Role of *cki-2* during development in *C. elegans*

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Abstract

Rapid progress has been made toward understanding the significance of CDK inhibitor proteins (CKIs) in the regulation of cell cycle progression. The overall goal of this study has been targeted to further expand our knowledge of CKI function through the investigation of a previously uncharacterized CKI named *cki-2* during development in *C. elegans*. The characterization of *cki-2* using a reverse genetic approach called co-suppression has revealed a novel mechanism that *cki-2* and its related cell cycle regulators are required for the appropriate elimination of centrioles during oogenesis. Loss of *cki-2* in the germ line caused perdurance of centrioles into the one-cell embryo, resulting in supernumerary centrosomes and aberrant cell divisions in the first cell cycle. This was significantly suppressed by reduction of cyclin E and a Cdk2 homologue, indicating that these cell cycle regulators are involved in this critical developmental process. In order to further understand the function of *cki-2*, a yeast two-hybrid screen was conducted which allowed us to identify three CKI-2 interacting proteins: orthologues of PCNA (PCN-1), SUMO (SMO-1), and a RING finger protein called RNF-1. CKI-2 has functionally separable domains in its amino (Cyclin/Cdk binding)- and carboxy (PCNA binding)-terminus and they exert distinct roles in cell cycle progression. It was observed that CKI-2 is covalently modified by SUMO on its N-terminus and this causes CKI-2 to relocalize to thr nucleolus, which is associated with its rapid degradation. Since many RING finger proteins act as components of the multi-subunit E3 ubquitin ligases, we speculated that RNF-1 might be involved in the CKI-2 degradation. This possibility was tested by co-expression of RNF-1 with CKI-2, revealing that co-expression of RNF-1 suppresses the embryonic lethality caused by the CKI-2 overexpression and moreover, this is correlated with an increased rate of CKI-2 degradation. In addition, western blot analyses performed on different genetic backgrounds suggested that the CKI-2 degradation occurs in an ubiquitin-dependent manner through the proteasome- mediated proteolysis pathway. Furthermore, a yeast-based assay developed to test a possible role of SUMO in modulating the CKI-2/RNF-1 interaction demonstrated that SUMO may antagonize the

interaction between CKI-2 and RNF-1, these highlighting an intriguing model that appropriate levels of CKI-2 are regulated through ubiquitin-dependent proteolysis mediated by RNF-1, and which may be modulated by SUMO.

Résumé

La compréhension de l'importance des inhibiteurs de CDK (CKI) dans la régulation de la progression du cycle cellulaire a connu un essor rapide. La présente étude a pour but général d'accroître encore notre connaissance de la fonction des CKI à travers l'étude de *cki-2*, un CKI qui n'a pas encore été caractérisé, durant le développement de *C. elegans*. Par le biais d'une approche génétique inverse appelée co-suppression, la caractérisation de *cki-2* a dévoilé un nouveau mécanisme par lequel *cki-2* et d'autres régulateurs du cycle cellulaire sont requis pour l'élimination des centrioles durant l'oogénèse. L'absence de *cki-2* dans la lignée germinale engendre la perduration des centrioles jusqu'au stade embryonnaire d'une cellule, produisant des centrioles surnuméraires et des divisions aberrantes durant le premier cycle cellulaire. Ces phénotypes sont significativement supprimés par une réduction des niveaux de cycline E ou ceux d'un homologue de Cdk2, indiquant que ces régulateurs du cycle cellulaire sont aussi impliqués dans ce processus important du développement. Afin de mieux comprendre le rôle de *cki-2*, un criblage à deux hybrides a été entrepris dans la levure. Celui-ci a révélé l'identité de trois protéines interagissant avec CKI-2 : PCN-1, un orthologue de PCNA, SMO-1, un orthologue de SUMO et une protéine en doigts de RING, appelée RNF-1. CKI-2 possède deux domaines fonctionnels distincts, l'un à son extrémité amino (interaction cycline/Cdk) et l'autre à son extrémité carboxy (interaction PCNA), qui jouent des rôles différents dans la progression du cycle cellulaire. Il a été montré que CKI-2 est modifié covalemment par SUMO à son extrémité amino, ce qui provoque sa relocalisation vers le nucléole et sa dégradation rapide. Étant donné que de nombreuses protéines en doigts de RING sont des composantes des ubiquitines ligases E3, nous pensons que RNF-1 pourrait être impliqué dans la dégradation de CKI-2. Cette possibilité a été testée par une co-expression de RNF-1 et CKI-2. Dans cette situation, l'expression de RNF-1 supprime la létalité embryonnaire causée par la surexpression de CKI-2 et de plus, elle est corrélée à une augmentation de la dégradation de CKI-2. Des analyses Western effectuées à partir de différents backgrounds génétiques suggèrent que la dégradation de CKI-2 se fait d'une manière dépendante de

l'ubiquitine à travers une cascade protéolytique impliquant le protéasome. Finalement, un essai dans la levure, développé pour tester un rôle possible de SUMO dans la modulation de l'interaction CKI-2/RNF-1 a démontré que SUMO serait capable d'antagoniser l'interaction de ces deux facteurs, révélant un modèle intéressant qui indiquerait que les niveaux normaux de CKI-2 sont régulés à travers la voie de protéolyse dépendante de l'ubiquitine par RNF-1 dont la fonction serait modulée par SUMO.

Preface

This thesis is a manuscript-based thesis and has been written by the candidate in collaboration with the candidate's thesis adviser.

This thesis consists of five chapters: Chapter I is a literature review, providing a comprehensive description of the knowledge behind this study, as well as rationale and objectives of the study; the following three chapters (Chapter II-IV) are composed of the manuscripts which have been accepted, or submitted, or will be submitted, where each chapter is presented in the following order: Abstract, Introduction, Results, Discussion (or combined results and discussion for chapter II), Materials and Methods, References, Table(s), Legends to Figures and Figures; Chapter V is a general discussion. Appendix includes supplemental data for the chapters (II-IV) and a published paper with a permission letter from the publisher (The Journal of Cell Biology).

This thesis has been written according to the "Guidelines for thesis preparation" from the Faculty of Graduate Studies and Research (http://www.mcgill.ca/gps/programs/thesis/guidelines/preparation/).

Contributions of Authors

Chapter II. This chapter has been accepted for publication to the Journal of Cell Biology as follows:
Dae Young Kim and Richard Roy. (2006). Cell cycle regulators control centrosome elimination during oogenesis in *C. elegans*.

I performed all of the work in this chapter.

Chapter III. This chapter has been submitted for publication as follows:
Dae Young Kim and Richard Roy. (2006). CKI-2 regulates embryonic cell divisions and is modulated by SUMO-mediated nucleolar localization and subsequent degradation.

I performed all of the work in this chapter except figure 3.3A which was performed by Shaolin Li, a research assistant in the Roy laboratory.

Chapter IV. A modified version of this chapter will be submitted for publication as follows:
Dae Young Kim, Yu Lu, and Richard Roy. (2006). RNF-1, a *Caenorhabditis elegans* RING finger protein, modulates CKI-2 through ubiquitin-dependent proteolytic pathway.

I performed the yeast two-hybrid screen with Simon Demers, a former independent study student in the Roy laboratory, and generated figure 4.1A. I also mapped the RNF-1 binding region on CKI-2 to generate figure 4.1B. Yu Lu, a graduate student in the Roy laboratory, performed western blot analyses to generate figure 4.2A and B and he also generated table 4.1. I performed a yeast-based competition assay and generated figure 4.3.

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List of Abbreviations

AD	Transcriptional activation domain
A/P	anterior/posterior
APC/C	Anaphase-promoting complex/cyclosome
ATP	Adenosine tri-phosphate
cdc	cell division cycle
CDK	Cyclin-dependent kinase
C. elegans	Caenorhabditis elegans
Cip1	Cyclin-dependent kinase-interacting protein 1
CKI	Cyclin-dependent kinase Inhibitor
CKI-2C	CKI-2 C-terminus
cki-2cs	cki-2 co-suppressed
CKI-2N	CKI-2 N-terminus
DAPI	4,6-diamidine-2-phenylindole
DBD	DNA binding domain
DTC	Distal tip cell
GFP	Green fluorescence protein
GST	Glutathione S-transferase
Emb	Embryonic lethality
hs	Heat shock promoter
INK4	Inhibitor of Cdk4
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilo base pair
KDa	kilo dalton
Kip1	Cyclin-dependent kinase-inhibiting protein 1B
MDM2	Mouse double minute 2
MTOC	Microtubule organizing center
PAR	Partitioning defective
PCM	Pericentriolar material
PCNA	Proliferating cell nuclear antigen
Pvl	Protruding vulva
Rb	Retinoblastoma-associated protein
RING	Really interesting new gene
RNAi	RNA-mediated interference
SAS	Spindle assembly
SCF	Skp/Cullin/F-box
SPD	Spindle defective
SUMO	Small ubiquitin-related modifier
UBC	Ubiquitin-conjugating enzyme
VPC	Vulva precursor cell
ZYG	Zygotic defective

Acknowledgments

First of all, I would like to thank my supervisor Richard Roy, who gave me an opportunity to study the wonderful field of Biology in his laboratory and who has never hesitated to spare his time to discuss all the things that I had to deal with during my study. I am so grateful to him for pushing me to attend so many scientific meetings, which have helped to expand my understanding of biology. I would also like to thank Rick for his generous understanding and advice on juggling the responsibilities of raising a family and undergoing my studies.

I would like to thank all of my past and present colleagues in the Roy laboratory. They have made me realize how important it is to be exposed to such fresh minds in life and in study. I would like to thank Shaolin Li for various technical assistances, Jimmy Ouellet for all the computer-related things, Michael Hebeisen for helping me with writing in French and for his sense of humor, Patrick Narbonne for wonderful birthday presents, Yu Lu for providing various biochemical data, Eileen Colella for general help with the laboratory, and Amelie Waldin and Shannon Ryan for help with thesis proofreading. I would like to thank Monique Zetka for her guidance and support during my study and I especially thank Kalun Law and Florence Couteau for their generous help. I am also grateful to both Laura Nilson and Howard Bussey for their supervision as my supervisory committee. I thank the people in the Bussey and the Lasko laboratory for their general help with experiments. I will not easily forget what has been provided to me from other members in the Department of Biology.

I would like to thank the people in the Onnoori Baptist church. I will never forget all the enjoyable memories that I have shared with the pastor Namsung Lee. I would like to thank my family, especially my mother and mother-in-law, for everything that they have provided to me. Lastly, I must appreciate the faithful support of my wife and the priceless smile of my kids. I do not believe that I could achieve all the things without their love.

Chapter I

Literature Review

1.1. Overview

DNA synthesis (S phase) and segregation of the synthesized DNA into daughter cells (Mitosis or M phase) represent two major events typical of most cell division cycles (Figure 1.1). In the past decade, a variety of genetic and biochemical studies in yeast and *Xenopus* have contributed to the expansion of our understanding of the molecular mechanisms implicated in the regulation of DNA replication and mitosis. It is now known that same sets of molecular machinery act in all eukaryotic animals and that their activities are differentially regulated in response to diverse intrinsic or extrinsic signals during development (Hartwell, 1991; Forsburg and Nurse, 1991; Murray and Hunt, 1993; Nurse, 1994; King et al., 1998; Mendenhall and Hodge, 1998; Masui, 2001; Nigg, 2001; Vidwans and Su, 2001).

During the synthesis phase, the replication of chromosomal DNA occurs through the assembly of multiple proteins which form the initiation complex at the origin of DNA replication (Kelly and Brown, 2000). Once this complex is assembled, DNA synthesis is triggered by the catalytic activity of protein kinases called Cyclin-dependent kinases (CDKs), which are also required to avoid the reassembly of the initiation complex during DNA synthesis. This is accomplished through the phosphorylation of key players involved in the assembly of the initiation complex (Jallepalli and Kelly, 1997; DePamphilis et al., 2006). During prophase in mitosis, the two daughter chromosomes begin to condense and attach each other along their length to form sister chromatids which remain firmly attached to each other via proteinaceous structures. In the end of prophase, the nuclear envelope breaks down, removing the nucleus/cytoplasm boundary, while a microtubule organizing center (MTOC), known as a centrosome in animals or as a spindle pole body (SPB) in yeast, begins to nucleate microtubules leading to the formation of the mitotic spindle. Between prometaphase and metaphase of mitosis, the chromosomes attach to the mitotic spindles via specialized regions on the chromosome called kinetochores and align midway between two MTOCs, forming a metaphase plate. Anaphase begins when the two sister chromatids are physically separated through a complex interplay of regulatory molecules and each

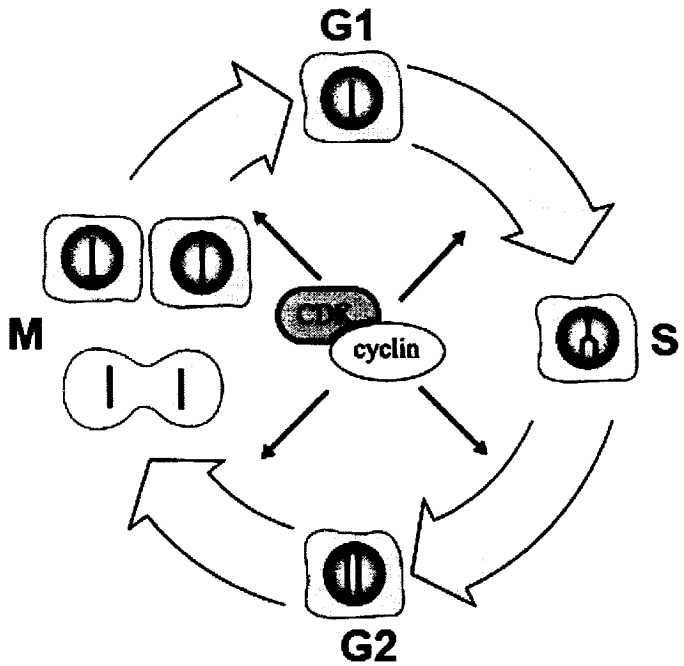

Figure 1.1. **The canonical mitotic cell cycle.** During G1 phase, cells grow by the accumulation of genetic materials and synthesis of organelles. When a cell reaches a certain size and receives an appropriate developmental cue, it is committed to enter DNA synthesis or S-phase during which the chromosomal DNA is duplicated. Between G2 phase and mitosis, the chromosomes separate and segregate into daughter cells, ensuring each daughter cell receives an exact copy of the genome. Cyclin/Cdk2 complexes are central in driving the cell cycle from one phase to another in response to developmental and environmental signals. It has been established that this driving force of the cell cycle is conserved among evolutionarily divergent eukaryotic organisms (from Murray and Hunt, 1993; from http://nobelprize.org).

chromatid moves back toward the MTOC. Following segregation of the complete set of genetic material and an MTOC, daughter cells are created through a physical division called cytokinesis (Murray and Hunt, 1993; Nasmyth et al., 2000). To ensure a perfect partition of the duplicated genome during this division, different stages of the cell cycle must be tightly coordinated to avoid mitotic division prior to the completion of DNA synthesis and the initiation of DNA synthesis prior to the completion of the previous mitotic division. This strict regulation of events ensures that the quantity of the genetic material (ploidy) remains intact and is maintained following each division.

In eukaryotic cells, two gap phases, G1 and G2, occur between DNA synthesis and mitosis (G1 before S phase and G2 before M phase). These serve to coordinate S phase and M phase by transducing the internal and external signals provided by growth factors and other signaling molecules which emanate from the cellular environment. During G1 phase, the cell prepares for S phase by accumulating cellular mass (proteins and RNAs) and synthesizing organelles that will be eventually partitioned between the two daughter cells. Developmental signals play a critical role in timing entrance into S phase (the G1/S transition), which determines whether the cell will progress to mitosis or arrest division. After this transistion, most cells are no longer responsive to extracellular signals and will be thereafter committed to completing mitotic division. This important cell cycle boundary is referred to as START in yeast or the restriction point in vertebrates (Murray and Hunt, 1993; Sherr, 1994; Pardee, 1989).

Initially, START was defined as the position in the cell cycle at which cell cycle events such as budding, DNA synthesis, and duplication of SPB became irresponsive to the loss of Cdc28, the CDK in buding yeast (Mendenhall and Hodge, 1998). Cdc28 has been identified from a genetic screen performed in budding yeast (Hartwell et al., 1974). Since cell division is essential, the first cell cycle mutants isolated were conditional mutants, which can grow at permissive temperatures (23°C) but not at non-permissive (or restrictive) temperatures (36°C). The cell division-specific temperature-sensitive mutants (or cell division cycle (*cdc*) mutants) were distinguished from other temperature-sensitive

mutants because such *cdc* mutants arrest at a specific position in the cell cycle, and as such all share the same morphology, suggesting that the mutated gene product is required to transit through a specific point in the cell cycle. Among the *cdc* mutants, the $cdc28^{ts}$ mutant showed a dramatic phenotype: no budding, no DNA synthesis, and no duplication of spindle pole body (SPB). This indicated that Cdc28 was a key determinant in control of these stages and was thus critical for cell cycle progression. In fission yeast, genetic screens have been performed in a similar manner as with budding yeast, and were pivotal in the identification and characterization of Cdc2 as a key molecule in mitotic entry (Nurse and Thuriaux, 1980). Genetic studies have revealed that *cdc2* encodes a protein kinase and that $cdc28^{+}$ (wild type of *cdc28*) genetically complements the $cdc2^{ts}$ mutant in fission yeast (Beach et al., 1982; Simanis and Nurse, 1986). Subsequent studies have shown that both genes are highly similar in their protein sequences (63% identity) and play a critical role in both START and in the onset of mitosis in both budding and fission yeasts. Furthermore, it has now been established that all eukaryotic organisms have a functional equivalent of *cdc2/cdc28*, indicating that the mechanisms that drive the cell cycle engine are strongly conserved among evolutionarily divergent organisms (Morgan, 1997).

Physical and biochemical studies have revealed that the monomeric form of the CDK is catalytically inactive and that it is only through cyclin binding that the enzyme acquires its catalytic activity. The different cell cycle stages are driven by various combinations of cyclins/CDKs. Therefore, the activity of the cellular CDK in cell cycles is a key point of regulation and is thus subject to a series of both positive and negative influences, which respond to signals that promote proliferation or, alternatively, quiescence (Morgan, 1995; Morgan, 1997).

