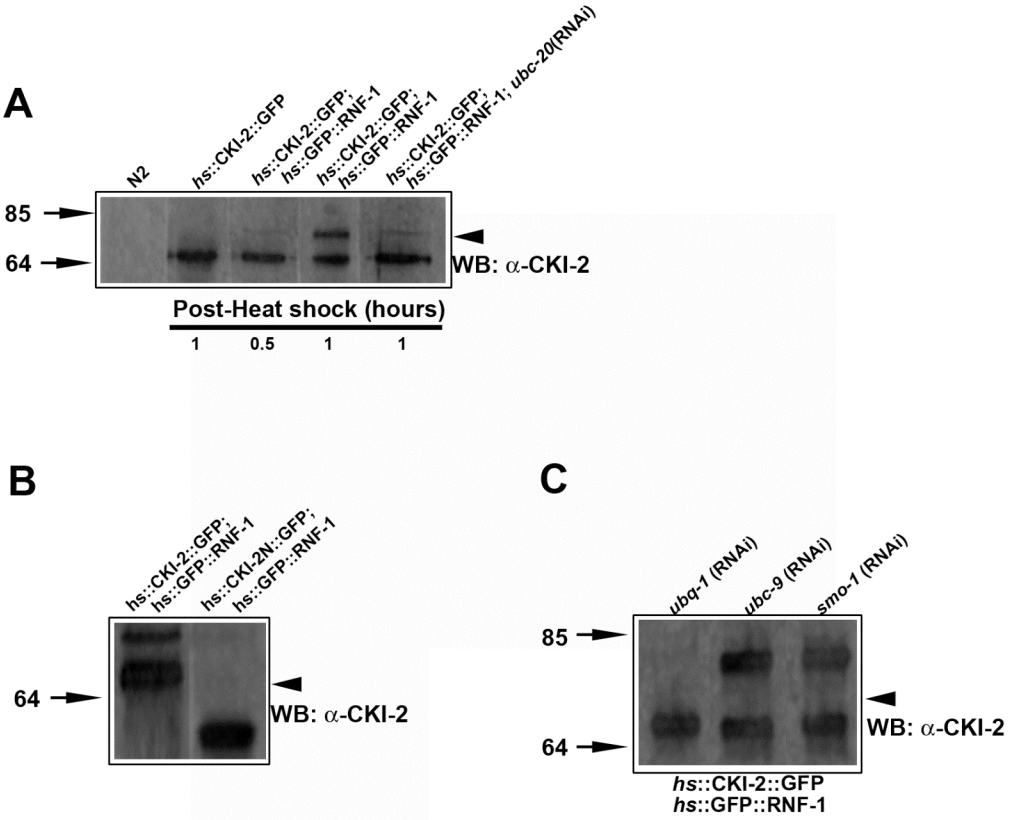

A
N2
hs::CKI-2::GFP
hs::CKI-2::GFP; hs::GFP::RNF-1
hs::CKI-2::GFP; hs::GFP::RNF-1
hs::CKI-2::GFP; hs::GFP::RNF-1; ubc-20(RNAi)
85
64
WB: α-CKI-2
Post-Heat shock (hours)
1
0.5
1
1
B
hs::CKI-2::GFP; hs::GFP::RNF-1
hs::CKI-2N::GFP; hs::GFP::RNF-1
64
WB: α-CKI-2
C
ubq-1 (RNAi)
ubc-9 (RNAi)
smo-1 (RNAi)
85
64
WB: α-CKI-2
hs::CKI-2::GFP
hs::GFP::RNF-1

Figure 3.4. RNF-1 promotes the degradation of CKI-2. (Top) Time course analysis of CKI-2 levels using western blotting with anti-CKI-2 or anti-tubulin serum. Protein extracts were prepared from a mixed population of the transgenic animals expressing CKI-2 ([*hs*::CKI-2::GFP]), or co-expressing CKI-2 and RNF-1 ([*hs*::CKI-2::GFP; *hs*::GFP::RNF-1]) at various times after heat-shock (2 to 6 hours). **(Bottom)** A western blot analysis performed using protein extracts prepared from the *pas-4* (*RNAi*)-treated transgenic animals co-expressing CKI-2 and RNF-1 ([*hs*::CKI-2::GFP; *hs*::GFP::RNF-1]) at various post heat-shock hours (2 to 5 hours). α-tubulin was used as a loading control. The arrows indicating 64 (KD) are the size markers.

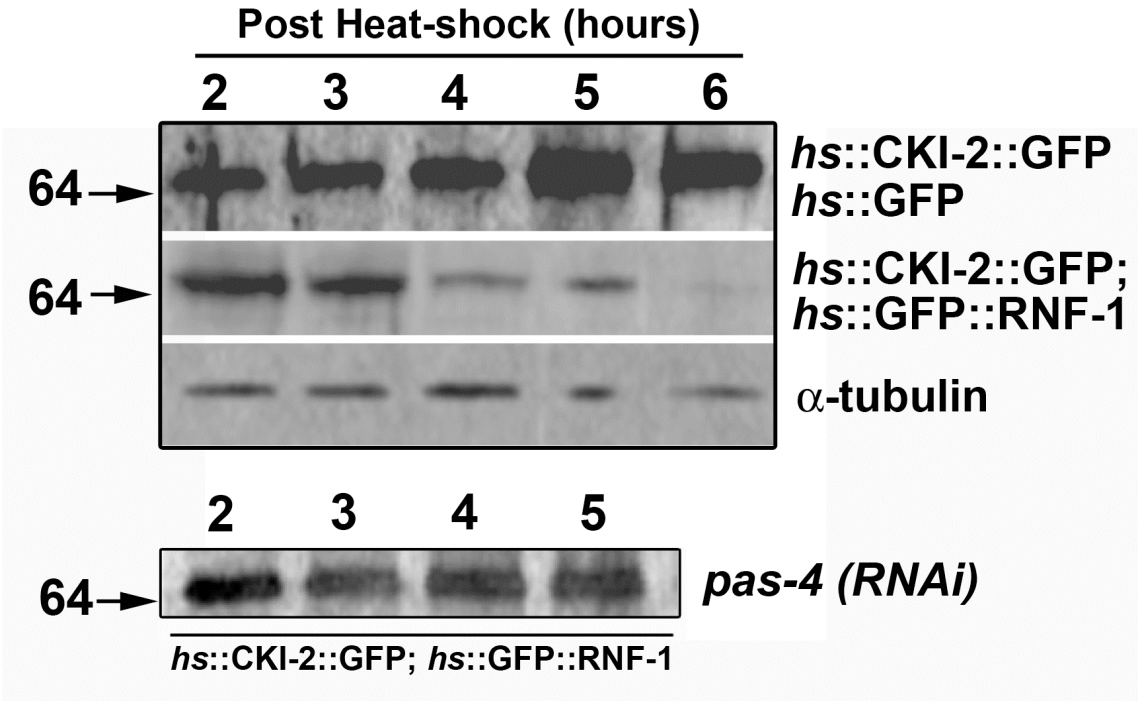

Post Heat-shock (hours)
2
3
4
5
6
64
hs::CKI-2::GFP
hs::GFP
64
hs::CKI-2::GFP;
hs::GFP::RNF-1
α-tubulin
2
3
4
5
64
pas-4 (RNAi)
hs::CKI-2::GFP; hs::GFP::RNF-1

Table 3.1. Coexpression of RNF-1 suppresses the embryonic lethality associated with misexpression of CKI-2 but not the the N-terminal variant or CKI-1. For the embryonic lethality (%), embryos from young adult animals carrying each heat-shock constructs were heat shocked and examined 30 hours later for the embryonic lethality determined by the number of L1 larvae present on the plate. Non-heat shocked embryos were used as controls. For each of the individual genotypes, three independent tests were carried out and the corresponding standard deviations were then calculated. The values represent the percentage of unhatched embryos that arise from the initial population of embryos (n).

	Embryonic lethality (%)	
Genotype	No Heat-shock	Heat-shock
N2	0 (n=433)	2.6±0.5 (n=382)
hs::GFP	0 (n=471)	3.2±0.8 (n=472)
hs::GFP::RNF-1	0.6±0.2 (n=498)	5.4±0.7 (n=463)
hs::CKI-2::GFP	1.4±0.3 (n=439)	20.6±1.5 (n=402)
hs::CKI-2::GFP;*hs*::GFP	1.1±0.6 (n=437)	19.4±2.3 (n=458)
hs::CKI-2::GFP;*hs*::GFP::RNF-1	1.2±0.6 (n=365)	6.6±1.2 (n=426)
hs::CKI-2N::GFP	1.2±0.7 (n=410)	18.3±1.4 (n=437)
hs::CKI-2N::GFP;*hs*::GFP	1.0±0.3 (n=396)	18.4±1.7 (n=425)
hs::CKI-2N::GFP;*hs*::GFP::RNF-1	1.1±0.5 (n=372)	19.8±2.6 (n=414)

CONNECTING TEXT (III TO IV)

In the previous chapter, I showed a series of biochemical experiment to identify the roles of RNF-1 as a CKI-2 interacting partner. My results suggest that RNF-1 antagonizes CKI-2 level through ubiquitin-proteasome protein degradation pathway, which represents a key regulatory pathway for cell cycle regulators.

In the next chapter, I will present the changes in γ-tubulin function and localization during the germ cell progression from mitosis to meiosis, which would subsequently facilitate the further characterization of the genes responsible for these changes.

CHAPTER IV

Re-distribution of γ-tubulin during the switch from mitosis to meiosis in the *C. elegans* germ line

4.1. ABSTRACT

Highly conserved γ-tubulin is the key microtubule nucleating factor in many species. In animal somatic cells, γ-tubulin is localized at the centrosome and enables it to function as the major microtubule organization center (MTOC). During germ cell progression from mitosis to meiosis in *C. elegans*, γ-tubulin re-distributes from its centriolar localization and thereafter, centrioles no longer act as MTOCs. How and why this redistribution occurs remain largely unknown.

We examined the changes in γ-tubulin localization and function during this developmental stage and found that γ-tubulin re-localizes to the germ cell membrane, triggered by the mitosis-meiosis transition. We plan to perform forward and/or reverse genetic screens to identify potential genes responsible for this γ-tubulin re-distribution and the change in MTOC in meiotic cells.

4.2. INTRODUCTION

γ-tubulin is a highly conserved molecule across eukaryotic species and it functions as the essential microtubule-nucleating protein in yeast, plant and animal cells (Raynaud-Messina and Merdes, 2007). During DNA segregation in acentrosomal cells, e.g. meiotic cells and plant cells, γ-tubulin is found at the spindle poles and contributes to spindle formation (Canaday et al., 2000; Palacios and Joshi, 1993). In most animal cells, γ-tubulin is a component of PCM and enables the centrosome to function as the primary MTOC (Stearns et al., 1991). Furthermore, γ-tubulin plays important roles in centriole duplication in animal cells. In fact, γ-tubulin has been found at the core of the centriole in mammalian cells (Fuller et al., 1995), and is involved in nucleating the A tubule of the microtubule triplet, which further guides the growth of the B and the C tubules (Guichard et al., 2010).