These CDK regulators act through several mechanisms: 1) protein-protein interaction (association or dissociation with Cyclins and CDK-inhibitory molecules (CKIs)) (Morgan, 1995; Sherr and Roberts, 1999), 2) transcriptional control (periodic fluctuation in the level of Cyclins in different phases of cell cycle) (Koch and Nasmyth, 1994; Nasmyth, 1996), 3) post-translational modification (activating or inhibitory

phosphorylation by CAK (CDK-Activating Kinase) (Kaldis, 1996; Espinoza et al., 1996), or Wee-1 family kinases(Fattaey and Booher, 1997; Lee and Yang, 2001; Kellogg, 2003); removal of inhibitory or activating phosphorylation by Cdc25 phosphatase (Nilsson and Hoffmann, 2000) or KAP (CDK-associated protein phosphatase) (Poon and Hunter, 1995)), 4) spatial control (localization of cell cycle regulators (Wee1, Cdc25, and CKI) to different subcellular compartments) (Pines, 1999), and 5) protein degradation (ubiquitin-dependent proteolytic degradation of Cyclins and CKIs) (Cardozo and Pagano, 2004).

Although these mechanisms implicated in the regulation of CDK activity are largely mediated in a temporal manner, achieved through the timely control of synthesis, modification, or degradation of cell cycle regulators, a growing body of studies has indicated that this temporal control in cell cycle must be coordinated with the spatial localization of the cell cycle regulators to ensure proper progression of cell cycles (Pines, 1999). In mammalian cells, this spatial control allows different members of cell cycle regulators arising from multigene families to adopt non-redundant or non-overlapping functions at the same cell cycle stage: cyclin B1 shuttles between nucleus and cytoplasm and, during mitosis, it translocates to the nucleus, while cyclin B2 is present in the ER and Golgi (Jackman et al., 1995); Wee1 kinase is nuclear (McGowan and Russell, 1993), whereas Myt1, a related Wee1 kinase, is localized to the ER and Golgi (Liu et al., 1997); Cdc25B is mostly cytoplasmic (Gabrielli et al., 1996), but Cdc25C accumulates in the nucleus (Girard et al., 1992).

These differential localizations of cell cycle regulators are acquired through several mechanisms: 1) intrinsic signals involved in protein sorting into organelles, such as nuclear import or export signals (Reynisdottir and Massague, 1997); 2) protein/protein interactions, such as Jab1 mediating the cytoplasmic translocation of p27Kip1 (a mammalian CKI) (Tomoda et al., 1999); 3) post-translational modifications, such as the cytoplasmic localization of NEMO through its SUMO (small ubiquitin-related modifier)-dependent modification (Melchior, 2000; Huang et al., 2003); 4) anchoring proteins, such as the membrane tethering of Smad2 (a transcriptional activator of TGF-β transduction pathway)

through its association with SARA (Smad anchor for receptor activation) in a TGF-β-dependent manner (Tsukazaki et al., 1998).

A considerable body of knowledge towards understanding cell cycle regulation has been obtained through genetic studies in yeast, however, given the importance of understanding the cell cycle progression in a developmental context where cells respond to diverse intrinsic and extrinsic developmental signals to mediate cell cycle-related events such as cell proliferation, cell growth, and cell differentiation, many recent studies have instead focused on understanding how cell cycle control is achieved in multicellular organisms.

In most multicellular organisms, cell proliferation must occur during periods of development where tissue generation is critical such as during embryogenesis or during organogenesis (Edgar and Lehner, 1996; Edgar et al., 2001; Vidwans and Su, 2001). However, it must also be arrested in a very timely manner prior to terminal differentiation. In the absence of appropriate controls, uncontrolled cell proliferation can lead to developmental abnormalities or to diseases such as cancer. CKIs are upregulated during the initiation of differentiation in most tissues when cell division must be arrested in conjunction with the onset of specific gene expression. CKI misregulation has been observed in many types of transformed cells, suggesting that CKIs may be critical to appropriately arrest cell division at this critical stage. CKIs arrest cell division mainly at G1/S, where cell cycle effectors including CKIs respond to growth promoting or impeding signals from the environment and from the developmental program. As such, the activity of CKIs must be tightly regulated in accordance with numerous inputs to coordinate cell growth and proliferation with cell differentiation (Lehner and Lane, 1997; Hong et al., 1998; Sherr and Roberts, 1999; Lehner et al., 2001; Lee and Yang, 2001; Raff et al., 2001).

Caenorhabditis elegans (*C. elegans*) has been useful to study various aspects of cell division in a developmental context, mostly due to its fully documented invariant pattern of cell division from one-cell zygote to the adult animal. This provides the basis for

the use of this powerful genetic system to identify cell division-defective mutants in a forward or reverse genetic manner. Moreover, its transparent body facilitates the use of fluorescence markers such as GFP (green fluorescence protein), which allow for more sensitive and quantitative genetic screens. To date most cell cycle regulators identified in *C. elegans* are highly conserved and play similar roles to their mammalian counterparts (Lambie, 2002; Fay, 2005; Kipreos, 2005).

The focus of this literature review is to describe the role of CDK inhibitors (CKIs) in the developmental regulation of cell cycle progression. The first part will focus on the mechanism of CKI-mediated inhibition of CDKs, largely based on knowledge obtained from the crystal structures of CDKs associated with various CKIs. The second part will concentrate more on how post-translational levels of CKIs are regulated by the proteolytic degradation pathway, which is highly conserved among diverse organisms. The third part will review the developmental control of cell proliferation by CKIs and the relationship between CKIs and cancer, followed by a review of some emerging roles of CKIs acting in transcription, cell migration, DNA replication, and centrosome biology. Finally, I will describe the *C. elegans* system focusing on early embryonic cell divisions where my research interest resides.

1.2. Mechanistic Basis of Inhibitory Activity of CKI

1.2.1. Overview

Based on sequence comparisons and protein structures obtained from CKI/CDK complexes, CDK inhibitors are largely divided into two families in mammalian cells: the INK4 (Inhibitors of CDK4) family and the CIP/KIP family. INK4 family CKIs specifically inhibit the catalytic subunits of CDK4 and CDK6, in which four INK4 proteins are present in mammalian cells: INK4a (p16), INK4b (p15), INK4c (p18), and INK4d (p19). This family of proteins does not interact with other CDK proteins or with D-type cyclins. On the other hand, the CIP/KIP family CKIs associate with a broader range of CDKs, which include cyclin D-, cyclin E-, and cyclin A-dependent CDKs. CIP/KIP CKIs also inhibit the activating phosphorylation of cyclin/CDK complex by CAK (CDK-activating kinase) (Pavletich, 1999; Sherr and Roberts, 1999).

In mammalian cells, it has been shown that there are three CIP/KIP family CKI proteins: p21Cip1, p27Kip1, and p57Kip2 (Sherr and Roberts, 1999). Typically, these CKIs share a conserved cyclin/CDK inhibitory domain at the amino terminus (N-terminus), while their carboxyl terminal (C-terminus) regions are divergent. It is now established that these CIP/KIP family CKIs are conserved among diverse animals and plants: in *Drosophila* (*D. melanogaster*), a single CIP/KIP family member named Dacapo (Lane et al., 1996); in *C. elegans*, two CIP/KIP CKIs called CKI-1 and CKI-2(Hong et al., 1998; Fukuyama et al., 2003); in *Xenopus* (*X. laevis*), four CIP/KIP family CKIs, p27Xic1(Su et al., 1995), p28Kix1(Shou and Dunphy, 1996), p16Xic2(Daniels et al., 2004), and p17Xic3 (Daniels et al., 2004); in *Arabidopsis* (*A. thaliana*), two CDK inhibitor proteins, ICK1/KRP1(Wang et al., 1998) and ICK2/KRP2(Lui et al., 2000). Although they have adopted differential roles in their developmental process during the passage of evolution, they all share the typical function of CIP/KIP family CKIs of inhibition of the catalytic function of CDK through their association with cyclin/Cdk complexes which act at G1/S (cyclin E/Cdk2), S-phase (cyclin A/Cdk2), and G2/M (cyclin B/Cdk1). In addition, there are non-canonical CKIs, which share little similarity in their primary sequnce but

inactivate the catalytic function of CDKs, in budding (p40Sic1) (Schwob et al., 1994) and fission yeast (Rum1) (Labib and Moreno, 1996), as well as in *Drosophila* (Roughex) (Foley et al., 1999; Foley and Sprenger, 2001). Unlike the CIP/KIP family CKIs, these CKIs do not inhibit the G1/S-Cdk function. Instead, they block the Cdk activity acting at S-phase and mitosis. It has been known that they are present in high concentrations during G1, which blocks the S- or M-phase Cdk activity, thereby preventing S-phase entry or mitosis from occurring during the period. In late G1, the CKIs are targeted by a phosphorylation-dependent proteolysis, allowing the S-phase entry and subsequent mitosis (Pagano, 1997). However, unlike Sic1, Far1 (a CKI in budding yeast) shows a distinct function. Far1 induces G1 arrest through the inactivation of the G1/S-Cdk activity in response to a mating pheromone (Valtz et al., 1995), which is reminiscent of p21Cip1, which induces G1 arrest in response to DNA damage (Harper and Elledge, 1996).

Intriguingly, the INK4 family CKIs inactivate the G1-Cdks (Cdk4 and 6) through their association with the monomeric form of Cdk4 or Cdk6 thereby blocking their association with cyclin D. The CIP/KIP CKIs are known to associate with the G1-Cdk complexes without blocking their activity, or in a manner to promote the assembly (Zhang et al., 1994; Blain et al., 1997; LaBaer et al., 1997). In proliferating cells, p27Kip1 is sequestered by cyclin D/Cdk4 complex in the cytoplasm, resulting in decreased levels of nuclear p27Kip1 thereby allowing S-phase progression (Toyoshima and Hunter, 1994). However, when the cells are exposed to TGF-β, INK4b (p15) which is transcriptionally induced by the TGF-β transduction pathway binds to the cyclin D/Cdk4 complexes thereby forcing p27Kip1 to associate with cyclin E/Cdk2 in the nucleus resulting in cellular arrest at G1. It has been shown that this coordination of INK4b (p15) and p27Kip1 is mediated by a differential localization of the two CKIs: INK4b (p15) is mostly cytoplasmic, while p27Kip1 is localized in the nucleus. When both INK4b (p15) and p27Kip1 are forced to be expressed in the same compartment, either cytoplasmic or nuclear, INK4b (p15) cannot displace p27Kip1, suggesting that the coordination of INK4b (p15) with p27Kip1 occurs through their spatial localizations (Reynisdottir and Massague, 1997). These studies indicate that two different types of CKIs are coordinated to exert their role in response to

developmental signals, which ensures the timely control of cell proliferation which is eventually coupled with timing of cell differentiation. There is little conservation within the INK4 family of CKIs among evolutionarily divergent animals and plants. This suggests that the role of the INK4 family CKIs has been specialized for mammalian animals.

1.2.2. Structural aspects of CDK regulatory mechanisms

To understand how CKIs inhibit the activity of CDKs, the crystal structures of the CDK and cyclin/CDK complexes (monomeric CDK2 (De Bondt et al., 1993), cyclin A/CDK2 (Jeffrey et al., 1995), phosphorylated cyclin A/CDK2 (Russo et al., 1996(a)), and phosphorylated cyclin A/CDK2-p27Kip1(Russo et al., 1996(b)), were determined, all of which have advanced our understanding of the mechanistic basis of how CKIs regulate CDK activities (Figure 1.2).

Monomeric CDK contains three major structural domains: an N-terminal lobe rich in β-sheets; a C-terminal lobe rich in α-helices; and the ATP binding and catalytic pocket present between the two domains. The C-terminal α-helical region possesses a characteristic signature sequence (PSTAIRE; PLSTIRE in CDK6) only present in the CDK family proteins, which performs a key role in cyclin/CDK contact and acts as a secondary regulatory element in addition to the activating phoshorylation site present at the T loop (De Bondt et al., 1993; Pavletich, 1999).

When cyclin A binds the PSTAIRE helix, this contact induces a conformational change in CDK2, moving the PSTAIRE helix into the catalytic cleft and thereby causing a 90° rotation of the helix. This results in the relocation of a glutamic residue (Glu51), which is normally located outside the catalytic site. The movement of this key amino acid residue into the catalytic site results in the formation of a catalytic triad (Glu51, Asp145, Lys33) and renders the protein capable of catalysis. Cyclin binding also causes a conformational change in the T loop. In absence of cyclin A binding, the T loop is located in front of the catalytic pocket, impeding the entry of protein substrates into the ATP-bound catalytic pocket. Once cyclin A binds, however, the T loop moves away from the catalytic cleft,

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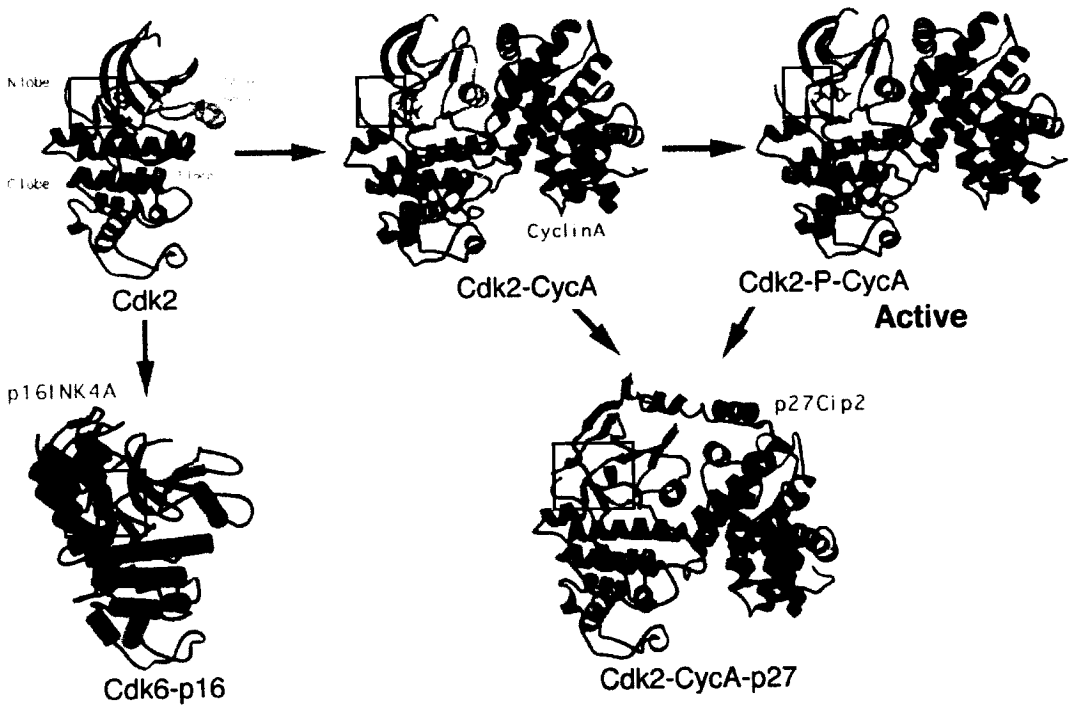
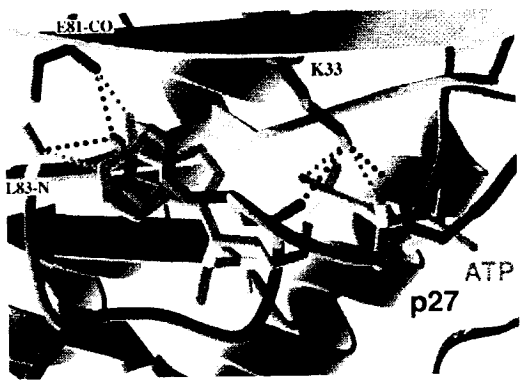

N lobe
C lobe
Cdk2
CyclinA
Cdk2-CycA
Cdk2-P-CycA
Active
p16INK4A
p27Cip2
Cdk6-p16
Cdk2-CycA-p27

B


E81-CO
K33
L83-N
ATP
p27

C

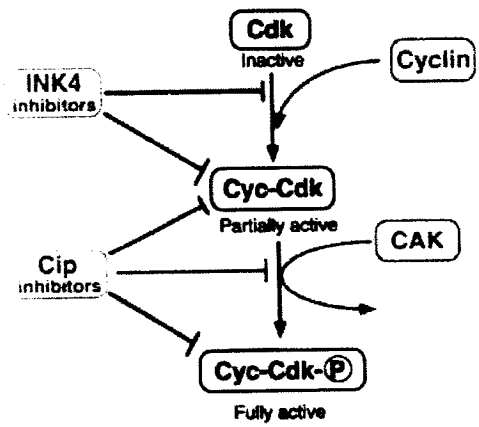

Cdk
Inactive
Cyclin
INK4
inhibitors
Cyc-Cdk
Partially active
CAK
Cip
inhibitors
Cyc-Cdk-P
Fully active

Figure 1.2. **The catalytic activities of Cdks are regulated by multiple mechanisms.** (A) Monomeric Cdk (Cdk2) is partially activated through its assoctiation with cyclin (Cdk2-CycA). This occurs through conformational changes of Cdk2 induced by the cyclin. Cyclin A binding causes the T loop to move away from the catalytic cleft, allowing substrates to access the ATP-bound pocket (marked by open rectangular boxes; ATP is shown in ball-and-stick representation). Cyclin A binding also exposes the activating phosphorylation site (Thr160) on the T loop. Phosphorylation of the CDK at this site on the T loop (Cdk2-P-CycA) causes additional structural changes to fully activate the CDK. p27Kip1 inhibits the catalytic activity of cyclin A/Cdk2 complex through its association with both cyclin A and Cdk2 (cdk2-CycA-p27). (B) A helical element present in p27Kip1 mimics binding of the protein substrate to the catalytic cleft, effectively inhibiting ATP binding. (C) Summary of Cdk regulation through its association with cyclin and CKIs. Cdk is partially or fully activated by cyclin binding (Cyc-Cdk; partially active) and subsequent phosphorylation with CAK (Cyc-Cdk-P; fully active). Whereas INK4 family CKIs associate with both the monomeric form of Cdk and the cyclin/Cdk complex (Cyc-Cdk; partially active form) to inhibit their activity, CIP/KIP family CKIs associate only with the cyclin/Cdk complex (Cyc-Cdk or Cyc-Cdk-P), but not with the monomeric form of Cdk. CIP/KIP family CKIs also block the phosphorylation of Cdk2 by CAK, thereby preventing the complex from becoming fully activated (from Pavletich, 1999).

allowing substrates to access the ATP-bound pocket. Cyclin A binding also exposes the activating phosphorylation site (Thr160). Phosphorylation of the CDK at this site on the T loop causes additional structural changes involved in reorganising the substrate binding site to fully activate the CDK (Jeffrey et al., 1995; Russo et al., 1996(a); Pavletich, 1999).

p27Kip1 inhibits the cyclin A/CDK2 complexes by interacting with both cyclin and CDK. There are two proposed mechanisms for this interaction. In the first, a structural element (3_{10}-helix) present in p27Kip1 mimics binding of the protein substrate to the catalytic cleft. When p27Kip1 binds CDK2, a tyrosine residue (Tyr88) in its 3_{10}-helix interacts with the catalytic cleft of CDK in a similar manner to ATP, effectively inhibiting ATP binding. In the second mechanism, p27Kip1 binding causes the β sheet rich catalytic cleft to be flattened, resulting in a corresponding loss of ATP contacting sites. Thus, the effects of CKI binding to its target are bipartite and highly effective in blocking CDK activity (Russo et al., 1996(b); Pavletich, 1999).

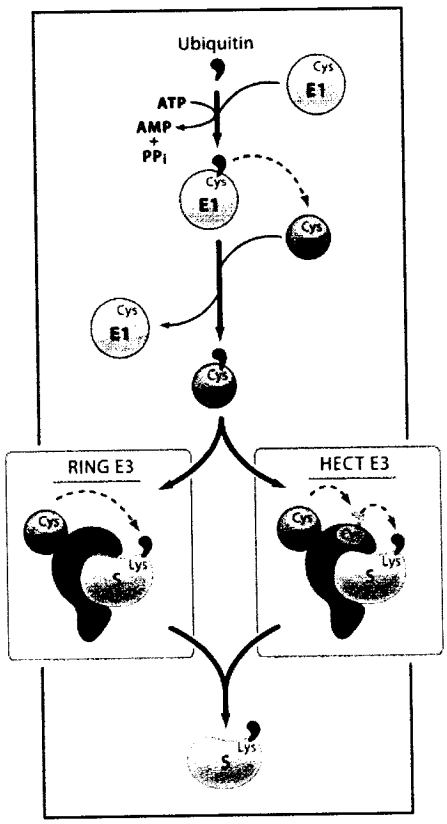
1.3. Regulation of CKIs by the Proteasome-Mediated Degradation

1.3.1. Overview

In eukaryotic cells, the transition from one cell cycle phase to another is irreversibly driven by proteolytic degradation of positive and negative regulatory proteins (cyclins and CDK inhibitors, respectively). This temporal destruction of key cell cycle regulators is mediated by the ubiquitin-proteasome system, a non-lysosomal protein degradation pathway in which target proteins are covalently modified by attachment of ubiquitin chains in an ATP-dependent manner. These ubiquitinated-target proteins are subsequently recognized by the proteasome complex and are thereafter proteolytically digested (Baumeister et al., 1998; Tyers and Jorgensen, 2000; Cardozo and Pagano, 2004).

Ubiquitin, a highly conserved small polypeptide composed of 76 amino acids, is transfered to target proteins by an enzymatic cascade that consists of separable catalytic steps executed by three enzymes (E1, E2, E3) (Figure 1.3) (Hochstrasser, 1996; Hershko and Ciechanover, 1998; Hochstrasser, 2006). Initially, ubiquitin is activated by an ubiquitin-activating enzyme (UBA or E1) whereby a high energy thioester bond between the carboxy end of ubiquitin and a cysteine residue of the E1 enzyme is formed. Secondly, the activated ubiquitin is transfered to a ubquitin-conjugating enzyme (UBC or E2) through the formation of a thioester linkage between the E2 and ubiquitin. Finally, the ubiquitin becomes covalently linked to a lysine residue in the target protein. This step is mediated by an ubiquitin ligase (E3) that specifically recognizes target substrates and recruits the E2 to this target site. There are two classes of E3 ligases: enzymatically active E3 ligases (HECT (Homology to E6AP Carboxy Terminus) domain E3s), which mediate the ubiquitin transfer to target proteins, and RING E3 ligases, which recruit E2 to ubiquitinate the target protein (Joazeiro and Weissman, 2000). The multiprotein E3 ligase complexes involved in cell cycle regulation can be classified into two groups: SCF (Skp/Cullin/F-box) complex and APC/C (anaphase promoting complex/ cyclosome) (Townsley and Ruderman, 1998; Cardozo and Pagano, 2004; Vodermaier, 2004). While the APC/C is required for the metaphase-to-anaphase transition and for mitotic exit, the

A


Ubiquitin
Cys
E1
ATP
AMP
+
PPi
Cys
E1
Cys
Cys
E1
Cys
RING E3
HECT E3
Cys
Lys
Cys
Cys
Lys
Lys

B

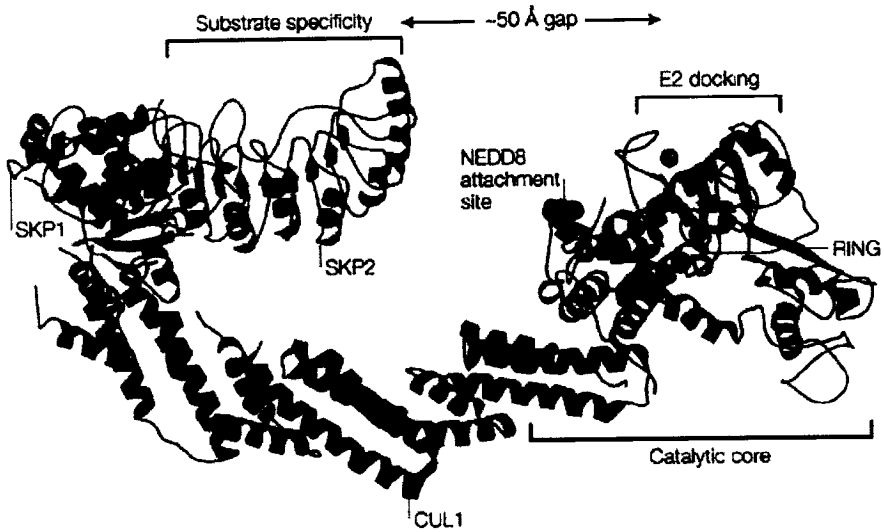

Substrate specificity
~50 Å gap
E2 docking
NEDD8
attachment
site
SKP1
SKP2
RING
Catalytic core
CUL1

Figure 1.3. **An ubiquitination pathway and the crystal structure of the canonical SCF.** (A) ubiquitin is activated by an ubiquitin-activating enzyme (E1) whereby a high energy thioester bond between ubiquitin (red comma-like shape) and a cysteine residue (Cys) of the E1 enzyme is formed. The activated ubiquitin is then transfered to an ubquitin-conjugating enzyme (E2) through a thioester linkage (Cys-Cys) between the E2 and ubiquitin. Finally, the ubiquitin becomes covalently linked to a lysine residue (Lys) in the target protein (S). This step is mediated by an ubiquitin ligase (E3) that recruits the E2 to this target site. Two classes of E3 ligases have been characterized: HECT domain E3 ligases and RING E3 ligases (from Hochstrasser, 2006). (B) SKP2, a F-box protein, acts as substrate receptor to provide the substrate-binding module. While the F-box binds to the adaptor protein SKP1, the E2 ubiquitin-conjugating enzyme is recruited by the RING protein to form the catalytic core of SCF. CUL1, a cullin protein, connect the substrate binding module with the catalytic core through its association with both the SKP1 and the RING protein, thereby physically separating the substrate-binding region from the E2 docking site in SCF, which is estimated to be ~50 Å in distance. Yellow spheres indicate zinc molecules, and the NEDD8 conjugation site on CUL1 protein is marked as red spheres (from Petroski and Deshaies, 2005).

canonical SCF complexes play an important role in determining the onset of S-phase and the mitotic entry.The canonical SCF E3 ubiquitin ligase complexes are composed of Skp1, Cullin, F box protein, and a RING domain protein such as Rbx1/Roc1 (Cardozo and Pagano, 2004). Skp1 mediates substrate binding through the F box protein, which specifically binds the target protein through the WD40 or leucine repeats (in Grr, a different F box protein). *cul-1*, the first cullin gene identified, was isolated in a *C. elegans* genetic screen to identify mutants showing a defect in cell cycle exit (Kipreos et al., 1996). Loss of *cul-1* causes hyperplasia in multiple tissues of larvae and embryos. It is known that CUL-1-based *Drosophila* and mammalian SCF targets G1 cyclins (Petroski and Deshaies, 2005). Moreover, since the loss of *cul-1* rescues the phenotypes associated with low levels of maternal CYE-1 (Fay and Han, 2000), it has been suggested that CUL-1 may be involved in the degradation of cyclins acting at G1 or G1/S. Cullin proteins along with a RING domain protein act to link the E2 to the E3 ligase complex (Petroski and Deshaies, 2005). RING domain proteins constitute a protein family that carries a cysteine-rich fold, which makes up two zinc finger-like structures that can bind E2 enzymes through this novel domain (Zheng et al., 2000; Joazeiro and Weissman, 2000). In higher eukaryotic animals, the RING finger protein Rbx1 associates with different cullin proteins (Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, Cul7). Each cullin protein adopts a different substrate adaptor module, which provides the basis to further classify SCF: in SCF1, SKP1/ CUL1/F-box/Rbx1; in SCF2, Elongin B/C/CUL2/SOCS-box/Rbx1; in SCF3, BTB (broad complex/tramtrack/bric-a-brac)/ CUL3/Rbx1; in SCF4, (unknown adaptor)/CUL4A/ DDB1 (DNA-damage-binding protein1)/Rbx1; in SCF5, Elongin B/C/CUL5/ SOCS-box/ Rbx1; and in SCF7, SKP1/CUL7/F-box/Rbx-1 (Petroski and deshaies, 2005). Both Elongin B/C and the BTB domain are structurally homologous to Skp1. Although these non-Skp1-based SCFs are not well characterized, it has been demonstrated that CUL2 and CUL3 play a role in meiosis in *C. elegans* (Feng et al., 1999; Pintard et al., 2003; Liu et al., 2004). Cullin family proteins are regulated by an ubiquitin-like covalent modifier called NEDD8 (Parry and Estelle, 2004). Neddylation causes the dissociation of CAND1, an inhibitor of SCF, from CUL1, thereby promoting the binding of Skp1 and F box proteins such as Skp2 to CUL1, resulting in the promotion of the assembly of the SCF ligase

complexes. On the other hand, deneddylation occurs through COP9-signallosome complex (CSN) (Bowerman and Kurz, 2006).

The APC/C complex was identified as an E3 ubiquitin ligase involved in the degradation of mitotic cyclins and thereafter is known to have a secondary role in the promotion of the metaphase/anaphase transition through an ubquitination-dependent proteolysis of securine, which is an inhibitory subunit of separase that cleaves the cohesive linkage between the two chromatids at the metaphase/anaphase transition. It has been characterized that APC/Cs are activated by two essential proteins: Cdc20/Fizzy (Fz) protein and Cdh1/Fizzy-related (Fzr) protein (Twonsley and Ruderman, 1998; Vodermaier, 2004). Although both Cdc20 and Cdh1 have conserved WD40 repeats in their protein sequence, it is unclear whether they can act as a substrate adaptor, as is the case of F boxes in SCF complexes. Several proteins implicated in the inhibition of Cdc20 and Cdh1 have been identified, one of which is Emi1(early mitotic inhibitor)/Rca1(regulator of cyclin A) (Reimann et al., , 2001a; Reimann et al., 2001b). It has been shown that Emi1 is involved in the entry into S-phase and mitosis through the inactivation of APC/C in late G1 and at the point of mitotic entry, which results in accumulation of cylin A and mitotic cyclins, respectively. During the metaphase/anaphase transition, Emi1 is targeted by the $SCF^{\beta\text{-}TrCP}$ via Cdk1- and Plk1 (polo-like kinase)-dependent phosphorylation, providing intriguing evidence that the SCF and the APC/C crosstalk to promote mitotic entry (Margottin-Goguet et al., 2003).

1.3.2. Regulation of the cell cycle through degradation of CKIs

In budding yeast, DNA replication is initiated by the S phase Cyclins (Clb5 and 6) in association with Cdc28 (Cdk1). These complexes are inactivated by a CKI called p40Sic1, which is abundant during G1 and inhibits B-type Cylins (Clb1-Clb6). During late G1 phase (the G1/S transition), the G1 cyclin (Cln1 and Cln2)-Cdc28 complexes phosphorylate p40Sic1 and the yeast CKI is subsequently eliminated by ubiquitin-mediated proteolysis, catalyzed by a protein complex (SCF^{Cdc4}) containing Skp1, Cdc4 (a F box protein), Cdc53 (Cul1), and Cdc34 (or Ubc3). This degradation of p40Sic1 allows the S phase cyclins

(Clb5 and 6) to initiate DNA synthesis (Sheaff and Roberts, 1996; Tyers and Jorgensen, 2000). Another CKI, Far1, which inhibits the G1 cyclins (Cln1 and Cln2)-Cdc28 complexes in response to mating pheromones, is also degraded by the SCF^{Cdc4} complex through phosphorylation by the Cln1/2-Cdc28 kinase activity (Henchoz et al. 1997).

In fission yeast, Rum1 CKI prevents active Cdc2/Cdc13 (mitotic cyclin) complexes from forming prior to START, thereby avoiding premature S phase and mitotic entry. The Cdc2/Cig2 complex, which remains inactivated by Rum1 until the minimal size threshold for division is achieved, promotes START and the onset of S phase, afterwhich Cdc2/Cig2 inactivates Rum1 through phosphorylation (Labib and Moreno, 1996). Rum1 is targeted by $SCF^{Pop1/2}$ (Pop1/2 are Cdc4 homologues and make a heterodimer), in which Pcu3 is a Cdc53 homologue (Kominami et al., 1998). The degradation of Rum1 allows the cell to pass through START and progress into S-phase.

This SCF/Cullin-based protein degradation pathway is also conserved in higher animals. In *C. elegans*, the SCF/Cullin-based degradation pathway is also conserved where CKI-1 appears to be eliminated in a CUL-2-dependent manner. In the *cul-2* mutant, germ cells arrest at G1, which correlates with an increased level of CKI-1 (Feng et al., 1999). In *Drosophila*, *Dacapo* is also known to be degraded by a Cullin-based E3 ligase complex. In the absence of CUL4B in *Drosophila*, cells arrest in G1 phase, which correlates with the post-transcriptional accumulation of Dacapo (Higa et al., 2006).

In *Xenopus*, p27Xic1 is degraded in an ubiquitin-dependent manner during the initiation of DNA synthesis, where Cdc34 (E2 enzyme) is required to initiate DNA synthesis through the degradation of p27Xic1. xSkp2, a frog Skp2 homologue, interacts with p27Xic1 and promotes its destabilisation when it is associated with Skp1. The SCFxSkp2-depdendent degradation of p27Xic1 does not need cyclin/Cdk2 kinase activity (Lin et al., 2006).

In mammalian cells, the level of p27Kip1 is high in quiescent cells, and decreases

upon cell cycle entry. This balanced level of p27Kip1 is maintained largely through timely degradation of p27Kip1, which is strictly controlled in response to diverse developmental signals (Firpo et al., 1994; Nourse et al., 1994; Pagano et al., 1995; Bloom and Pagano, 2003). p27Kip1 is phosphorylated (Thr187) by cyclin E/Cdk2 kinase activity and the phosphorylated p27Kip1 is recognized by the SCF^{Skip2} complex (Carrano et al., 1999; Tsvetkov et al., 1999). p27Kip1 is stabilized in $skp2^{-/-}$ mice (Nakayama et al., 2000) and conversely, exogenous Skp2 promotes the degradation of p27Kip1 which induces S phase entry (Sutterluty et al., 1999). In addition, the loss of CUL4 (CUL4A) also causes accumulation of p27Kip1 (Higa et al., 2006). These data indicate that p27Kip1 is targeted by the conserved phosphorylation-dependent, SCF/Cullin-based, ubiquitin-dependent degradation pathway.

The post-translational levels of p21Cip1 are also regulated by proteasome-mediated degradation (Cayrol and Ducommun, 1998). Although it is unclear whether ubiquitin is required for the proteolytic degradation of p21Cip1 (Touitou et al., 2001; Jin et al., 2003), recent evidence suggests that p21Cip1 degradation is also mediated by a conserved SCF^{Skp2}/Cullin-based proteolytic pathway (Yu et al., 1998; Bloom and Pagano, 2004). SCF^{Skp2} plays an important role in the degradation of p21Cip1 specifically during S phase of the cell cycle (Bornstein et al., 2003).

The SCF/Cullin-based ligases are also involved in the degradation of another CIP/KIP family member, p57Kip2. p57Kip2 coimmunoprecipitates with Skp2 and accumulates abnormally in $Skp2^{-/-}$mice. Overexpression of Skp2 promotes the degradation of p57Kip2, while dominant negative Skp2 stabilises p57Kip2, suggesting that SCF^{Skp2} mediates the degradation of p57Kip2. Moreover, cyclin E/Cdk2-dependent phosphorylation of p57Kip2 is also required for the degradation of p57Kip2 (Kamura et al., 2003).

Taken together, these studies indicate that the cullin-based SCF pathway is conserved and involved in the degradation of CKI family members among evolutionarily

divergent animals. Therefore, future investigation will focus on gaining more insight as to how these conserved degradation pathways have evolved to exert their distinct role to regulate CKI functions during development.

1.4. Developmental Control of CKIs and Its Relationship with Cancer

1.4.1. Overview

Cancer is a very complex disease that almost always includes some aspect of misregulated control of cell division. This can occur through hyperactivation/ overexpression of positive regulators such as Cyclins or hypoactivation/ underexpression of negative factors including human tumor suppressors such as p53, Rb or CKIs (Sherr and Roberts, 1999; Lee and Yang, 2001; Bloom and Pagano, 2003; Nakayama and Nakayama, 2006). Since CKIs are capable of responding to diverse developmental signals which impinge on the G1/S transition, considerable study has been dedicated to understanding how misregulation of CKIs can cause uncontrolled cell proliferation, hyperplasia and cancer.