In *C. elegans*, γ-tubulin stabilizes the centriolar localization of SAS-4, a key factor required for centriole duplication because of its ability to promote the assembly of centriolar microbutules (Dammermann et al., 2008). Consistent with these important roles, defects in γ-tubulin result in malfunction of spindles and/or failure of centriole duplication (Fuller et al., 1995; Haren et al., 2006).

In cycling cells, centrosome duplication and MTOC capacity are tightly coupled with cell cycle progression (Nigg and Raff, 2009). Similarly, the dynamic levels of γ-tubulin are under cell cycle-dependent control. γ-tubulin localizes at the centriole throughout the cell cycle at a low level, yet it begins to accumulate substantially at the G2/M transition. Its centrosomal concentration peaks during metaphase and is maintained at high levels through anaphase and telophase, accompanied by a peak in microtubule nucleating ability (Julian et al., 1993; Strome et al., 2001). This accumulation is largely regulated by M-phase kinases, including Aurora A kinase and polo-like kinase (PLK)-1 (Hannak et al., 2001; Sumara et al., 2004).

During *C. elegans* meiosis, γ-tubulin is reduced to an undetectable level at centriolar foci following the transition from mitosis to meiosis (Bobinnec et al., 2000). As a result, the remaining centrioles no longer organize the germ cell

microtubule network. These changes coincide with a change in the positioning of ZYG-12, a gene product required for appropriate centrosome attachment to the nuclear membrane during mitosis, to distinct patches around the envelope (Malone et al., 2003). The germ cell membrane replaces the centrosome as the major microtubule-nucleating site and ultimately mediates germ cell nuclear positioning, alone with homologue pairing through ZYG-12 (Zhou et al., 2009). How γ-tubulin re-localizes to the germ cell membrane from the centrioles and how this re-localization during meiosis affects germ cell function or meiosis *per se* remains elusive.

Here, we show the fate of γ-tubulin during germ cell progression into meiosis. Consistent with previous studies by others, we observed that γ-tubulin begins to re-distribute from its centriolar foci to the cell membrane at the onset of pachytene. This dispersal appears to be dependent on the transition from the mitotic cycle typical of the distal germ cells to the meiotic cell cycle that occurs at or around the transition zone in the developing adult gonad.

4.3. MATERIALS AND METHODS

4.3.1. Nematode Strains

The following *C. elegans* strains were used: N2 Bristol was used as the wild-type strain throughout. *gld-1*(*q485*). All *C. elegans* strains were cultured using standard techniques and maintained at 15˚C (Brenner, 1974).

4.3.2. Antibodies and immunological methods

The following primary antibodies were used: rabbit polyclonal anti-γ-tubulin (Sigma LL17), mouse monoclonal anti-γ-tubulin (Sigma T5326), mouse monoclonal anti-α-tubulin (Sigma T9026), and Cys5-conjugated rabbit polyclonal anti-SAS-4 (a gift from K. Oogema Lab). Secondary antibodies were anti-rabbit Alexa 488 (Invitrogen), anti-rabbit Alexa 555 (Invitrogen) and anti-mouse Alexa 488 (Invitrogen). Germ lines were fixed and stained as described elsewhere (Couteau et al., 2004). DAPI (4,6-diamidine-2-phenylindole, Sigma) was used to counterstain slides to reveal DNA/nuclei. Indirect immunofluorescence microscopy shown in Figure 1 was performed using a Leica DMR compound microscope (60X) equipped with a Hamamatsu C4742-95 digital camera, imaging ~0.5 μm-thick optical section (Z scan). Image analysis and pseudocolouring were performed using Openlab 4.0.2 software (Improvision, UK). Experiments shown in Figure 2 and Figure 3 were performed using the 100X oil-immersion objective lens in a DeltaVision Image restoration system (Applied Precision). Data were collected as a series of 13-27 optical sections in increments of 0.2 μm under standard parameters with the softWoRx3.0 software (Applied Precision). All microscopy was performed at 20C. Images were processed with Adobe Photoshop (version 10.0).

4.4. RESULTS

4.4.1. The level of γ-tubulin is greatly reduced at the centrosome during meiosis

Others have shown that γ-tubulin is no longer detectable at the centriole in the pachytene region of the adult hermaphrodite germ line (Bobinnec et al., 2000). We first wanted to confirm the exact stage in which γ-tubulin dissociates from this structure and to determine whether the disappearance of γ-tubulin is due to complete centrosome elimination or instead due to the relocalization of γ-tubulin from the centriole to another cellular localization. We stained gonads of N2 hermaphrodite animals with antibodies against γ-tubulin and a centriolar component, SAS-4 (Pelletier et al., 2006), and found that the γ-tubulin signal overlaps with SAS-4 in the mitotic region (Figure 4.1). However, in the region where germ cells have entered meiotic prophase (pachytene), γ-tubulin can still be found at SAS-4 positive foci, although its intensity is significantly reduced (Figure 4.1). Interestingly, a considerable portion of γ-tubulin accumulates at the germ cell membrane (Figure 4.1). Our observations suggest that γ-tubulin modifies its cellular localization from being predominantly centriole-bound to the cell membrane at the onset of meiosis.

4.4.2. γ-tubulin nucleates microtubules in mitotic germ cells.