Genetic surveillance mechanisms called checkpoints evolved to ensure that all cellular progeny receive an "error-free" genome (Abraham, 2001). The most common function of checkpoints is to coordinate cell cycle progression with genome integrity (Zhou and Elledge, 2000; Canman, 2001; Nigg, 2001), which is frequently misregulated in cells undergoing tumorigenesis. Intriguingly, and especially true in yeast, most genes implicated in the checkpoints are usually non-essential in a normal condition. They exert their role only under a genetically perturbed condition such as DNA damage. Under genotoxic stress, the ATM/ATR kinases initiate a cascade of checkpoint responses mediated by two protein kinases called CHK1/2 kinases in addition to downstream effector molecules such as p53, MDM2 (mouse double minute 2), and p21Cip1 (Abraham, 2000). p53 is a tumor suppressor protein that acts as a transcription factor to induce a number of target genes in response to genotoxic stress (Levine, 1997). In response to DNA damage, p53 is phosphorylated by ATM/ATR and CHK1/2 kinases leading to its stabilization. In addition to this, MDM2, which normally targets p53 for degradation, is also targeted by ATM/ATR and CHK1/2, thereby further stabilizing p53 and resulting in its increased transcriptional activity. p21Cip1 is a key transcriptional target protein induced by p53 which causes G1 arrest through inhibition of the cyclin E/CDK2 complex (Levine, 1997; Rotman and Shiloh, 1999). The ATM/ATR-p53 pathway is also involved in G2/M

checkpoint, which is mediated by transcriptional induction of downstream effectors including p21Cip1, GADD45 (Growth arrest and DNA-damage-inducible 45), and 14-3-3 sigma proteins. This prevents mitotic entry following DNA damage (Taylor and Stark, 2001).

In mammalian cells, mitogenic growth signals induce the assembly of cyclin D-dependent G1 kinases (cdk4 and cdk6) and the CIP/KIP family CKIs. One of the most influential paradigmes of mammalian cell cycle progression posits that the active cyclin D/Cdk4/6 kinase complex will then trigger Rb phosphorylation, resulting in the dissociation of E2F family transcription factors from Rb. The unbound form E2Fs then transcriptionally activate a number of genes required for S phase entry, including cyclin E and cyclin A. The cyclin E/Cdk2 kinase further phosphorylates Rb while also triggering the phosphorylation of p27Kip1. Following this, p27Kip1 is degraded in an ubiquitin-dependent manner. Therefore, through this positive feedback loop that includes a complex interaction of kinases, CKIs, transcription factors and the proteolytic degradation system, the onset of S-phase entry becomes linked to cellular growth during G1. (Sherr and Roberts, 1999).

1.4.2. Developmental understanding of CKIs: Regulators and developmental signals

Considering their crucial role in coordinating cell proliferation with cell differentiation, which is often compromised during tumorigenesis, it is not surprising that in many cancer cells, CKIs have been the target of genetic alterations that affect their expression through either deletion or mutation.

In many cancer cells, the levels of CKIs are frequently reduced both at the transcriptional or the post-translational level. Moreover, the reduced expression of CKIs is correlated with poor prognosis in patients. Therefore, maintenance of appropriate levels of CKIs appears critical to limit the rate of cancer progression or tumorigenesis. The CIP/KIP CKIs are also transcriptionally silenced by inappropriate methylation in their promoters. In rhabdomyosarcomas (RMSs), the p21Cip1 promoter is inappropriately methylated,

thereby resulting in downregulation of p21Cip1 (Chen et al., 2000). Also, the methylation of the promoter (5'CpG island) of p27Kip1, seen in some malignant melanomas, is an important way to control the transcriptional levels of p27Kip1 (Worm et al., 2000). Moreover, it is found that inappropriate methylation of the promoter region of p57Kip2 represses the mRNA level of p57Kip2 which is an important aspect of genomic imprinting to promote growth of tumors (Shin et al., 2000). These results indicate that transcriptional repression or silencing through inappropriate methylation in promoters provides an important mechanism to downregulate various members of this CKI family in cancer cells, thereby affording a considerable growth advantage.

The transcriptional and post-translational levels of CKIs are tightly controlled under diverse mitogenic stimuli to prevent overproliferation. Since these signals are often targets of known growth factors or oncogene products, it is not surprising that many of these gene products converge on CIP/KIP CKIs to confer growth advantages to transformed cells. p21Cip1 is downregulated by a c-Myc oncogenic signal, while overexpression of c-Myc represses the transcriptional level of p21Cip1 thereby permitting S-phase entry in a c-Myc-dependent manner (Claassen and Hann, 2000). On the other hand, TGF-β upregulates the transcriptional level of p21Cip1, which seems to be mediated by downregulation of c-Myc (Datto et al., 1995a; Datto et al., 1995b). STAT1 (signal transducers and activators of transcription 1) also induces p21Cip1 through binding to the p21Cip1 promoter in response to diverse growth factors and cytokines such as γ-IFN (interferon), thereby mediating growth suppression (Chin et al., 1996). Moreover, it is known that hypermethylation in the p21Cip1 promoter blocks the binding of STAT1 in rhabdomyosarcomas (RMSs), which compromises the STAT/γ-IFN signaling pathway resulting in reduced p21Cip1 expression (Chen et al., 2000). p27Kip1 is also negatively regulated by c-Myc. c-Myc sequesters p27Kip1 through the induction of D-type cyclins (cyclin D and D2), thereby activating cyclin E/CDK2 (Vlach et al., 1996). c-Myc also transcriptionally represses p27Kip1 through binding to the promoter region of p27Kip1(Yang et al., 2001). It is known that p57Kip2 is downregulated by TGF-β through proteolytic degradation (Nishimori et al., 2001).

HER2/neu is a member of the epidermal growth factor receptor (EGFR) family and plays a notable role in the pathogenesis of breast cancer. It is a membrane-bound tyrosine kinase and promotes cell growth (Slamon et al., 1989). HER-2 phosphorylates p21Cip1 (Thr145) through the activation of AKT kinase via PI3K (phosphatidylinositide 3-kinase), which causes p21Cip1 to be relocated to the cytoplasm, resulting in a reduction of function (Zhou et al., 2001). Her2- overexpressing cancers also show downregulated p27Kip1, which suggests a link between HER2 oncogenic signalling and the level of p27Kip1 (Newman et al., 2001; Yang et al., 2000). HER2 downregulates p27Kip1 by affecting its subcellular localization, through relocalization of the p27Kip1/JAB1 (an exporter of p27Kip1) complex from nucleus to cytoplasm via activation of the mitogen-activated protein kinase (MAPK) (Tomoda et al., 1999; Lee and Yang, 2001), thereby proteolytically degrading p27Kip1 in an ubiquitin-dependent manner.

PTEN (phosphatase and tensin homolog) is a tumor suppressor which antagonizes the AKT kinase pathway by removing the 3' phosphate group of phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Simpson and Parsons, 2001). PTEN is one of the most frequently lost tumor suppressor genes in human cancers (Lee and Yang, 2001). PTEN mediates cell cycle arrest by regulating p27Kip1 protein stability through its effect of reducing Skp2, an important SCF component (F-box) required for p27Kip1 degradation (Mamillapalli et al., 2001). Depletion of p27Kip1 by antisense oligonucleotides suppresses PTEN-induced cell cycle arrest, demonstrating that p27Kip1 is a downstream regulator of the PTEN pathway.

VHL (Von Hippel-Lindau) protein is a tumor suppressor which acts as an E3 ubiquitin ligase. Loss of VHL protein is correlated with many cancers and causes upregulation of angiogenic factors which result in uncontrolled blood vessel growth required for tumor development (Ivan and Kaelin, 2001). VHL negatively regulates cell cycle progression by mediating the upregulation of p27Kip1. A study using a *vhl*-deficient cell line shows that the accumulation of p27Kip1 requires VHL protein in serum-free conditions, suggesting that p27Kip1 acts downstream of the VHL pathway. It is unclear,

however, how VHL causes upregulation of p27Kip1 (Pause et al., 1998; Kim et al., 1998).

IGF (Insulin-like growth factor)-II has an important role during embryonic development, where misregulation of IGF-II correlates with the BWS (Beckwith-Wiedemann syndrome) and different tumors. Exogenous IGF-II in embryonic fibroblasts or a high level of IGF-II in serum causes reduced expression of p57Kip2, suggesting that p57Kip2 and IGF-II may play antagonistic roles (Caspary et al., 1999; Grandjean et al., 2000).

Mice lines lacking the CKIs have been generated to test the knowledge that has been accumulated using transformed cell line systems and have greatly enhanced our understanding of the role of CKIs in a developmental context. p21Cip1-deficient mice ($p21^{-/-}$) develop normally although they show an impaired response to DNA damage (Deng et al., 1995). Moreover, unlike p53-deficient animals ($p53^{-/-}$) (Elson et al., 1995), $p21^{-/-}$ mice show no effect on tumor formation. This observation indicates that the role of p21Cip1 in the G1 control might be genetically redundant and moreover, p21-independent p53 functions such as programmed cell death might be more significant during tumorigenesis than the G1/S checkpoint.

Mice lacking p27Kip1 activity ($p27^{-/-}$) are viable and show dosage-dependent enlargement of organismal size with higher number of cells in most organs without showing apparent morphological defects. Unlike p21Cip1 which has an unclear role in tumorigenesis, p27Kip1 is clearly downregulated in a transcriptional and/or post-transcriptional manner in many cancer cells. The $p27^{-/-}$ mice are susceptible to carcinogenesis, suggesting that the level of p27Kip1 must be tightly maintained to prevent cellular transformation (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Although there has been no evidence that p27Kip1 is transcriptionally induced by p53, a recent study shows that p27Kip1 transcriptional and/or post-transcriptional levels are downregulated in tumors of $p53^{-/-}$ mice and that genetic alterations of p27Kip1 in $p53^{-/-}$ mice, for example chromosomal deletions, enhance tumor development. These results

suggest that p27Kip1 cooperates with p53 in tumor suppression, although the basis of this cooperation is currently unclear (Philipp-Staheli et al., 2004). p57Kip2 null mice ($p57^{-/-}$) show abnormal overgrowth and differentiation, which correlates with the BWS, a childhood overgrowth syndrome (Yan et al., 1997; Zhang et al., 1997).

Taken together, diverse signal transduction pathways and their downstream mediators are required to maintain the appropriate transcriptional or post-transcriptional levels of CKIs. Since reduced levels of CKIs are frequently observed in many cancer cells, it appears critical that the levels of these CKIs be tightly controlled and very responsive to diverse growth stimuli.

1.5. Developmental Control of Cell Proliferation by CKIs

During growth periods in multicellular organisms, cells proliferate until the fields in which they occur reach an appropriate size, after which, a cue signals the onset of terminal differentiation and instructs the cell to stop cycling. It is at this stage, where the CKIs seem to play a key role. Upregulation of the levels of CKIs is accompanied by initiation of differentiation in a broad range of tissues, therefore CKIs must be controlled in response to extrinsic developmental signals as well as cell autonomous cues. Otherwise, the uncoordinated regulation of cell proliferation and differentiation would ultimately result in developmental catastrophe or could potentially give rise to hyperplasia and/or tumorigenesis. Therefore, it is pivotal to better understand how these developmental cues impinge on the cell cycle machinery to mediate cell cycle regulation in developing multi-cellular organisms (Edgar and Lehner, 2001;Edgar et al., 2001; Raff et al., 2001).

Drosophila and *C. elegans* are representative metazoan model organisms that have the same advantages as yeast in that they are genetically tractable and relatively easy to manipulate. They have been useful in pioneering forays into understanding cell cycle regulation in a developmental context, mostly due to their amenability to powerful genetic approaches which have allowed the identification of detailed genetic pathways involving cell cycle regulators and their downstream targets and effectors.

1.5.1. Developmental regulation of cell cycle in *Drosophila*

During embryonic development in *Drosophila*, maternally-loaded cell cycle regulators drive rapid syncitial divisions (mitotic cycle 1-13) which are typified by repetitive S/M cycles without any intervening gap phases (G1 or G2 phase) (Edgar, 1995). As such, these divisions are reductional and have no real growth phase. As development proceeds, the stores of maternal regulators are exhausted and/or degraded and begin to be replaced by their zygotic counterparts. During interphase of mitosis 14, syncytial nuclei start to cellularize and the rapid syncytial divisions are arrested through inactivation of Cdk1. These early rapid divisions cause depletion of maternal S-phase regulators and result in an

increase in unreplicated DNA during the later divisions, thereby triggering the DNA replication checkpoint. The *Drosophila* DNA replication checkpoint response requires the activities of *mei-41* (ATM/MEC1/Rad3 homologue) and *grapes* (CHK1 homologue), which together inactivate Cdk1, which is then further inactivated through degradation of maternal Cdc25 phosphatases ($Cdc25^{String}$ and $Cdc25^{Twine}$) beginning after mitosis 13 (Sibon et al., 1997; Sibon et al., 1999; Edgar and Datar, 1996). The degradation of these phosphatases (String and Twine) mediates the maternal-to-zygotic transition (MZT) after which, the majority of mitotic divisions in many embryonic tissues, including the epidermis, are regulated at G2/M by the distinctive and pulsed transcription of $Cdc25^{String}$. This transcription is mediated by a complex spatio-temporal interplay of cell type-specific patterning proteins, which bind the upstream cis-regulatory region of $Cdc25^{String}$. This suggests that $Cdc25^{String}$ acts as a patterning sensor to time cell division with transcriptional cues provided by individual cell types (Edgar, 1995; Lehman, et al., 1999).

After embryogenesis, *Drosophila* larvae prepare for metamorphosis during which the total mass of larvae is highly increased (~200 fold). This increase of the mass is due to increased cell growth, which arises from a modified cell cycle known as an endocycle in endoreduplicative tissues (ERTs) including most larval tissues such as the gut, salivary glands, and muscles. During late embryogenesis, the endocycle is dependent on inactivation of Cdk1 caused by the loss of $Cdc25^{String}$ and other mitotic cyclins (cyclin A and B) as well as the cyclic expression of cyclin E (Royzman et al., 1997). Consistantly high levels of cyclin E inhibit the endocycle, suggesting that the periodicity of cyclin E expression is important. This periodiocity seems to be dependent on E2F, a family of transcription factor responsible for the transcriptional activation of cyclin E (Edgar et al., 2001).

The endocycle is regulated in response to nutrients (Britton and Edgar, 1998). Under starvation conditions, DNA replication is not initiated in most ERTs due to reduced expression of cyclin E and E2F. Ectopic cyclin E or E2F in starved larvae induces entry into endocycle, suggesting that these proteins have a key role in triggering the endocycle.

Intriguingly, ectopic expression of cell growth-related genes such as the d-Myc transcription factor (Johnston et al., 1999) and PI3K (Weinkove et al., 1999) can trigger DNA replication in starved larvae, suggesting that cell growth in response to these important factors is linked to nutritional/environmental status and is mediated most likely by key cell cycle regulators (Lehner et al., 2001).

In *Drosophila*, not all the tissues of the larvae undergo endocycles. Embryogenesis generates approximately 1000 imaginal disc cells which proliferate during larval and early pupal development to form most adult structures (wings, antennae, legs, and eyes) (Bryant and Simpson, 1984; Lehner et al., 2001). During mid-embryogenesis, imaginal cells arrest in G1 until they are exposed to nutrients after hatching, suggesting that the imaginal disc cell cycle is also coupled to nutrient status and the environment. During late imaginal development, cyclin E and $Cdc25^{String}$ are limiting factors in the control of the G1/S and the G2/M transitions, respectively. In late imaginal disc cells, G2/M is not coupled to cell growth but, instead, the cis-acting transcriptional enhancers of $Cdc25^{String}$ mediate cell type-specific patterning (Edgar et al., 1994; Edgar et al., 2001). They do this through interaction with numerous patterning genes in response to a wide spectrum of developmental signals that include *decapentalplegic* (*dpp*), *wingless* (*wg*), *Notch*, and *EGFR* (Johnston and Edgar, 1998; Edgar et al., 2001). On the other hand, unlike the G2/M, G1/S in the disc cells is coupled by cell growth-related factors such as d-Myc, PI3K, and Ras (Neufeld et al., 1998). Ectopic growth factors increase the levels of cyclin E in a post-transcriptional manner in which 5'-untranslated region of cyclin E mRNA seems to be playing a key role as a growth sensor to couple cell growth with G1/S progression (Polymenis and Schmidt, 1997; Prober and Edgar, 2000). Intriguingly, cell patterning genes such as *wg* and *dpp* also stimulate cell growth and proliferation (Edgar et al., 2001). As such, in imaginal disc cells, cell patterning signals coordinate cell growth with cell proliferation which leads to appropriate morphology and size.

1.5.2. Developmental role of CKIs in the cell cycle regulation in *Drosophila*

In *Drosophila*, endodermal cells switch to endocycles after a brief pause in G1 (Edgar,

1995). Embyronic epidermal cells, in contrast, arrest in G1 after mitosis 16 and begin to differentiate raising the intriguing question of how the cell cycle knows the precise point at which to arrest. This cell cycle arrest is accompanied by a reduction in the level of cyclin E (Knoblich, 1994) and moreover, ectopic cyclin E causes extra rounds of the mitotic cell division in the embryo and the eye imaginal disc, suggesting that a reduction of cyclin E is important for mitotic exit. However, since some cyclin E is still detectable after the final mitosis, it was suggested that another factor may be required to ensure the mitotic exit.

Genetic screens to identify molecules which are involved in the timely arrest of the embryonic cell cycle have isolated numerous mutations, one of which was a gene named *dacapo* which encodes a CIP/KIP family CKI in *Drosophila* and is essential for embryogenesis. *dacapo* mutant embryos die in the late embryo or early larval stages with several extra cells as a result of their inability to arrest at the appropriate time in response to the switch to larval development. *dacapo* is expressed in various tissues including the embryonic epidermis, postembryonic CNS (central nervous system) and PNS (peripheral nervous system). In these cells, *dacapo* expression is upregulated just prior to the mitotic exit (de Nooji et al., 1996; Lane et al., 1996).

Dacapo protein (DAP) interacts with cyclin E/Cdk2 complexes and inhibits their associated kinase activity *in vitro*. Overexpression of DAP inhibits cell cycle progression in the eye imaginal disc and genetically interacts with G1 regulators such as *Drosophila* Rb (retinoblastoma) homologue (Rbf) and Cyclins. Moreover, premature expression of DAP causes precocious cell cycle arrest in G1 phase, consistent with the role of CKIs in G1 control (Knoblich et al., 1994; de Nooji et al., 1996; Lane et al., 1996).

dacapo is transiently expressed before mitosis 16 during embryogenesis when many cells exit the mitotic cell cycle, differentiate or prepare for larval development. During postembryonic development, in the eye imaginal discs, it was shown that upregulation of DAP is associated with mitotic exit prior to differentiation. High levels of DAP have been observed in the differentiating post-mitotic cells posterior to the

morphogenetic furrow, which traverses the disc in a posterior to anterior manner during development, and in doing so, synchronizes cells at the G1 phase of the cell cycle prior to differentiation (Lane et al., 1996; de Nooji et al., 1996).

dacapo mutants execute an extra round of mitotic division after mitosis 16 during embryogenesis and this also occurs in some postembryonic cells, including the central nervous system (CNS) and the peripheral nervous system (PNS), where an extra mitotic division occurs before mitotic withdrawal (de Nooji et al., 1996). It is unclear how the mutant is eventually capable of exiting the cell cycle after the extra round of mitosis, although successful mitotic exit might require the activity of *Dacapo* in concert with a reduction in the levels of positive G1 regulators such as cyclin E. Although the *dacapo* mutant fails to exit mitosis at the appropriate time, no apparent defects in the embryonic morphology or cell fate determination are observed in *dacapo* mutants, suggesting that the embryonic lethality of the *dacapo* allele may be due to some additional essential role of *dacapo* in the embryo.

Dacapo is also involved in *Drosophila* oogenesis, where it regulates meiotic progression and also distinguishes the oocyte from the developing cells (Hong et al., 2003). During oogenesis, the oocyte develops in the germline cyst that is comprised of 16 cells, of which, only the oocyte executes meiosis and remains in prophase of meiosis I, while the remaining 15 cells (nurse cells) go on to execute endocycles. *dacapo* is differentially expressed in the oocyte and the nurse cells, in which high levels of DAP present in the meiotic oocyte block DNA synthesis and help to maintain meiotic prophase. Interestingly, the level of DAP oscillates in the nurse cells, probably through each of the sequential rounds of DNA replication, in order to allow the nurse cells to become polyploid. In *dacapo* mutants, all cells in the developing egg chambers enter the endocycle, which forces all of the cells including the prospective oocyte to become nurse cells, suggesting that *dacapo* may be directly or indirectly required for the maintenance of oocyte differentiation. Since the sequential rounds of DNA synthesis require cyclic changes in cyclin E activity, DAP may be critical to achieve this through its ability to inhibit cyclin E/Cdk2 activity.

DAP does not work alone during *Drosophila* embryogenesis to regulate cell cycle progression. The *roughex* (*rux*) gene encodes a CKI which specifically associates with mitotic cyclin (cyclin A or B)/Cdk1 complexes, thereby inhibiting Cdk1-dependent mitosis and S phase function (maintenance of G1 state) in the cyclin A/Cdk1-dependent manner (Foley et al., 1999; Foley et al., 2001). Rux does not inhibit the cyclin E/Cdk2 complex, but rather it inhibits the Cdk1 kinase by two different mechanisms: 1) through preventing the activating phosphorylation of Cdk1 by CAK (Thr161); and 2) Rux also inhibits the activated cyclin A/Cdk1 complex, suggesting that Rux can directly inhibit the Cdk1 activity. Overexpression of cyclin E causes downregulation of Rux, suggesting that Rux is controlled post-translationally by cyclin E/Cdk2-mediated phosphorylation during the G1/S transition (Foley et al., 1999).

In budding yeast, mitotic exit is initiated by the inactivation of the mitotic kinase (Cdk1) by degradation of mitotic cyclins, and also by association with p40Sic1. However, in higher eukaryotes, the destruction of mitotic cyclins has been found to be an essential mechanism for the inactivation of Cdk1 activity during mitotic exit. Consistent with this, *rux* mutants show a delayed metaphase-to-anaphase transition. In addition, overexpression of Rux is sufficient to drive mitotic cells arrested at metaphase into interphase, while *rux* mutants show a reduced capacity to overcome the arrest induced by ectopic expression of non-destructible form of cyclin A. These results suggest that Rux is the first CKI that is necessary to inhibit mitotic kinase activities to mediate mitotic exit in a manner comparable to p40Sic1 in *S. cerevisiae*. In fact, although there is no sequence homology between Rux and p40Sic1, p40Sic1 specifically inhibits the *Drosophila* mitotic cyclin/Cdk1 complexes but not the cyclin E/Cdk2 complex (Foley et al., 2001).

1.5.3. Developmental regulation of cell cycle in *C. elegans*

Unlike *Drosophila*, where nuclear divisions occur without cytokinesis during early embryogenesis, in *C. elegans*, the early embryonic divisions occur asymmetrically by cytoplasmic cleavages without any obvious G1 and G2 phase. Cell divisons occur asynchronously and division timing is mostly due to difference in the length of S-phase

(Edgar and McGee, 1988). Cell cycle control by timing S-phase occurs even at the two-cell embryo and perturbation of this division timing causes a defect in the embryonic polarity arising from missegregation of cell fate-determining proteins such as PIE-1 (germline determinant) (Encalada et al., 2000; Brauchle et al., 2003).

Most somatic cells generated during embryogenesis stop dividing and terminally differentiate, while some somatic and germline blast cells continue dividing through cell lineage-specific patterns to form characteristic larval and adult structures during post-embryonic development (Lambie, 2002; Kipreos, 2005). In contrast to the embryonic divisions, most of the post-embryonic divisions that occur during four larval stages (L1 to L4) undergo canonical cell cycles; that is they undergo two gap phases (G1 and G2) that separate the DNA synthesis and mitotic stages of the cell cycle. Unlike during embryonic development, during post-embryonic development, cell division is mostly controlled at the level of G1/S progression, which responds to external developmental signals in addition to cell-intrinsic cues (Ambros, 2001).

Heterochronic pathways control the timing or patterning of the developmental cell cycle during post-embryonic development (Ambros, 2000; Ambros, 2001). Three heterochronic genes play a crucial role in the specification of developmental fates during the first two larval stages (L1 and L2). *lin-4*, which encodes a small RNA (microRNA) and is induced in response to food at hatching and negatively regulates the translation of *lin-14* (encoding a novel nuclear protein) and *lin-28* (encoding a cytoplamic RNA binding protein) mRNAs through its ability to associate with their 3'-untranslated region (UTR) (Ruvkun and Guisto, 1989; Wightman et al., 1991; Lee et al., 1993; Wightman et al., 1993; Euling and Ambros, 1996; Moss et al., 1997). LIN-14 protein is abundant in the early L1 stage and specifies the L1 stage-specific fates. As *lin-4* accumulates during the L1, the level of LIN-14 progressively declines, however, LIN-28 protein persists until the early L2 to promote L2 stage-specific fates. The loss of *lin-14* or *lin-28* causes the precocious onset of vulval cell divisions. Normally, vulva precursor cells (VPCs) are formed in the L1 stage and become quiescent through an extended G1 arrest which continues until the L3 stage

when they begin to divide again (Sulston and Horvitz, 1977; Euling and Ambros, 1996). In *lin-14* or *lin-28* heterochronic mutants, however, the VPCs divide prematurely in mid L2. Genetic studies show that *lin-14* acts through *lin-28* and that the loss of *lin-14* is correlated with the loss of *cki-1* expression in the VPC (Hong et al., 1998; Ambros, 2001). However, LIN-14 does not seem to be a direct regulator of the *cki-1* expression because the embryonic expression of *cki-1* is not affected by the loss of *lin-14* (Hong et al., 1998). Moreover, loss of *cki-1* causes precocious VPC divisions (Hong et al., 1998), suggesting that the VPC division timing is largely dependent on the activity of the heterochronic genes which impinge on *cki-1*.

Cell cycle progression is also developmentally controlled at the level of G1 progression in response to extrinsic signals. Post-embryonic development stops or is suspended under unfavorable developmental conditions during which cells arrest in G1 for extended periods of time: For example, in absence of food, newly hatched L1 larvae arrest and cell cycle progression does not occur until food is provided (Hong et al., 1998; Baugh and Sternberg, 2006). Similary, in response to poor growing conditions, later post-embryonic development can be temporarily suspended in the L2 stage to become an alternative L3 stage called "dauer", which is accompanied by substantial morphological changes in addition to global arrest of the cell cycle progression in response to changes in developmental signals that include transforming growth factor-β (TGF-β) and/ or insulin-like molecules (Riddle and Albert, 1997).

Like the endocycle in *Drosophila* larvae, two tissues (the intestine and the hypodermis) in *C. elegans* undergo endoreplication, in which DNA synthesis occurs without subsequent mitosis, resulting in a sort of controlled polyploidy. During the L1/L2 transition, most intestinal nuclei (14 of 20) undergo an extra round of nuclear division without cytokinesis (karyokinesis) and thereafter undergo successive cycles of endoreplication, which increases the ploidy of these nuclei to 32C. Loss of SCF^{LIN-23} causes a transition from the first endocycle to a second nuclear division without affecting the ensuing endocycles (Hedgecock and white, 1985; Kipreos et al., 2000), suggesting that

the nuclear division-endocycle transition involves the same SCF-like activity.

In hypodermal cells, endoreplication occurs in two steps: 1) the hypodermal seam cell divides to generate a daughter cell which duplicates its DNA (becoming 4C ploidy) which thereafter fuses with the large syncytial cell hyp7; 2) during the adult stage, the hyp7 syncytial nuclei undergo endoreplication. The endoreplication in the hypodermis is stimulated by the TGF-β signaling pathway, which controls body size (Flemming et al., 2000).

Two germline precursor cells generated during embryogenesis divide throughout the entire post-embryonic life of the organism to give rise to approximately 1000 germ cells in the adult (Schedl, 1997; Seydoux and Schedl, 2001). Two somatic cells called distal tip cells (DTCs) located at the distal ends of the somatic gonad are essential for the mitotic proliferation of germ cells, during which asynchronous mitotic division is triggered by Cdk1 kinase (Kimble and White, 1981; Ashcroft and Golden, 2002; Lamitina and L'Hernault, 2002). The DTCs maintain the mitotic proliferation of germ cells through LAG-2, a membrane-bound delta homologue, which activates the Notch receptor GLP-1 in the germ cells. The activated GLP-1 prevents germ cells from entering into meiosis and this occurs through inhibition of GLD-1 (an RNA binding translational repressor) and the GLD-2/GLD-3 complex (a cytoplasmic poly (A) polymerase), which promote meiotic entry and/or antagonize mitotic proliferation of germ cells (Eckmann et al., 2004; Crittenden et al., 2002; Hansen et al., 2004).

Sheath cells, which are descendants of somatic gonadal lineage and form the basement membrane surrounding the gonad, provide another source for the maintenance of the mitotic proliferation of germ cells by a yet unknown mechanism (Hall et al., 1999). Laser ablation of the sheath cell precursor does not eliminate all the germ cells but results in a reduced proliferation rate, indicating that sheath cells are not essential for the germ cell proliferation but are required to maintain optimal proliferation rate (McCarter et al., 1997; Killian and Hubbard, 2005).

One intriguing question regarding developmental cell cycle control is how cell cycle decisions are linked with cell fate specification. In *C. elegans*, the timely specification of cell fate in most somatic cells is largely invariant. However, these cell fate decisions can be influenced by several parameters including cell cycle status. In *C. elegans*, the determination of VPC fates demonstrates a link between the cell cycle phase and the final cell fate decisions adopted by these cells. The VPC lineage (P5.p, P6.p, and P7.p) gives rise to the cells that will make up the vulva cells. In G1 or early S-phase, P6.p receives the highest levels of the inductive signal LIN-3, an EGF (epidermal growth factor)-like signal that is produced in the anchor cell (AC) to adopt the primary vulva fate (1°). Then, P6.p produces a Notch ligand (a delta homologue) to activate a Notch receptor LIN-12 on P5.p and P7.p, which keep them from adopting the primary vulva fate. In late S or G2 phase however, the Notch ligand activates P5.p and P7.p and the VPCs adopt the secondary vulva fate (2°). This demonstrates that two different cell fates can be adopted in response to a single differentiation signal (Notch) depending on the cell cycle phase of the signal-receiving cells (Ambros, 1999; Ambros, 2001; Fay, 2005; Kipreos, 2005).

Most cell cycle regulators originally identified in mammalian cells have also been found and characterized in *C. elegans*, with the notable exception of the INK4 family CKIs, showing that nearly all G1 regulators are strongly conserved and share similar roles to their mammalian counterparts (Fay, 2005; Kipreos, 2005; Koreth and van den Heuvel, 2005).

In higher eukaryotes, progress through G1 is driven by the cyclin D/Cdk4/6 complexes coupled with cyclin E/Cdk2 being regulated to transit into S-phase (Morgan, 1997). In *C. elegans*, the D- and E-type cyclin homologues, CYD-1 or CYE-1, have been identified and appear to play similar roles. On the other hand, the Cdk4/6 homologue CDK-4 has been identified and characterized, although the identity and function of the Cdk2 orthologue has not been clearly determined. CYD-1/CDK-4 are mostly required for cell cycle progression during larval stages, while CYE-1 is required for both embryonic and post-embryonic cell divisions (Park and Krause, 1999; Fay and Han, 2000; Boxem and van den Heuvel, 2001).

cye-1 was isolated from genetic screens to identify mutations causing Pvl (protruding vulva)-sterility in *C. elegans* (Seydoux et al., 1993; Fay and Han, 2000). Following genetic studies have revealed that maternal, but not zygotic, CYE-1 is essential for embryonic development and that CYE-1 plays an important role in cell divisions in multiple larval tissues, vulva patterning, fertility, and intestine-specific endoreplication (Fay and Han, 2000; Brodigan et al., 2003). Moreover, recent data showed that LIN-35/Rb and the RNAi pathway cooperate to regulate the nuclear divisions occurring in *C. elegans* intestine and that this regulation appears to be mediated by the control of *cye-1* transcription (Grishok and Sharp, 2005). RNAi (RNA-mediated interference) is a post-transcriptional gene silencing mechanism induced by dsRNA in both germ line and soma of *C. elegans* (Fire et al., 1998; Grishok and Mello, 2002). It has been established that RNAi-related pathways including microRNA (miRNA) pathway are mediated through the degradation of mRNA (Elbashir et al., 2001), the transcriptional repression of target genes (Volpe et al., 2002), or the perturbation of mRNA translation (Olsen and Ambros, 1999). Intriguingly, genetic studies have shown that the RNAi pathways are also mediated through the repression of chromatin modifications (Volpe et al., 2002). Therefore, it is probable that LIN-35/Rb is involved in the transcriptional repression of genes in *C. elegans* soma, as is *cye-1* in the intestine, likely through chromatin modification. In mammalian cells and *Drosophila*, it has been known that cyclin E is a major target induced by E2F transcription factors and negatively regulated by Rb protein (Duronio and O'Farrell, 1995; Geng et al., 1996). Thus, to gain more insight about cyclin E function, it will be critical to understand the regulatory mechanism that is exerted by these cell cycle regulators.

In mammalian cells, Rb proteins associate with E2F transcription factors, which form heterodimers with DP transcription factors (Frolov and Dyson, 2004). E2F transcription factors can function as transcriptional activators or repressors, while Rb members bind to both forms of E2F and thus actively repress the trancription of S-phase genes. *C. elegans* has a single Rb family member, LIN-35, which was identified by class B synthetic multivulva (SynMuv) mutations (showing a multivulva phenotype only when associated with class A SynMuv mutations) (Lu and Horvitz, 1998). Although *lin-35* is not

essential for cell cycle progression and viability, inactivation of *lin-35* partially rescues the mutant phenotypes of *cyd-1* and *cdk-4* (Boxem et al., 2001), suggesting that LIN-35 is a negative regulator acting downstream of CDK-4 and CYD-1. Two E2F homologues (*efl-1/2*) and a DP homologue (*dpl-1*) have been identified in *C. elegans* (Ceol and Horvitz, 2001; Boxem and van den Heuvel, 2002). LIN-35 interacts with EFL-1 as well as DPL-1 *in vitro* and *efl-1 (RNAi)* partially rescues the cell cycle defect of *cyd-1* mutant, suggesting that EFL-1 functions in combination with LIN-35 (Boxem and van den Heuvel, 2002). The role of EFL-2 is unclear. On the other hand, DPL-1 functions to promote or repress cell cycle progression depending on the developmental context, likely through its differential association with other interacting partners.

1.5.4. Developmental role of CKIs in cell cycle regulation in *C. elegans*

The *C. elegans* genome project has identified two CIP/KIP family CKIs on chromosome II, which were named CKI-1 and CKI-2 (Hong et al., 1998; Feng et al., 1999; Fukuyama et al., 2003). While *cki-1* has been well characterized, little was known about *cki-2*, mostly due to the fact that it shows no apparent RNAi phenotype.

Ectopic expression of CKI-1 causes G1 arrest in a cell-autonomous manner, which is reminiscent of most other known CKIs (Hong et al., 1998). The developmental expression of *cki-1* is mediated by regulatory elements present in the 5' upstream sequences through which *cki-1* is turned on and off in a spatial and temporal fashion in response to diverse developmental cues including heterochronic control (Ambros, 2001), GON-2 a TRP channel protein in the somatic gonad during L1 (West et al., 2001), and downstream of insulin-like signaling at the onset of larval development (Hong et al., 1998; Baugh and Sternberg, 2006).

cki-1 is dynamically expressed in a broad range of embryonic and post-embryonic tissues, in cells beginning to terminally differentiate or executing transient developmental cell cycle arrest. The developmental expression of *cki-1* correlates with the developmental arrest of the cell cycle in G1: *cki-1* is not detectable in dividing vulva cells, while the

expression of *cki-1* is increased after these final divisions; *cki-1* is highly expressed in animals undergoing diapause such as at eclosion or during dauer (Hong et al., 1998). This suggests that *cki-1* may link nutritional status with cell cycle changes very similar to the role of p27Kip1 where its levels are elevated in the absence of serum (Pause et al., 1998). Therefore, this may be a conserved function of this CKI family.