Since γ-tubulin is a key microtubule-nucleating factor, we wanted to analyze the function of γ-tubulin during meiosis. Not surprisingly, microtubules in mitotic germ cells are nucleated and organized by γ-tubulin bound to the centriole (Figure 4.2). However, upon entry into meiosis, the microtubule network is no longer nucleated around γ-tubulin foci at the centriole, and α-tubulin distributes ubiquitously throughout the cytoplasm. Thereafter, a significant amount of γ-tubulin is distributed around the nuclei during diakinesis, whereas α-tubulin remains cytoplasmic throughout meiosis I prophase (Figure 4.2). Our data suggests that γ-tubulin functions as the microtubule-nucleating factor in the mitotic germ cells, but then dissociates from the centriole while its capacity to

organize the microtubule network is concomitantly attenuated upon entry into meiosis.

4.4.3. The centrosomal localization and the microtubule-nucleating capacity of γ-tubulin in germ cells is mitosis-dependent

We next wanted to test if the changes in γ-tubulin function and localization are regulated through spatial control or via the mitosis-meiosis transition. To distinguish between these possibilities, we used homozygous *gld-1* (*q485*) animals, in which the function of an RNA-binding protein GLD-1 is disrupted (Lee and Schedl, 2001). Homozygous *gld-1* (*q485*) animals fail to continue meiosis and re-enter into mitosis, although they are able to initiate meiosis and demonstrate visually normal polarized meiotic nuclei (Francis et al., 1995).

We performed immunostaining with anti-α- and γ-tubulin in *gld-1* (*q485*) homozygous animals to monitor the microtubule-nucleating capacity and the localization of γ-tubulin. In the mitotic distal tip, γ-tubulin foci are obvious and are able to nucleate α-tubulin (Figure 4.3, A and A'). More proximally, the intensity of γ-tubulin is reduced, accompanied by the ubiquitous distribution of α-tubulin in the transition zone (Figure 4.3, B and B'), suggesting that γ-tubulin loses its microtubule nucleating capacity.

Germ cells fail to continue meiosis and subsequently re-enter into mitosis. In this region, the intense γ-tubulin foci and microbule-nucleating capacity that are typical for mitosis appear to be regained (Figure 4.3, C and C'). Following re-entry into mitosis, the germ cells in *gld-1* (*q485*) homozygous animals continue to undergo mitotic cycles and γ-tubulin maintains its typical mitotic localization and function throughout the rest of the germ line (Figure 3 D, E, D' and E'). Taken together, our analysis suggests that the centrosomal localization of γ-tubulin and its microtubule-nucleating capacity can be re-established with a renewal of mitotic activity, even in cells that have already entered meiosis.

4.5. DISCUSSION

The microtubule-nucleating capacity of the centriole is mainly enabled by γ-tubulin and other pericentriolar proteins. During mitosis, the recruitment of γ-tubulin to the centrosome is coupled with the initiation of M phase by the mitotic kinases PLK-1 and Aurora A kinase (Barr et al., 2004). The accumulation of γ-tubulin significantly increases the MTOC capacity of the centrosome, which contributes to the establishment of proper mitotic spindles.

Our immunostaining indicates that in the pre-meiotic region, strong γ-tubulin foci are usually associated with DNA with a typical mitotic metaphase appearance (Figure 1 and 2), suggesting that the γ-tubulin in these cells is very similar to that seen in somatic cells undergoing the same stages of cell division. This accumulation of γ-tubulin and other PCM proteins is part of the maturation process that occurs in an oscillatory manner as cells cycle in and out of mitosis.

During the onset of meiosis, γ-tubulin becomes incapable of nucleating microtubules and re-distributes from the centrioles to the membrane as cells enter into pachytene. This localization change is likely associated with a key triggering event that coincides with the onset of meiosis.

Interestingly, a recent study suggests that although PLK-1 is associated with the centrosome in the pre-meiotic tip in the *C. elegans* germ line, it dissociates from the centriole in pachytene (Harper et al., 2011). Whether PLK-1 is responsible for the γ-tubulin dissociation from the centrioles of germ cell nuclei as they approach the transition zone is still an open question.

During the course of γ-tubulin re-distribution, centrosomes lose their association with ZYG-12 and are no longer responsible for nuclear anchoring in germ cells (Zhou et al., 2009). ZYG-12 interacts with SUN-1, a KASH domain protein, in a PLK-2 dependent manner and this interaction is essential for homologue synapsis during meiosis (Labella et al., 2011). In future, we want to further elucidate the role of γ-tubulin re-distribution may play a role in the ZYG-12 re-localization that ultimately affects homologue pairing during meiosis.

In order to identify the important factors responsible for the observed dynamic changes in γ-tubulin localization and function during germ cell progression from mitosis to meiosis, we plan to perform forward and reverse genetic screens monitoring any alteration of γ-tubulin localization or stability in various mutant backgrounds. By identifying genes that modify this process, we hope to characterize the mechanisms behind γ-tubulin redistribution and the means by which signals initiated in meiosis affect γ-tubulin function during meiosis, while perhaps shedding some light on the functional changes to the centriole and the PCM that occur during interphase in cycling mitotic cells.

4.6. ACKNOWLEDGEMENTS

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4.8. FIGURES

Figure 4.1. γ-tubulin level is reduced at centrosome after mitosis. Gonads dissected from N2 hermaphrodite were stained with anti-SAS-4 (red), anti-γ-tubulin (green) and DAPI (blue). Pre-meiotic and TZ represent the mitotic germ cell region and the transition zone, respectively. Germ cells at the mitotic stage, leptotene/zygotene stage and pachytene stage are shown from top to the bottom. Scale bar, 5 μm.