These conditional cell cycle arrests described above are compromised following *cki-1* (RNAi) (Hong et al., 1998): in the starved L1 larvae, *cki-1* (RNAi) causes hypodermal cells and M cells to undergo S phase; *cki-1* (RNAi)-treated hypodermal cells and gonadal cells divide during developmental arrest in dauer larvae (*daf-7* or *daf-2*). A recent study reveals that DAF-16/FOXO is involved in the transcriptional control of *cki-1* and other genes important for diverse aspects of post-embryonic development, which mediate cell cycle arrest in response to unfavorable environmental conditions (Baugh and Sternberg, 2006).

cki-1 (RNAi) causes a precocious extra round of cell division in VPCs in a cell autonomous manner, which results in extra VPCs that give rise to pseudovulvae in a gain of function mutant of *lin-12* (encoding a membrane-bound Notch receptor), suggesting that the extra VPCs that arise due to these supernumerary cell divisions maintain vulval potential (Greenwald, 1998; Hong et al., 1998). Although *cki-1* (RNAi) causes extra VPCs due to the precocious divisions of VPCs, more than one extra division does not occur, suggesting that there may be other negative regulators acting in parallel to *cki-1*. In fact, genetic studies reveal that *cki-1* acts in parallel to *lin-35/Rb*. In *C. elegans*, the CYD-1/CDK-4 complex positively regulates G1 progression, where LIN-35 acts downstream of CYD-1/CDK-4 (Boxem and van den Heuvel, 2001). It has been shown that *cki-1* inactivation causes the precocious S phase entry and an extra cell division in *cyd*-1 or *cdk-4* mutants, while *lin-35* mutation does not compromise S phase timing, although *lin-35* does cause multiple rounds of DNA synthesis. In addition, CKI-1 interacts with CYD-1 in yeast two-hybrid system (Boxem and van den Heuvel, 2001). Therefore, these results argue that CKI-1 cooperates with LIN-35 in the G1 progression, which is under control of

the CYD-1/CDK-4 kinase activity.

In addition to its cell-autonomous activity at G1 arrest, *cki-1* also plays a cell-nonautonomous role in the somatic gonad to affect germ cell divisions. *cki-1* (RNAi) causes germline hyperplasia after the L3 stage, where the affected gonad is disorganized due to the appearance of extra distal tip cells (DTCs), or anchor cells, and even ectopic gonad arms (Kostic et al., 2003). Unlike the extra VPCs in the *cki-1* (RNAi)-affected animals, which are due to precocious extra division of the vulva precursor cells, the extra DTCs do not arise from the duplication of pre-existing DTCs, but rather from defective cell fate determination during their formation. Genetic studies show that *cki-1* negatively controls cell divisions in the somatic gonadal precursor cells (Z1/Z4 lineage) around the time that the DTC cell fate is acquired. However, in *cki-1* (RNAi), a somatic cell type that arises from the divisions of the somatic gonadal precursors is transformed to the DTC fate due to aberrant divisions in the precursors. Similar results have also been shown in the asymmetric division of *C. elegans* somatic gonadal precursor cell (SGP) where the loss of cyclin D delays the SGP division thereby disrupting the asymmetry of SGP daughters (Tilmann and Kimble, 2005). Although this study argued that simple delay of the SGP division is unlikely a cause of the disruption of the SGP asymmetry, these studies suggest that an alteration of cell division timing may play a critcal role for the appropriate specification of these key cells.

Under mitogenic signals, CKIs such as p27Kip1 are degraded by ubiquitin-dependent proteolysis at the G1/S transition after which cells irreversibly undergo S phase entry (Slingerland and Pagano, 2000). Recent studies have revealed that a similar mechanism might also be conserved in *C. elegans*. In *C. elegans*, *cul-2* is involved in the G1/S transition and mitosis. In the *cul-2* mutant, germ cells undergo G1 arrest which correlates with an increased level of CKI-1 in the nucleus. This suggests that *cul-2* mediates CKI-1 degradation during G1 progression. Mitotic chromosomes in the mutant embryos are not correctly condensed resulting in aneuploidy (Feng et al., 1999).

Since the SCF-mediated protein degradation mostly occurs in a phosphorylation-dependent manner, it has been postulated that the stability of CKI-1 can be regulated in a similar fashion. Inactivation of the *C. elegans cdc-14* phosphatase causes extra divisions in many tissues with no defect in mitosis, morphogenesis or cell fate determination (Saito et al., 2004). Genetic studies show that *cdc-14* acts upstream of *cki-1*, to maintain CKI-1 in a hypophosphorylated form. This form is less efficiently recognized by the ubiquitin-dependent proteolysis machinery resulting in the accumulation of CKI-1 in the nucleus. In budding yeast, Cdc14p dephosphorylates p40Sic1, stabilizing it (Visintin et al., 1998). p27Kip1 is also known to be dephosphorylated by Cdc14A *in vitro* (Kaiser et al., 2002). These findings suggest that CDC-14 may play an evolutionarily conserved role in stabilizing CKIs through maintenance of the hypophosphorylated form.

Although CKI-1 and CKI-2 appear quite similar in their N-termini, they are divergent in their C-terminal domains (Feng et al., 1999). Furthermore, a study using the regulatory elements in the 5' upstream sequences of *cki-2* uncovered considerable differences in the developmental expression patterns between the two CKIs (Hong et al., 1998; Fukuyama et al., 2003). While *cki-1* begins to express in the late stage embryo when the cells become post-mitotic, *cki-2* is expressed at much earlier embryonic stages (at approximately the 64-cell stage) and its expression is maintained throughout embryogenesis. Similar to CKI-1, overexpression of CKI-2 causes an embryonic arrest with large blastomeres, suggesting that overexpression of CKI-2 may lead to premature cell cycle arrest (Fukuyama et al., 2003). These observations imply that *cki-2* might play a role during embryogenesis, and which does not overlap with *cki-1*.

1.6. Other Functions of CKIs: Emerging Roles

The characterization of many CKIs as cell cycle regulators has provided a means of better understanding how intrinsic and extrinsic developmental cues are coupled to cell cycle progression. Although CKIs exert their role through their ability to inhibit the catalytic activity of cyclin/Cdk complexes during specific stages of the cell cycle, recent studies show that CKIs do not only play a role in the inhibition of Cdks and in the induction of cell cycle arrest in response to various developmental cues, but are also involved in other biological processes in a Cdk-dependent or -independent manner, including cell migration, apoptosis, DNA replication, and centrosome duplication (Coqueret, 2003; Denicourt and Dowdy, 2004).

1.6.1. Non-canonical functions of the CIP/KIP family CKIs

While p21Cip1 mediates cell cycle arrest in the p53-dependent programmed cell death following DNA damage, p21Cip1 also provides a mechanism for transformed cells to survive the p53-dependent apoptotic pathway (Gorospe et al., 1997). Upon neuronal differentiation, p21Cip1 relocalises to the cytoplasm where it inhibits SAP and ASK1 pro-apoptotic kinases to block cell death (Shim et al., 1996; Asada et al., 1999; Tanaka et al., 2002). p21Cip1 also binds to procaspase-3 in mitochondria to inhibit caspase-3 activation. Caspase-3 inhibits nuclear localization of p21Cip1, forcing it to reside in the cytoplasm, where it plays a pro-survival role to protect the cell against apoptosis (Levkau et al., 1998).

p27Kip1 also exerts its function depending in a subcellular localization-dependent manner. The cytoplasmic detection of p27Kip1 is found in many cancers including some breast and colon cancers where it correlates with poor prognosis (Slingerland and Pagano, 2000). During tumorigenesis the activated form of AKT kinase mediates the cytoplasmic localization of p27Kip1 through phosphorylation on T157. Its non-phosphorylated form (p27Kip1-T156A) accumulates in the nucleus and arrests cell cycle progression in an AKT-independent manner. Since it is known that the proteolytic degradation of p27Kip1

occurs through phosphorylation on T187 (Ganoth et al., 2001), it is unlikely that the cytoplasmic localization would be linked to its ubiquitin-dependent proteolysis, although it could block programmed cell death, as observed with p21Cip1 (Reed, 2002; Viglietto et al., 2002). Unlike p27Kip1, there is no evidence of the cytoplasmic relocalization of p21Cip1 in tumors, however, it is known to be relocalized to the cytoplasm in response to the HER2/Neu and E7-mediated oncogenic signals (Zhou et al., 2001). These findings show that the biological functions of CKIs can be modified by specific cellular signals and that their function is often governed by their cellular localization.

In budding yeast, the function of Far1 also relies on its cellular localization. In the nucleus, Far1 associates with Cdc28/Cln complexes to cause G1 arrest. Interestingly, upon pheromone binding to a receptor on the cell surface during mating, Far1 associates with a group of cytoplasmic cell polarity proteins, including Cdc24p, Cdc42p, and Bem1p, which reorganize the actin cytoskeleton, consequently polarizing the cell toward its mating partner. This observation indicates that cytoplasmic Far1 is involved in cell orientation in a Cdk-independent manner (Gulli and Peter, 2001).

In addition to its anti-apoptotic function in the cytoplasm as described above, cytoplasmic p21Cip1 also inhibits cell migration, or motility, in a manner similar to Far1 in budding yeast. p21Cip1-derived small peptides inhibit cell spreading through dissociation of an integrin receptor from adhesion contacts (Fahraeus and Lane, 1999). Ectopic expression of p21Cip1 in the cytoplasm affects the formation of actin structures and promotes neuronal growth and branching which occur through association with Rho-kinase to inhibit its function in actin reorganization (Tanaka et al., 2002). p27Kip1 also seems to play a role in cell motility (Boehm and Nabel, 2001). The vascular smooth muscle cells (VSMC) of adult arteries, which are normally quiescent, undergo G1 progress upon stimulation by growth factors and following mechanical injury. Interestingly, VSMC can also migrate in response to mitogenic signals and this is antagonized by treatment with rapamycin. It was shown that rapamycin affects the VSMC migration in a p27Kip1-dependent manner. In wild type ($p27^{+/+}$) mice, rapamycin inhibits VSMC

migration, while in mice lacking p27 ($p27^{-/-}$), this migration is no longer inhibited and cells are capable of movement. It is unclear however whether the effect on the cell migration is linked to cell cycle, although these observations imply that the CIP/KIP CKIs, p21Cip1 and p27Kip1, may have a regulatory role to limit cell spreading, a role that is particularily important for the metastatic potential spreading of cancer cells.

A recent study showed that DNA replication is regulated by p27Kip1 in a Cdk2-independent manner. p27Kip1 interacts with a DNA replication component called MCM7, which is a member of the minichromosome maintenance (MCM) domain protein family essential for the initiation of DNA replication and the maintenance of genome integrity (Nallamshetty et al., 2005). p27Kip1 binds to the conserved MCM domain of MCM7. This interaction inhibits its ability to license DNA replication which usually occurs in a growth factor-dependent manner, but is independent of the Cdk2 inhibitory activity of p27Kip1. This finding suggests that CIP/KIP CKIs are present on the chromatin and that they may be coupled with DNA replication, likely through growth factor-mediated signal transduction pathway.

CKIs also play a role in cell differentiation and/or cell fate determination, which seems to be independent of their inhibitory function. In *X. laevis*, p27Xic1 promotes primary neuron formation through stabilization of a proneural protein, X-NGNR-1 which upregulates the NeuroD transcription factor (Vernon et al., 2003). p27Xic1 is highly expressed in the cells that are destined to become primary neuronal cells. Loss of p27Xic1 prevents primary neural differentiation, while its forced expression promotes neurogenesis. Interestingly, overexpression of the N-terminus of p27Xic1 promotes neural differentiation, while overexpression of either the C-terminus or a p27Xic1 variant (p27Xic1 (35-96)) lacking Cdk2 inhibitory activity does not have any such effect. This is consistent with a previous result from glial cell differentiation, which showed that overexpression of p27Xic1 (35-96) does not induce glial cells (Ohnuma et al., 1999). These observations suggest that p27Xic1 is implicated in cell differentiation in a cell cycle-independent manner. p27Xic1 is also highly expressed in the developing myotome (tissue destined to

become muscle) and is involved in muscle differentiation in a similar manner to its role in primary neurogenesis, in which neither the C-terminus nor p27Xic1 (35-96) demonstrated any effect on muscle differentiation. This suggests that its effect on differentiation is separable from its cell cycle role (Vernon and Philpott, 2003).

Taken together, these data suggest that CKIs are not merely Cdk inhibitors but might be polyvalent regulators that act in a broad range of cell biological pathways including DNA replication, cell motility, apoptosis, cell differentiation and/or cell fate determination, both in a Cdk-dependent and –independent manner.

1.6.2. CKIs and centrosome duplication

In most animal cells, the mitotic spindle is bipolar and the segregation of the genetic materials into two daughters at a cell division requires pulling forces that are generated by microtubules that nucleate at the centrosomes. The centrosome, or spindle pole body as it is referred to in yeast, is composed of a pair of centrioles, where each centriole is an open cylinder consisting of nine sets of radially arrayed microtubules (triplets, doublets, or singlets depending on the cell type). These cylinders are surrounded by electrodense material called pericentriolar material (or PCM), which contains a number of proteins involved in the regulation of centrosome function and the nucleation of microtubules (O'Connell, 2000; O'Connell, 2002; Delattre and Gonczy, 2004; Leidel and Gonczy, 2005).

The centrosomes form the spindle poles and thus each cell possesses two. Following division the centrosome must therefore duplicate once per cell cycle in a manner that is coupled to DNA synthesis (Murray and Hunt, 1993). The resulting mother and daughter centrosomes segregate at mitosis into the daughter cells. Intriguingly, these processes occur through a similar manner to DNA replication, where duplication strictly relies on pre-existing centrioles and moreover, the centrosome cycle occurs in a semi-conservative manner such that the centriole of the centrosome is either a mother or daughter centriole. Centrosomal duplication and segregation must therefore be precisely

coordinated with other cell cycle events and when these processes are unsynchronized the effects are drastic. Inappropriate maintenance of centrosome number in the cell results in mitotic defects due to abnormal segregation of the genetic material, a characteristic feature of many tumor cells (Lingle and Salisbury, 2000; Sankaran and Parvin, 2006). Not surprisingly, understanding how cell cycle regulators impinge on the various mechanisms involved in centrosome assembly, duplication, and segregation has become a major focus in cell biology.

The process of centrosome duplication is initiated at/or around the G1/S transition, where the two centrioles are separated from each other in a process called "splitting", during which they undergo significant structural changes (Figure 1.4). Using an *in vitro Xenopus* system, it has been demonstrated that Cdk2 coupled with cyclin E and/or cyclin A is required for this centriole splitting beginning at the onset of S phase. The centriole splitting is inhibited by depletion of Cdk2, cyclin E, or cyclin A. This suggests that Cdk2 might mediate the phosphorylation-dependent proteolysis of proteins that are involved in the pairing of centrioles. Many studies have argued that this early step of centrosome duplication may be mediated through proteolytic degradation: inactivation of SCF E3 ligase activities in *Xenopus* blocks the splitting of centrioles (Freed et al., 1999); perturbation of $APC^{Cdc20/Fizzy}$ function in *Drosophila* embryos also affects centrosome duplication by causing a delay in centriole splitting (Vidwans et al., 1999).

During S phase, the two split mother centrioles begin to form daughter centrioles at which time, PCM accumulates around and on the centriole. Following S phase in G2 the centrosome begins to mature followed by the segregation of the newly formed centrosomes to their respective poles at mitosis, resulting in the formation of a bipolar mitotic spindle. It is now known that centrosome separation is mediated by a protein kinase Nek2 in G2 through its ability to phosphorylate C-Nap1 which connects mother centrioles within the two centrosomes, thereby generating the two individual centrosomes (Fry, 2002).

Recent studies in *Xenopus* and mammalian cells have revealed that cyclin E/Cdk2

activity is required for centrosome duplication during S phase (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Consistent with this observation, centrosomes undergo multiple rounds of duplication when the cycle is blocked in S phase following hydroxyl urea (HU) treatment. Moreover, this effect can be suppressed by treatment with the Cdk2 inhibitor roscovitine (De Azevedo et al., 1997; Lacey et al., 1999). Overexpression of p21Cip1 or p27Kip1 blocks centrosome duplication, while only the N-terminal Cdk inhibitory domain of p21Cip1 or p27Kip1 had an effect on duplication (Lacey et al., 1999), suggesting that this effect is dependent on their Cdk2 inhibitory activity. cyclin E has also been found to localize to the centrosome (Hinchcliffe et al., 1999; Matsumoto and Maller, 2004) and its overexpression causes premature onset of the centrosome duplication cycle, suggesting that the initiation of the centrosome duplication is coupled to the entry to S-phase which is thought to be mediated by cyclin E/Cdk2.

Since centrosome duplication occurs only once per cell cycle, it would be of great interest to understand how this process is regulated to maintain centrosome number during the cell division cycle. It has been shown that the continued activity of Cdk2 in S-phase arrest does not give rise to the re-duplication of centrosomes in the same cell cycle, suggesting the possibility that CDK2 activity alone is not sufficient, and that another mechanism may be involved in controlling unscheduled re-duplication of the centrosomes. A cell fusion assay showed that only G1 centrosomes, but not G2 centrosomes, maintain a potential to duplicate and that this discrepancy is intrinsic to the centrosome (Wong and Stearns, 2003). Moreover, an ultrastructural study showed that there is a structural difference between G1 and G2 centrosomes, where centrioles in G2 centrosomes remain tightly opposed or engaged, whereas they are disengaged in G1 centrosomes. Furthermore, it has been shown that centrioles are present as an engaged form throughout the cell cycle except the late stage of mitosis or early G1 (Kuriyama and Borisy, 1981). Taken together, these results argue that centrosome duplication may be licensed by the disengagement of centriole pairs, thereby ensuring the correct number of centrosomes.