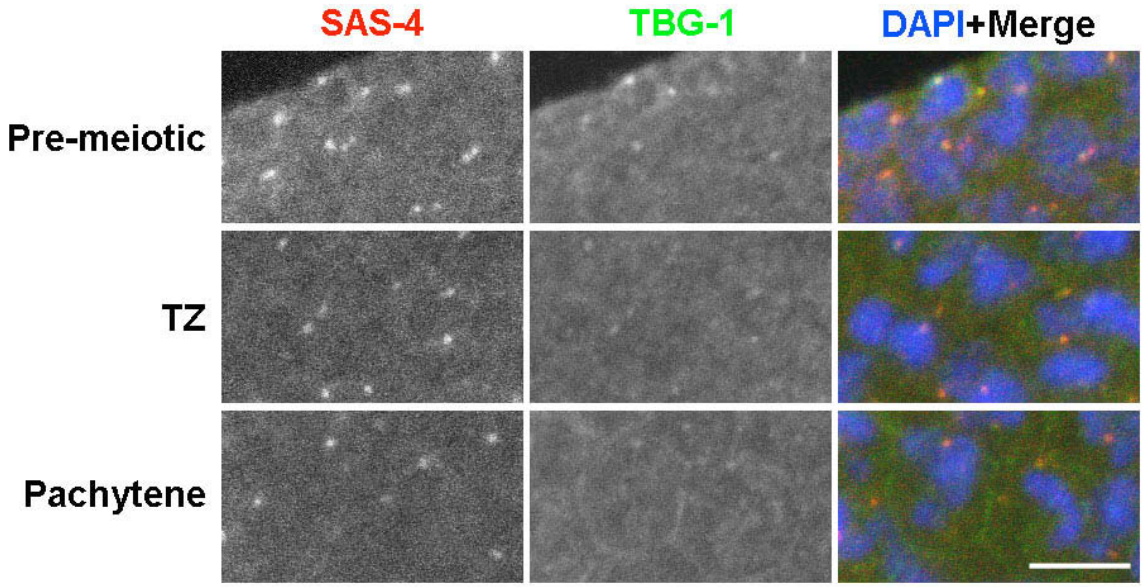

SAS-4
TBG-1
DAPI+Merge
Pre-meiotic
TZ
Pachytene

Figure 4.2. γ-tubulin loses its microtubule-nucleating capacity and relocates to cell membranes during meiosis. The N2 hermaphrodite gonad was dissected and stained with anit-α-tubulin (green), anti-γ-tubulin (red) and DAPI (blue). Germ cells at both boundaries of the pre-meiotic tip, transition zone, pachytene, late pachytene and diakinesis were shown. Arrow head, intense γ-tubulin foci that nucleate α-tubulin. Scale bar, 5 μm.