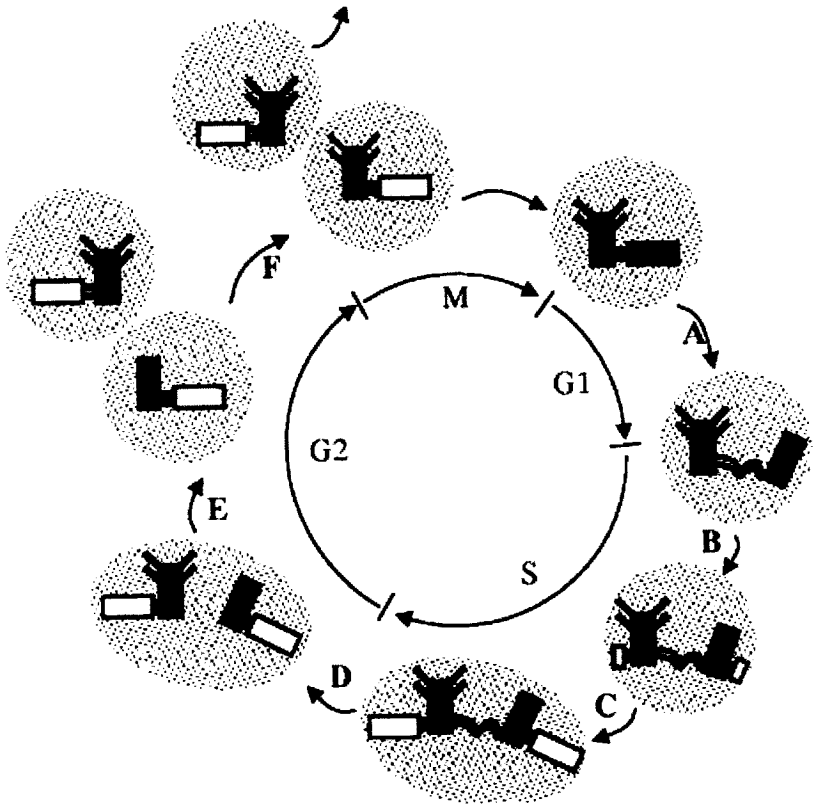


Figure 1.4. **The canonical centrosome cycle.** After mitosis, cells contain a single centrosome (a pair of centrioles; black and grey rectangle: mother and daughter centriole, respectively) and surrounding PCM (shaded circle). In the boundary between anaphase and early G1, the centrioles, tightly opposed, are disengaged (or disoriented) and licensed to duplicate. In the G1/S transition, the disengaged centrioles slightly split (A). In S-phase, new daughter centrioles begin to form at the distal ends of parental centrioles perpendicular to their proximal ends (B) and elongate (C). During mitosis, the duplicated centrosomes separate and segregate into daughter cells to generate (D-F). In the duplication cycle, mother centrioles are distinguished by their proximal appendages from daughter centrioles, where the appendages are completed at the end of each cycle (from Delattre and Gonczy, 2004).

Recent data have shown that the centriole disengagement is not dependent on cyclin E/Cdk2 but instead it requires APC/C and separase activity (Tsu and Stearns, 2006(a); Tsu and Stearns, 2006(b)) which is involved in the separation of sister chromatids through its ability to digest cohesin, a protein responsible for the two sister chromatid adhesion. The activity of separase is regulated through its association/ dissociation with securin depending on the various cell cycle stages: when the cell is not dividing, securin associates with separase, inactivating its activity; during anaphase in mitosis, securin is proteolytically digested to generate the active form of separase in an APC/C-dependent manner (Nasmyth et al., 2000). Therefore, this study suggests that the "only once per cell cycle" control of the centrosome duplication is ensured by such a temporal separation of centriole growth, depending on cyclin E/Cdk2 in S-phase from the centriole disengagement at the mitosis/G1 boundary, so that premature onset of centriole disengagement cannot occur before the onset of anaphase where active separase is present. Considering the importance of maintaining the correct number of centrosomes during cell cycle progression, it is not suprising that this control is often compromised in the process of tumorigenesis, often causing genomic instability, a hallmark of cancer.

A number of studies have linked loss or inactivation of p53 to genomic instability. $p53^{-/-}$ mice cells show a high incidence of aneuploidy, in part due to abnormal duplication of centrosomes (Tarapore et al., 2001; Tarapore and Fukasawa, 2002). p53 is involved in two mechanisms that affect centrosome biology: the initiation of centrosome duplication at G1 and the inhibition of extra duplication. Addition of p21Cip1 to $p53^{-/-}$ cells partially restores the centrosome duplication defect, while introduction of wild-type p53 almost completely restores the duplication cycle, suggesting that p53 controls centrosome duplication in a manner mediated at least in part by the p53 transcriptional target p21Cip1.

Taken together, centrosome duplication occurs through a canonical cycle that is tightly coupled to the cell division cycle so that the centrosome is permitted to duplicate only once per cell cycle. This seems to be acquired through a temporal separation of the centriole duplication licensing step that occurs at anaphase from the centriole growth step

that begins at S-phase. Since centriole growth occurs through a cyclin E/Cdk2-dependent manner, it is probable that CKIs are implicated in the control of this critical process, likely through their ability to inhibit Cdk2 activity.

1.7. *C. elegans* System: Early Embryonic Development

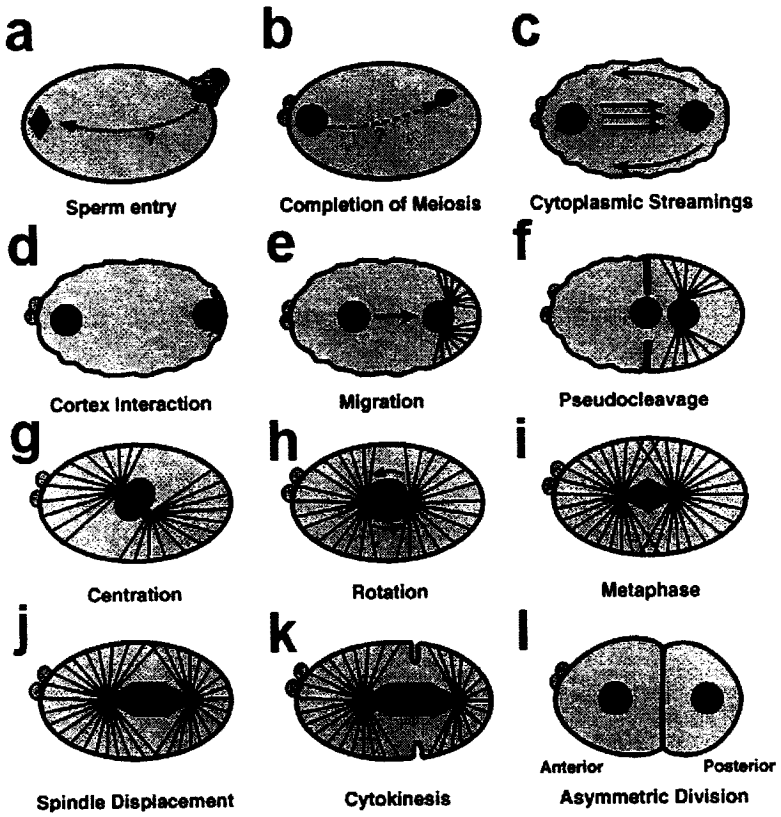
1.7.1. Overview

In *C. elegans*, one could conveniently divide the process of embryogenesis into three stages: 1) formation of the zygotic embryo at fertilization and the subsequent early cleavage divisions, including the generation of six founder cells which give rise to both the somatic and germ line cells (until ~120 minutes after fertilization), 2) completion of cell cleavages and the beginning of embryonic cell differentiation and organogenesis, including gastrulation (from ~120 minutes after the first cleavage until ~350 minutes), and then 3) completion of embryonic cell differentiation, morphogenesis, and organogenesis (~350 minutes after the first cleavage until hatching). At 22°C, it takes about 14 hours from fertilization to hatching (Wood, 1988). During embryogenesis, 671 cells are generated, of which, 113 cells undergo programmed cell death, leaving 556 somatic cells and two germ cell precursors (Z2 and Z3) (Lambie, 2002).

During post-embryonic development in *C. elegans*, the distally-located germ cells in each gonad are maintained in a mitotically active state by Notch signaling. The mitotic germ cells escape from the Notch signals as they move from distal to proximal, causing the cells to enter a long period of meiotic arrest (meiotic prophase I). As the meiotic germ cells approach the proximal gonad arm, they become cellularized which is followed by yolk accumulation and oocyte growth. The most proximal oocyte begins to mature in response to the signals from the spermatheca, during which nuclear envelope breaks down, meiotic progression occurs, and cytoskeletal structures reorganize. The oocyte is fertilized by sperm, which in turn specifies the posterior pole of the zygote and triggers the onset of serial events that will eventually specify the asymmetry typical of the first cell division in the *C. elegans* embryo (Schedl, 1997).

The sperm entry signals the completion of meiosis (I and II) during which two polar bodies are extruded while also providing a pair of centrioles forming the sperm pronucleus/centrosome complex (SPCC) which plays a major role in establishing the

A


a
Sperm entry
b
Completion of Meiosis
c
Cytoplasmic Streamings
d
Cortex Interaction
e
Migration
f
Pseudocleavage
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Centration
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Rotation
i
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Anterior
Posterior
Asymmetric Division

B

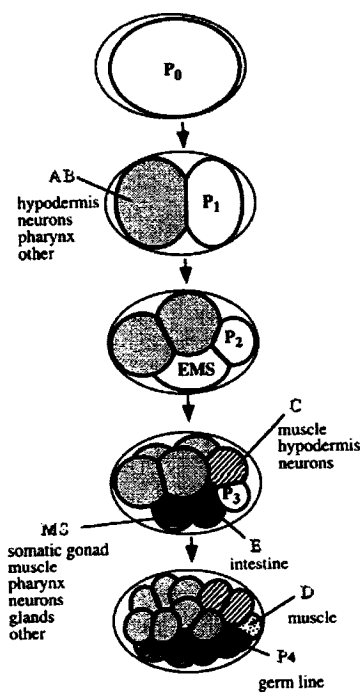
P0
AB
hypodermis
neurons
pharynx
other
P1
P2
EMS
C
muscle
hypodermis
neurons
P3
MS
somatic gonad
muscle
pharynx
neurons
glands
other
E
intestine
D
muscle
P4
germ line

Figure 1.5. **Asymmetric divisions of the *C. elegans* zygote.** (A) At fertilization, completion of meiosis is triggered by the sperm entry (a), which results in the serial extrusion of two polar bodies (b) while also specifying the presumptive anterior pole. The sperm-derived centrosome induces cytoplasmic streaming (c), which pushes the sperm pronucleus/centrosomal complex (SPCC) towards the opposite side subsequently causing the SPCC to interact with the cortical membrane and specifying the presumptive posterior pole. During this time the sperm-derived centrosome duplicates to generate two centrosomes (d). Following this period, the maternal pronucleus (M) migrates toward the paternal pronucleus (S) (e) which is accompanied by a pseudocleavage (f). In the mean time, the split paternal centrosomes migrate to opposite sides of the paternal pronucleus and nucleate microtubules (asters) (f). The two pronuclei meet in the posterior hemisphere and move back to the center (g), accompanied by centrosomal rotation causing the spindle axis to align along the established A/P axis (h). During metaphase, chromosomes are aligned midway between the two poles and interact with the kinetochore microtubules radiating from the centrosomes along the A/P axis (i). Thereafter, during anaphase, the mitotic spindle shifts slightly to the posterior (spindle displacement) (j) which causes an asymmetric cleavage resulting in a larger anterior blastomere (AB) and a smaller posterior blastomere (P1) (k,l). Broken and solid arrows indicate signaling events (unknown) and movement, respectively. Solid lines indicate spindles radiating from centrosomes. M and S mark maternal and paternal pronucleus, respectively. Two spheres on the cortex are extruded polar bodies (from Schneider and Bowerman, 2003). (B) After completion of the first mitotic division, the P1 blastomere and its descendants (EMS, P2, and P3) subsequently divide asymmetrically and generate an additional four somatic founder cells (E, MS, C, and D) and one germ line founder cell (P4). These founder cells ultimately give rise to diverse organ tissues to form intact body as indicated. P0 indicates a fertilized embryo. Anterior is to the left and posterior to the right (from Rose and Kempheus, 1998).

A/P polarity (Figure 1.5). The sperm-derived centrioles split and duplicate in the zygote to form two centrosomes that become aligned between the sperm pronucleus and the cortex in the presumptive posterior pole. This interaction initiates a series of early events, including cytoplasmic flux, pseudocleavage, and ruffling due to cortical movement, which results in the asymmetric distribution of cell fate-determining factors such as P-granules (germline-specific electrodense RNA containing complexes). During this period, the maternal pronucleus (which arises from the oocyte) migrates, initially slowly due to cytoplasmic components or flow, and then later quickly due to sperm-derived microtubules-mediated pulling force toward the paternal pronucleus (which originates from the sperm). The split and now duplicated paternal centrosomes migrate to opposite sides of the paternal pronucleus and nucleate microtubules (asters) finally making the spindle axis perpendicular to the A/P (anterior/posterior) axis. The two pronuclei meet in the posterior hemisphere and move back to the center, which is accompanied by centrosomal rotation causing the spindle axis to align along the now established A/P axis. The nuclear envelope breaks down following the alignment of the maternally and paternally-derived chromosomes at the metaphase plate. The centrosomes nucleate microtubules to form the first mitotic spindle along the A/P axis and thereafter, during anaphase, the mitotic spindle slightly shifts posteriorly which causes an asymmetric cleavage resulting in a larger anterior blastomere (AB somatic founder cell) and a smaller posterior blastomere (P1 cell) and effectively segregates the germ line from the soma. This asymmetry affects the cell division timing and the cell fate determination in the two blastomeres as well as, ultimately, their descendants (Golden, 2000; Schneider and Bowerman, 2003; Cowan and Hyman, 2004b; Lyczac et al., 2002).

After completion of the first mitotic division, the P1 blastomere and its descendants (EMS, P2, and P3) subsequently divide asymmetrically and generate an additional four somatic founder cells (E, MS, C, and D) and one germline founder cell (P4). During the asymmetric and asynchronous early cleavages, which produce the founder cells, intercellular signals including a Notch receptor GLP-1, the Wnt receptor MOM-5, and RAS/MAPK, in addition to others are known to play crucial functions in the ultimate

specification of cell fates. This indicates that cell/cell-interactions are required in addition to cell-autonomous signals for cell type-specific cell fate determination in the early embryo (Rose and Kempheus, 1998).

1.7.2. PAR Proteins and Their Role in Asymmetric Cell Division

1.7.2.1. Overview

In many cells, asymmetric cell divisions provide a means to increase complexity. The polarity of cell divisions therefore becomes an important step during the development of many organisms. Unlike in *Drosophila*, where polarity establishment occurs during oogenesis, *C. elegans* oocytes show no sign of asymmetry prior to fertilization. In *C. elegans*, the asymmetry begins following the entry of the sperm into the mature oocyte at fertilization, which ultimately triggers the uneven cortical localization of a group of conserved, cortical membrane-localizing molecules called PAR proteins (Rose and Kempheus, 1998). This results in asymmetric distribution of cell fate-determining factors in addition to the positioning of the mitotic spindle. In *par* (partitioning-defective) mutant embryos, the first mitotic division occurs symmetrically following the synchronous division of daughter cells. Genetic screens have identified six PAR proteins (Figure 1.6). After the SPCC-induced polarization, PAR-1 (encoding a serine/threonine kinase) and PAR-2 (a RING domain protein) localize to the posterior, while the PAR-3 (three PDZ domain-containing protein)/PAR-6 (single PDZ domain-containing protein) complex associated with PKC-3 (an atypical protein kinase C) localizes to the anterior. PAR-4 (a serine/threonine kinase) and PAR-5 (a 14-3-3 protein) are distributed evenly throughout the cortex (Watts et al., 2000; Morton et al., 2002). Genetic studies have shown that the SPCC signals exclude PAR-6 and other anterior PAR proteins from the posterior in order to establish the initial A/P polarity, while PAR-2 in the posterior cortex functions to maintain this polarity. Although it is largely unclear how the PAR proteins localize to the cortex, it is now known that the distribution of PAR proteins require an intact cytoskeletal microfilament (Schneider and Bowerman, 2003; Cowan and Hyman, 2004). In the absence of non-muscle myosin NMY-2, PAR-3 distributes evenly and PAR-2 cannot be detected in

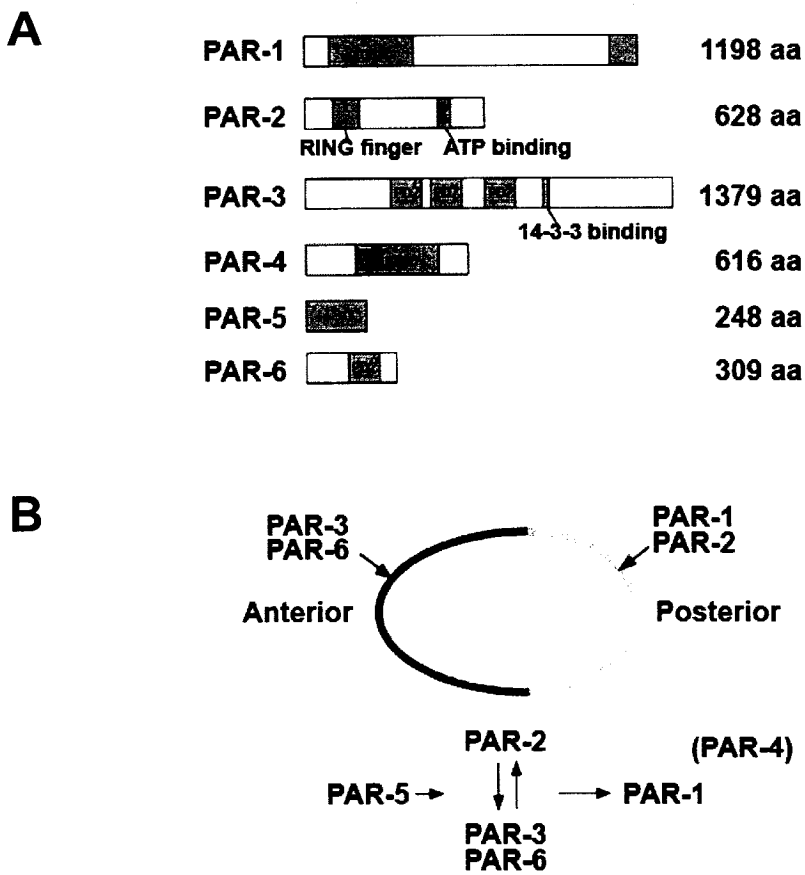

Figure 1.6. **Summary of PAR proteins and their distribution in the *C. elegans* zygote.** (A) Molecular nature of six PAR proteins (PAR-1 to 6). aa, amino acid. The shaded boxes indicate conserved domains found in their primary sequences (kinase, PDZ, 14-3-3). (B) After the SPCC-induced polarization, PAR-1 and PAR-2 localize to the posterior, while the two PDZ domain containing PAR proteins, PAR-3 and PAR-6, associate with PKC-3 (an atypical protein kinase C) and localize to the anterior. PAR-4 and PAR-5 are distributed evenly throughout the cortex. Genetic studies have shown that PAR-5 acts upstream of the PAR proteins and PAR-2 in the posterior cortex functions to maintain the anterior restriction of PAR-3/PAR-6/PKC-3. PAR-1 appears to be downstream of PAR-2. The cortical distribution of PAR-4 is not affected by other *par* mutations (from Rose and Kempheus, 1998).

the cortex. This suggests that microfilament dynamics play a role in the cortical distribution of the PAR proteins. Orthologues of most of the PAR proteins, with the exception of PAR-2, have been found to regulate various aspects of cell polarity from insects to mammals (Pellettieri and Seydoux, 2002), suggesting that the mechanism for establishing cell polarity through cortical localization of the PAR proteins and their interplay with cytoskeletal elements has been conserved throughout evolution.