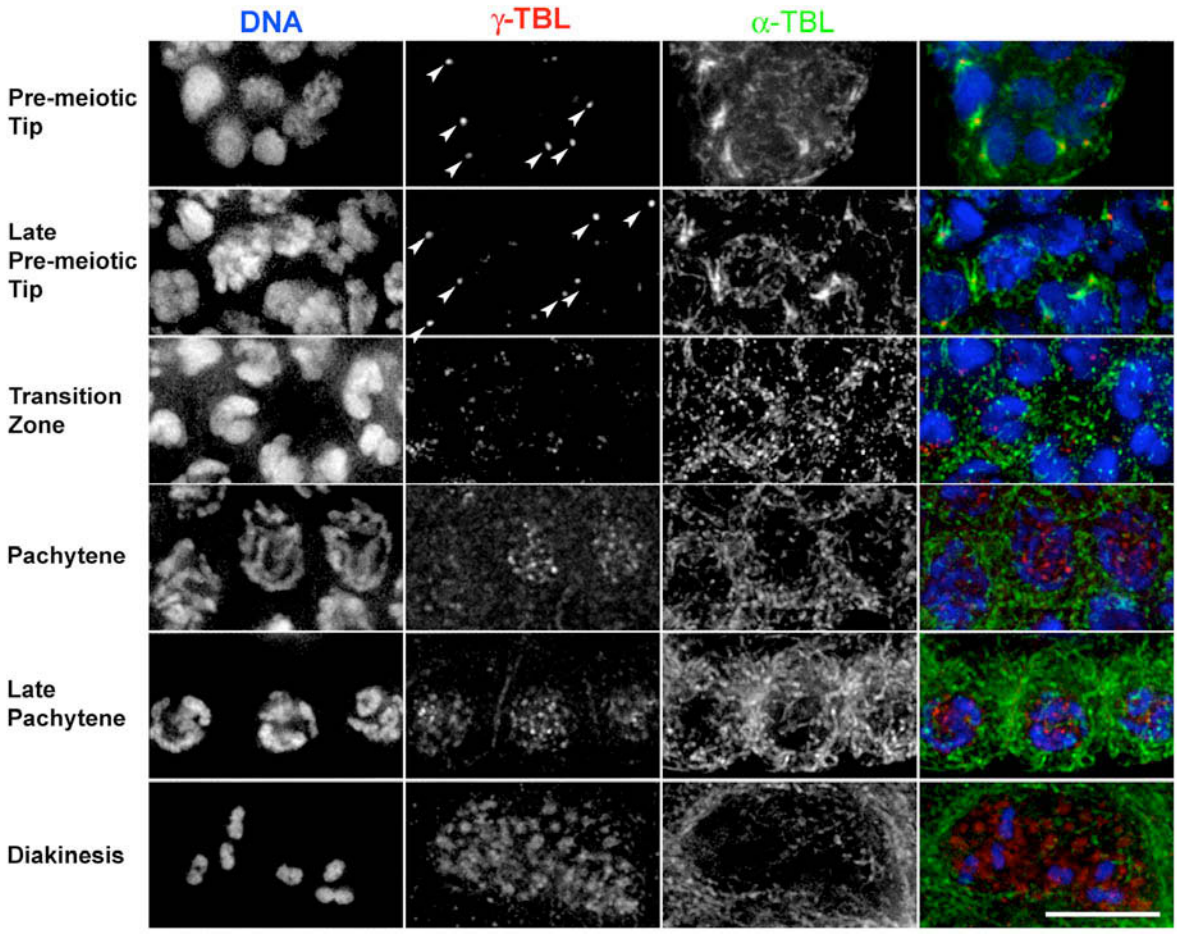
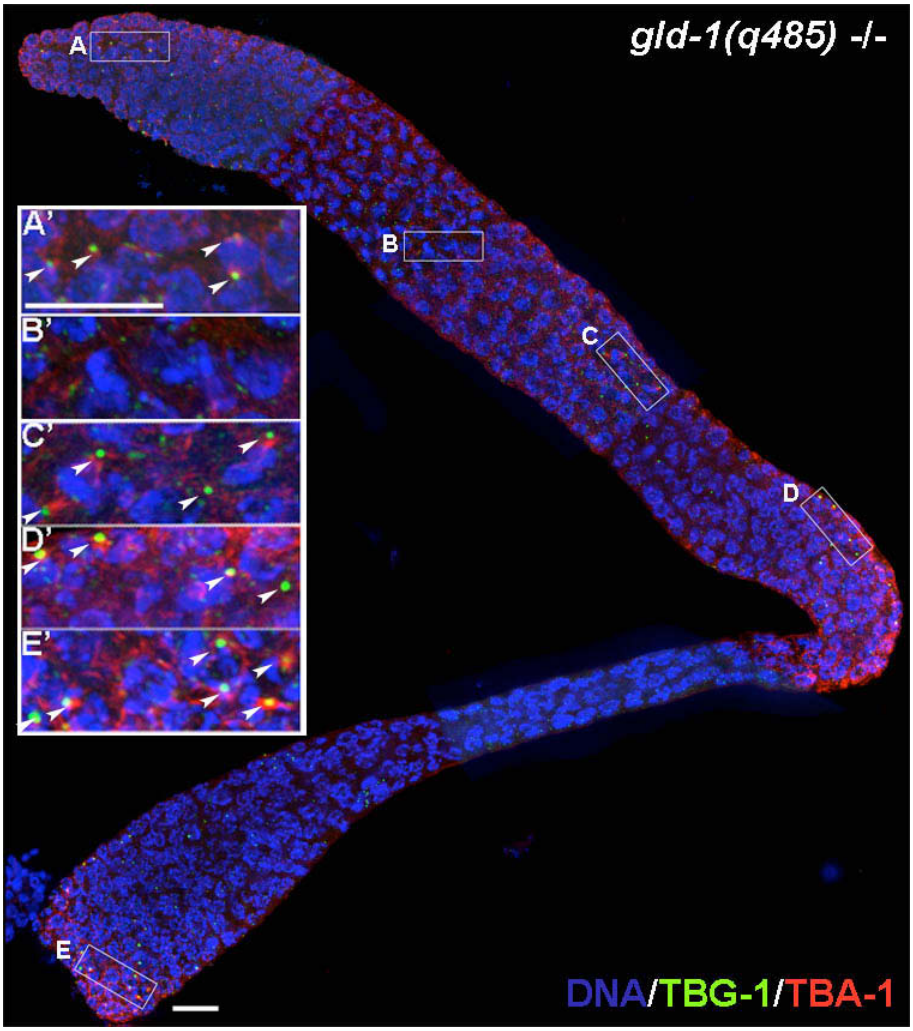

DNA
γ-TBL
α-TBL
Pre-meiotic Tip
Late Pre-meiotic Tip
Transition Zone
Pachytene
Late Pachytene
Diakinesis

Figure 4.3. γ-tubulin recovers its centriolar localization and microtubule-nucleating capacity upon the entry into mitosis in a *gld-1* mutant. The gonad dissected from homozygous *gld-1* (*q485*) hermaphrodite was stained with anti-α-tubulin (green), anti-γ-tubulin (red) and DAPI (blue). The germ cells shown in (**A**) correspond to the most distal pre-meiotic tip, (**B**) transition zone, (**C**) re-entry into mitosis, (**D**) the middle of the germ line and (**E**) the most proximal end of gonad are shown and highlighted in **A'-E'** respectively. **A'-E'** represent 3X zoom for **A-E**. Arrow head, intense γ-tubulin foci that nucleate α-tubulin. Scale bar, 5 μm.


gld-1(q485) -/-
A
B
C
D
E
A'
B'
C'
D'
E'
DNA/TBG-1/TBA-1

CHAPTER V

GENERAL DISCUSSION

The major goal of my research is to reveal how the function and stability of the centrosome are regulated in response to developmental cues in a variety of cell cycle contexts that deviate from the canonical mitotic cell division cycle. I used endocycling cells together with meiotic germ cells in *C. elegans* as a cellular model to analyze centriole/cell cycle coupling, duplication, elimination, and centrosome function by examining the quantity, stability and localization of centriolar or pericentriolar proteins in wild type and in various mutant situations. In parallel, I took advantage of a series of genetic and biochemical approaches in an attempt to understand the mechanisms involved in maintaining appropriate cellular levels of CKI-2, an important negative cell cycle regulator that may be important for centriole elimination during oogenesis. Overall, I believe my work has contributed to our current understanding of several aspects of centrosome biology from a more developmental perspective.

5.1. CENTROSOME UNCOUPLING, FUNCTIONAL CHANGE AND ELIMINATION IN ENDOCYCLING CELLS

As discussed in Chapter II, I showed that centriole duplication becomes uncoupled from S phase and subsequently centrioles are eliminated during the second larval stage in the endocycling intestinal cells of the *C. elegans* larva using SPD-2 as a marker for centrioles due to its role as the most upstream factor of the centriole biogenesis pathway (Delattre et al., 2006; Pelletier et al., 2006).

Electron microscopy (EM) has historically been used as the ultimate method to determine exactly when centriole elimination occurs in *Drosophila* nurse cells and follicle cells, as well as in the *C. elegans* germline (Mahowald et al., 1979; Mikeladze-Dvali et al., 2012). Unfortunately, the difficulty in fixing young larvae and their minute physical size make it very challenging to perform EM analysis (Hall, personal communication). Nevertheless, immunostaining using antibodies against centriole proteins is sufficient to indicate when centriole elimination occurs, as confirmed with EM in *C. elegans* (Mikeladze-Dvali et al., 2012). I therefore based the remaining portion of my analyses on the results obtained with these centriole markers.

Similar to mitosis, the endocycle S phase is also driven by CDK-2 activity, which has been shown to simultaneously drive centriole duplication (Edgar and Orr-Weaver, 2001). My observations suggest that the centriole becomes refractory to CDK-2 activity in endocycling cells. SPD-2 subsequently undergoes a unique transient nuclear localization that precedes its eventual elimination.

I also monitored the dynamics of γ-tubulin, the major microtubule-nucleating factor in the PCM (Stearns et al., 1991). Compared to other cell lineages that undergo conventional mitosis, intestinal cells`γ-tubulin undergoes a rapid dispersal during the last anaphase to becomes undetectable by the time endocycles begin, suggesting that centrosomes may lose their MTOC capacity right after the last nuclear division. Interestingly, recent studies indicate that γ-tubulin interacts with SAS-4 and plays a role in centriole duplication (Dammermann et al., 2008; Gopalakrishnan et al., 2012; Guichard et al, 2010). It is also possible that the quick dispersal of γ-tubulin may contribute to the uncoupling of centriole duplication from the endo S-phase.

5.1.1. Centriole uncoupling and elimination in the intestine is endocycle-dependent

After observing cell cycle uncoupling and the eventual elimination of centrioles, I sought to identify the mechanisms responsible for each of these events. I used a candidate approach, targeting the factors essential for endocycle or normal DNA replication to evaluate their impacts on the duplication and stability of centrioles and centrosomes. *lin-35*, the Rb orthologue in *C. elegans*, promotes the normal initiation of the endocycle program in the intestine and is also involved in the transcriptional regulation of many cell cycle-related genes (Kirenko and Fay, 2006; Ouellet and Roy, 2007). Centrioles undergo extra duplications in *lin-35* mutants, which I have proposed is partially due to misregulated transcription of essential centriole duplicating genes.

Furthermore, I have shown that centrioles do not respond to unscheduled DNA synthesis. Centriole duplication is clearly uncoupled from S-phase CDK activity in *cul-4* mutants, where un-quantized DNA re-replication occurs due to

stabilization of DNA replication licensing factor CDT-1 (Kim and Kipreos, 2007; Zhong et al., 2003). However, the centriole is not eliminated in *cul-4* mutants and γ-tubulin maintains its basic centrosomal level throughout the unscheduled DNA re-replication. This result suggests that simply re-replicating DNA is not sufficient to trigger centriole duplication nor does it result in centriole elimination.

5.1.2. S545, a potential sensor of centriole cycle/cell cycle uncoupling in endocycling cells?

The relationship between the centrosome and various kinases, including CDK, PLK and Aurora A kinase, has been characterized in mitotic cells (Nigg and Raff, 2009; Decker et al., 2011). Therefore, I wanted to address whether these kinases might also play a role in centriole elimination through SPD-2.

Performing bioinformatic analysis and data mining available mass spectrometric data, I identified several Serine or Threonine residues on SPD-2 as likely targets of CDK, PLK or Aurora A kinase. To determine if they indeed play any functional roles, I mutated the residues to both non-phosphorylatable (Serine to Alanine) and phospho-mimetic (Serine to Glutamic acid) variants and subsequently introduced them into a temperature-sensitive *spd-2* (*oj29*) mutant.

At 25°C, each of these variants can rescue the embryonic lethality typical of *spd-2* (*oj29*) animals, suggesting that the individual residues do not affect the essential function of SPD-2. However, the putative CDK target Serine 545 plays a unique role in coupling centriole duplication to DNA replication, as the non-phosphorylatable variant of S545 results in centriole duplication failure during the mitotic cycle in intestine, whereas the phospho-mimetic variant drives extra centriole duplication during L2. These results suggest that S545 may be essential for the coupling/uncoupling of centriole duplication with S-phase onset in endocycling cells.

5.1.3. S357 and ubiquitin-proteasome pathway contributes to the stability of SPD-2

The phospho-mimetic variant of S357 demonstrates an enhanced SPD-2 nuclear localization and the centriole persists beyond its normal period of elimination. The persistence of the SPD-2 S357E variant is likely due to the stabilization of SPD-2, as a similar phenotype is observed if key subunits of the proteasome are reduced by RNAi. Bioinformatic analysis suggests that S357 may be a PLK target, and recent work has shown that SPD-2 interacts with PLK-1 *in vitro* and contributes to the centrosomal localization of PLK-1 *in vivo*. PLK-1 in turn promotes the centrosomal recruitment of other proteins, such as γ-tubulin and SPD-2 (Decter et al., 2011). These data support that a functional association exists between SPD-2 and PLK-1.

5.2. RNF-1 REGULATES THE DEGRADATION OF CKI-2

Chapter III of my thesis showed my current work on understanding the role of RNF-1 as a CKI-2 interacting partner.

Since our antibodies against RNF-1 and CKI-2 were not able to detect the endogenous proteins, I carried out a series of experiments in which CKI-2 is overexpressed with or without RNF-1. The biochemical and genetic results of these experiments suggest that RNF-1 antagonizes CKI-2 through ubiquitin-dependent protein degradation, which is consistent with the typical role of RING-finger motif proteins as E3 ubiquitylation ligases. CKI-2 also interacts with SMO-1, the SUMO othologue in *C. elegans*. Since some RING-finger motif proteins regulate SUMOylation (Jackson, 2001), I tested if RNF-1 could be involved in the SUMOylation of CKI-2. However, my overexpression experiments suggest that RNF-1 is unlikely to be a key regulator of CKI-2 SUMOylation.

The importance of the ubiquitylation pathway in the regulation of CKI-1 in *C. elegans* has been well characterized (Feng et al., 1999; Kipreos et al., 1996; Nakayama and Nakayama, 2003; Wu et al., 2003). Our overexpression data nevertheless suggest that the ubiquitylation pathway is also involved in regulating the level of another CKI family member CKI-2 in *C. elegans*. Recently, a new *cki-2* allele that causes extra cell divisions in the VPC has been characterized (Buck et al., 2009). The availability of this allele will allow us to further verify how these proteins may affect the function of endogenous CKI-2.

Anti-RNF-1 immunostaining revealed that RNF-1 shows localization to, or around the nuclear envelope. Nuclear envelope E3s play critical roles in the nuclear ubiquitylation system. For instance, in yeast, Doa10 functions as a transmembrane ubiquitin ligase and is anchored to the inner nuclear membrane, which allows it to ubiquitylate target substrates in the nucleus (Deng and Hochstrasser, 2006). CKI-2 contains a nuclear localization signal and our recent transgenic line expressing CKI-2::GFP signal indicated that CKI-2 is indeed localized in the nuclus (unpublished data). It would be interesting to further understand if the nuclear envelope localization of RNF-1 is important for its role in controlling CKI-2 levels in the nucleus.

5.3. THE ROLE of γ-tubulin DURING GERM CELL DEVELOPMENT

In Chapter IV of my thesis, I presented my preliminary observations on the dynamic nature of the cellular distribution of the microtuble nucleating factor γ-tubulin during the mitosis-meiosis transition in the germ line of *C. elegans*. Upon initiation of meiosis, γ-tubulin becomes re-localized from the centrosomal foci to gradually accumulate at the germ cell membrane. This re-localization of γ-tubulin is not a result of centriole elimination, which occurs during diplotene, but is likely linked to the onset of meiosis.

Based on my data, this re-distribution is dependent on the initiation of meiosis. By performing a genome-wide RNAi screen we hope to identify genes that regulate or otherwise contribute to this re-distribution. Moreover, if the γ-tubulin re-distribution occurs prematurely or is delayed in certain animals treated with RNAi, observing these animals can help to further understand the biological significance of this re-distribution.

5.4. CONCLUSION

Overall, by following various centriolar or PCM proteins, I have been able to analyze centriole duplication and centrosome function in non-proliferating, yet nevertheless, cycling cells. Moreover, one part of my work was devoted to understanding the post-translational regulation of CKI-2, an important protein for cell division control and presumably for centriole elimination during oogenesis in *C. elegans*.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

As presented in Chapter II, taking advantage of various centriolar and pericentriolar markers, I revealed that during the endocycles in *C. elegans*, centrioles stop responding to S phase activity and lose pericentriolar materials to eventually become eliminated. The uncoupling and elimination represent a developmental adaptation of the centrosome duplication cycle to the unique features typical of this non-mitotic variation of the cell cycle. Subsequently, I carried out a candidate analysis to identify potential factors that might mediate this adaptation. I found that the proper initiation of the endocycle program is important for cell cycle/centrosome cycle uncoupling. Moreover, this uncoupling is independent of unscheduled DNA re-replication, suggesting it is a developmentally-regulated event associated with cells that undergo endocycle. Taking advantage of bioinformatic tools and Mass spectrometric analysis performed by others, I identified two amino acids that play distinct roles in centrosome regulation during the endocycle. S545 acts to couple the cell cycle with centrosome duplciation presumably through CDK-2 activity, whereas S357 may contribute to the stabilization of SPD-2 in a PLK-dependent manner. Moreover, the ubiquitylation-proteasome pathway may also participate in the final elimination of SPD-2 presumably via the proteasome.

In Chapter III, I demonstrated that RNF-1, a RING finger motif protein that interacts with CKI-2, plays an important role in the ubiquitylation of CKI-2, which further triggers its proteasome-mediated degradation. Consistent with this role, the co-expression of RNF-1 with CKI-2 can suppress the embryonic lethality caused by the misexpression of CKI-2, suggesting that RNF-1 may function as a negative regulator of CKI-2. Moreover, immunostaining experiments further indicated the endogenous RNF-1 is localized around nuclear periphery in early embryos, although further investigation is still required to reveal the biological significance of this localization.

As presented in Chapter IV, using centriolar and pericentriolar markers, I established a timeline of γ-tubulin re-localization from centrosome to the germ cell membrane during the various stages of germ cell developments. γ-tubulin is

displaced from the centriole at the onset of meiosis, while centriolar markers remain intact. This dispersal appears to occur in a meiosis-dependent manner; γ-tubulin will resume its centrosomal localization in addition to microtubule nucleating activity if the meiotic cells are forced to re-enter mitosis.