1.7.2.2. PAR proteins and spindle positioning in the first cell division

Our current understanding of anterior/posterior (A/P) polarity specification at the first division of the zygote has been greatly enriched from studies of PAR proteins. However, relatively less is understood about how this polarity mediated by PAR proteins is coupled with the characteristic asymmetric spindle positioning which results in an asymmetric mitotic division in the zygote. Recent studies are shedding light on how these critical developmental events are coordinated. It was shown that PAR proteins mediate the asymmetric positioning of spindles at the cortex which is in turn translated into differential pulling forces exerted on the two spindle poles (Grill et al., 2001). Through performing microtubule-severing experiments, they found that pulling forces external to the spindle mediate the separation of the spindle poles following severing. More intriguingly, it was shown that in the severed embryos, the posterior spindle pole migrates for a greater distance and at a higher velocity than its anterior counterpart following severing. This suggests that stronger net forces are exerted on the posterior pole, explaining overall displacement of spindles toward the posterior. Since disruption of actin filaments by cytochalasin treatment does not affect the spindle positioning, it is unlikely that the asymmetry is mediated by actin filaments, but more likely by astral microtubules (Aist et al., 1993). Though it is still unknown how the pulling force is generated by astral microtubules, it is predicted that the force generation may be mediated by microtubule depolymerization on the cortex (Hyman and White, 1987; Korinek et al., 2000; Lee et al., 2000). A similar experiment performed in *par* mutants (*par-2* and *par-3*) showed that in irradiated *par-2* mutant embryos, the peak velocity of the two spindle poles is similar to that of the anterior spindle pole after severing in wild type embryos, while in *par-3* mutant

embryos, it is similar to that of posterior spindle pole after severing in wild type embryos (remember that PAR-2 and PAR-3 localize to posterior and anterior, respectively). This suggests that in *par* mutants, the pulling forces are equally exerted on the two spindle poles due to equally positioned spindles, resulting in the symmetric mitotic division. Taken together, these results strongly argue that polarity cues mediated by PAR proteins are translated into the generation of differential pulling forces that act on the two spindle poles, resulting in the asymmetry of the zygotic division. However, this did not explain how PAR proteins mediate this difference in net pulling forces or what molecules generate such forces.

A global RNAi-based screen uncovered two genes, *gpr-1* and *gpr-2*, encoding coiled coil domain proteins carrying a GoLoco motif (Yu et al., 2000; Schaefer et al., 2000; Schaefer et al., 2001), whose inactivation caused symmetric zygotic division due to aberrant spindle positioning in the one-cell embryo, generating two blastomeres of identical size (Gonczy et al., 2000). Intriguingly, the initial A/P polarity mediated by PAR proteins seems to be undisrupted by *gpr-1/2 (RNAi)*, indicating that the equal zygotic division in *gpr-1/2 (RNAi)* is not due to abnormal A/P polaity, and that GPR-1/2 act downstream of PAR proteins. As other GoLoco motif proteins regulating Gα subunits (De Vries et al., 2000; Kimple et al., 2001; Natochin et al., 2001), a number of observations suggest that GPR-1/2 might act through Gα signaling in the one-cell embryo: inactivation of *goa-1 (RNAi)* or *gpa-16 (RNAi)* causes similar defects as *gpr-1/2 (RNAi)*; inactivation of both *goa-1* and *gpa-16* by RNAi does not enhance the *gpr-1/2 (RNAi)* phenotype; *gpr-1/2* genetically interacts with *goa-1* and *gpa-16* which encode G_α subunits GOA-1 and GPA-16, respectively; GPR-1/2 physically interacts with GOA-1 through its GoLoco motif; inactivation of *gpb-1* (G_β subunit) or *gpc-2* (G_γ subunit) does not rescue the *gpr-1/2 (RNAi)* phenotype. Microtubule- severing experiments demonstrated that after severing in *gpr-1/2 (RNAi)* or *goa-1/gpa-16 (RNAi)* embryos, the two spindle poles have identical velocities which were considerably lower than that of the anterior or posterior spindle poles after severing in wild type embryos. Since the astral microtubules remain intact, this argues that G_α signaling mediates the generation of pulling forces exerted on the two spindle poles

during the first division. Moreover, through immunostaining using GPR-1/2 antibodies and GFP fusions of GPR-1/2, it was shown that GPR-1/2 asymmetrically enriches at the posterior cortex during mitosis. Intriguingly, the cortical asymmetry of GPR-1/2 was disrupted in *par* mutants, where in *par-2* or *par-3* mutant embryos, GPR-1/2 are evenly distributed at the cortex.

Taken together, these results argue that G_α signaling is differentially activated at the cortex, likely due to asymmetric enrichment of GPR-1/2 during mitosis, which generates distinct pulling forces exerted on the two spindle poles, resulting in two blastomeres of different size. Given that G_α subunits such as $G_{\alpha i}$ in *Drosophila* (Cai et al., 2003) are involved in spindle positioning, these factors may be components of an evolutionarily conserved mechanism to dictate spindle positioning through a G_α signaling pathway.

1.7.3. Centrosome biology in *C. elegans*

1.7.3.1. Molecules involved in the centrosome reproduction: Duplication and maturation

Knowledge regarding centrosome reproduction has accumulated from studies carried out on the *C. elegans* embryo, mostly due to availability of powerful genetic tools in *C. elegans*. Moreover, the transparency of the one-cell embryo of *C. elegans* has facilitated live imaging and immunofluorescence microscopy at high resolution. Although serious concerns have been proposed regarding generalization of the knowledge acquired in *C. elegans*, mostly due to its atypical structure of centriole and absence of several proteins, such as ε-tubulin and centrin, known to be important for centrosome reproduction in other species, it is widely believed that the core molecules identified in *C. elegans* may play a conserved role among evolutionarily divergent species. Since aspects of this thesis deal with the biogenesis of centrioles, I will describe centrosome reproduction while focusing on a set of proteins identified to be essential for the centriole duplication in *C. elegans* (Figure 1.7).

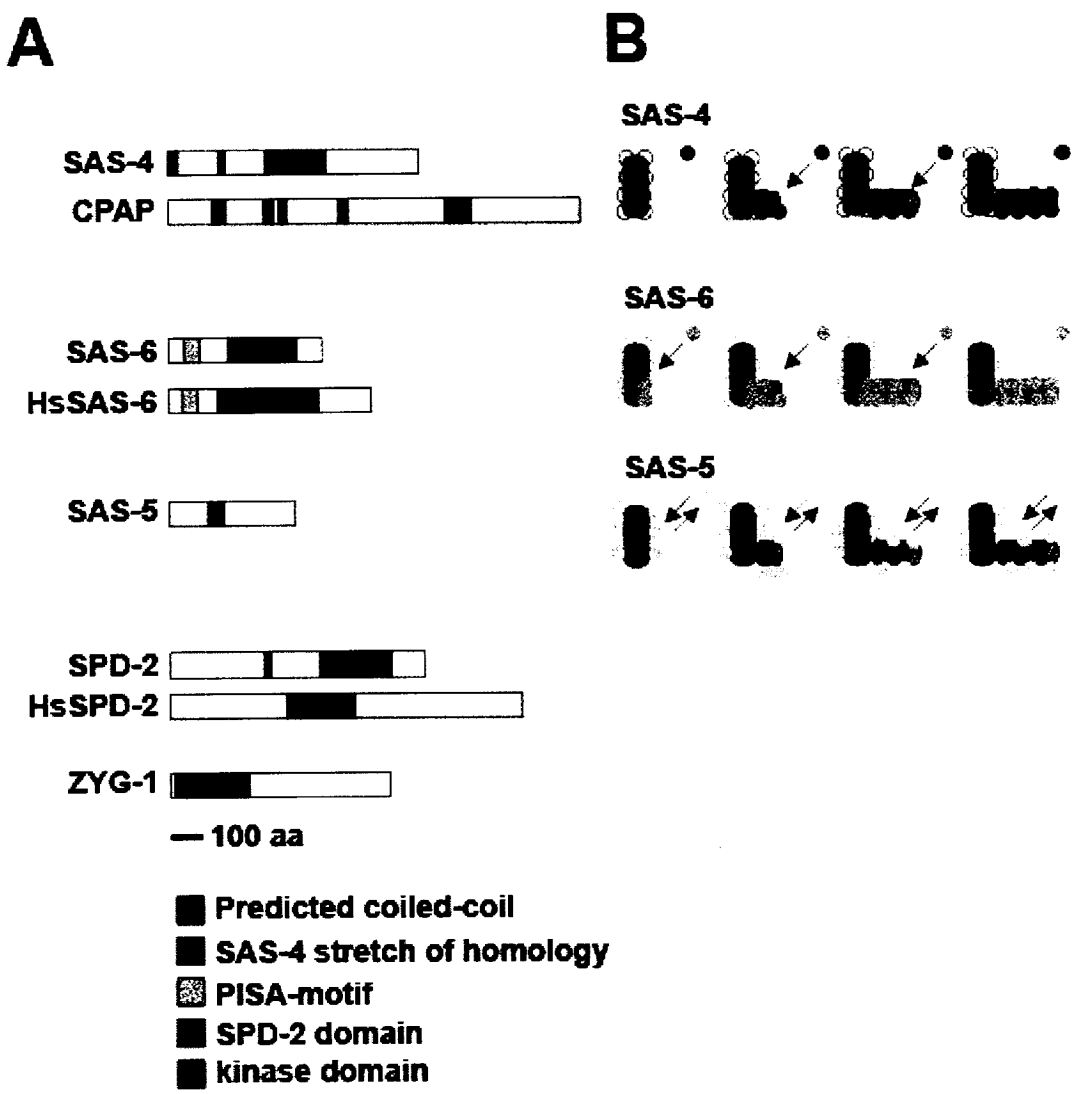

Figure 1.7. **Proteins required for centriole formation in *C. elegans* and their dynamic recruitment into centrioles.** (A) Proteins required for centriole formation in *C. elegans* embryos were schematically represented, where conserved domains or motifs on each protein were indicated by colored boxes. CPAP, HsSAS-6, and HsSPD-2 indicate human homologues of SAS-4, SAS-6, and SPD-2, repectively. (B) Dynamic localization of SAS-4 (red), SAS-6 (orange), and SAS-5 (yellow) to centrioles. Black and gray barrel indicate mother and daughter centrioles, respectively. Open or closed circles indicate proteins recruited into centrioles in previous (open circles) or current (closed circles) cell cycles (from Leidel and Gonczy, 2005).

zyg-1 (for zygotic defective) was uncovered as a conditional allele from forward genetic screens to identify maternal-effect embryonic lethal mutants (Wood et al., 1980; Kemphues et al., 1988) or mutants showing defects in both embryonic and post-embryonic cell lineages (O'Connell et al., 1998), where *zyg-1* alleles form a monopolar mitotic spindle at restrictive temperature. Genetic studies carried out in the one-cell embryo of *C. elegans* uncovered an essential role of *zyg-1* as a protein kinase in centriole duplication (O'Connell et al., 2001). The study revealed that *zyg-1* exerts its role through differential parental contributions to form centrioles in the one-cell embryo. In absence of paternal activity of *zyg-1*, only a single centriole is formed during spermatogenesis due to a defect in the centriole formation. After fertilization, the centriole duplicates to form a centrosome by adopting maternal activity of *zyg-1* present in oocyte during the first division. Since only a single centrosome is present in the zygote, the embryo fails to form a bipolar spindle, rather forming monopolar spindles which cause a cytokinesis defect. Although the centrioles separate and duplicate to form bipolar spindles during the second division, the embryo eventually arrests due to aneuploidy. If the maternal activity of *zyg-1* is lacking, a pair of centrioles is normally donated from the sperm. Although the centrioles are not able to duplicate due to the absence of maternal *zyg-1*, the centrioles still separate and each centriole recruits PCM componenets to form an aberrant centrosome during the first division. During the second division, however, the centrosome cannot be duplicated, resulting in an embryonic arrest with monopolar spindles at the two-cell stage. Taken together, ZYG-1 has a dual maternal and paternal activity to ensure proper formation of the centriole in the embryo. Although ZYG-1 is a protein kinase known to be auto-phosphorylated, its substrates still remained to be identified.

RNAi-based global scale genetic screens uncovered two genes, *sas*-4 and *sas*-6 (for spindle assembly), in which *sas-4 (RNAi)* or *sas-6 (RNAi)* causes an embryonic arrest at the two-cell stage with monopolar spindles (Dammermann et al., 2004; Kirkham et al., 2003; Leidel et al., 2005; Leidel and Gonczy, 2003; Sonnichsen et al., 2005). SAS-4/6 are coiled-coil proteins present at the center of the centrosome, suggesting that they are associated with the centriole. Using fluorescence recovery after photobleaching (FRAP)

(Leidel and Gonczy, 2003; Leidel et al., 2005) and GFP-SAS-4/6 incorporation assay (Leidel and Gonczy, 2003; Kirkham et al., 2003; Leidel et al., 2005), it was revealed that SAS-4 is recruited into daughter centriole once per cell cycle, while SAS-6 is firstly incorporated into mother centriole and thereafter recruited into daughter centriole depending on SAS-4 activity.

Another SAS family protein, SAS-5, was indentified by forward and reverse genetic screens (Dammermann et al., 2004; Delattre et al., 2004). Like SAS-4/6, SAS-5 is a coiled coil protein incorporated into centrioles throughout the cell cycle. SAS-5 has a dual paternal and maternal role like *zyg-1*. However, unlike SAS-4/6, FRAP analysis showed that SAS-5 shuttles between the cytoplasm and the centrioles. SAS-5 physically associates with SAS-6 which seems to be required for their centriole localization. Although it is still unclear how SAS family proteins act in the centriole formation, genetic studies have suggested that they might exert their role in controlling the size of PCM (Kirkham et al., 2003; Delattre et al., 2004; Leidel et al., 2005). It was shown that incomplete depletion of *sas-4*, *sas-5*, or *sas-6* by RNAi causes smaller spindle poles due to reduced PCM, suggesting that the centriolar proteins might control the recruitment of PCM by regulating centriole size. While centrioles are required for the accumulation of PCM proteins, some PCM components also play an important role in normal centriole formation. Lack of the PCM protein γ-tubulin or SPD-5 (for spindle defective; Hamill et al., 2002) causes partial formation of the centriole, likely due to aberrant loading of SAS-4 at the centrioles.

SPD-2 (for spindle defective) was identified as a conditional allele from a genetic screen to find mutants with defects in mitotic spindle assembly, where the mutation caused absence of early events involved in A/P polarity specification such as cortical ruffling, pseudocleavage (PC), and cytoplasmic streaming, resulting in mislocalization of P-granules and PAR proteins (O'Connell et al., 1998). Since A/P polarity is lost in *spd-2* mutants, it has been suggested that *spd-2* might act at very early step of the process. SPD-2 is a coiled coil protein, which enriches at the centrosome in an Aurora kinase and cytoplasmic dynein-dependent manner. SPD-2 is in turn required for the centrosome

recruitment of almost all known PCM proteins such as γ-tubulin, ZYG-9, SPD-5, an aurora kinase (Aurora-A), and a polo-like kinase (PLK-1), indicating that SPD-2 acts at an early step of the centrosome assembly/maturation pathway through the centrosome recruitment of PCM proteins. Although SPD-5 is required for the centrosome association of SPD-2, the centriole localization of SPD-2 occurs independently of SPD-5. More intriguingly, lack of *spd-2* causes loss of centriole duplication in the second cell division, suggesting that SPD-2 also has a role in the centriole formation as well as its role in the centrosome maturation. In fact, *spd-2* genetically interacts with *zyg-1*, where the heterozygous mother for both *spd-2* and *zyg-1* (*spd-2/+; zyg-1/+*) produces dead embryos forming monopolar spindles, similar to that of the *zyg-1* homozygote, suggesting that both SPD-2 and ZYG-1 act in a common pathway. Given that SPD-2 has a dual PCM assembly and centriole duplication role, it has been proposed that SPD-2 might act as a scaffold protein to localize and modulate ZYG-1 and its catalytic substrates present in the pericentriolar region (Kemp et al., 2004; Pelletier et al., 2004; Leidel and Gonczy, 2005).

Recent studies revealed the epistatic relationship that exists between the centriolar proteins (Delattre et al., 2006; Pelletier et al., 2006). It was shown that SPD-2 acts at the earliest step and is required for the centriole localization of ZYG-1 and SAS-4/5/6. Thereafter, three SAS proteins are recruited depending on ZYG-1, where SAS-5 and SAS-6 are required for the SAS-4 recruitment. Therefore a complex interplay between components of the centrioles and the PCM seems critical to ensure appropriate centriole formation (Kemp et al., 2004; Pelletier et al., 2004). Since these proteins, essential for centriole formation, seem to have homologues in other species, they might play an evolutionarily conserved role in this critical developmental process (Hamill et al., 2002; Andersen et al., 2003; Leidel and Gonczy, 2003; Leidel et al., 2005).

1.7.3.2. Centrosome and the asymmetric cell division

As previously discussed, sperm entry triggers cortical polarity through the formation of two types of polarity domains along the A/P axis: PAR domains (uneven distribution of PAR proteins) and contractile polarity (such as anterior cortex ruffling and posterior

smooth domain). Many genetic studies have hinted or shown that the centrosome plays a central role in this developmental process (Sadler and Shakes, 2000; Goldstein and Hird, 1996; Cuenca et al., 2003; Cowan and Hyman, 2004(a)); Cowan and Hyman, 2004(b); O'Connell et al., 2000; Hamill et al., 2002). It has been established that sperm entry is correlated with the posterior domain in *C. elegans* (Goldstein and Hird, 1996). However, it is unclear whether the sperm entry position provides a predetermined domain that attracts the centrosome or whether the centrosome is attracted to a random position of the posterior cortex in order to initiate cortical polarity. The role of the centrosome in polarity establishment in *C. elegans* appears to be mediated by microtubules (Wallenfang and Seydoux, 2000), although recent data showed that depletion of microtubules did not abolish polarty establishment (Cowan and Hyman, 2004(b)). Therefore, it is still debatable whether this event occurs in a microtubule-dependent or -independent process. Taken together, these data show that the centriole pair provided by the sperm plays a critical role during the period that establishes the intial polarity along the anterior/posterior axis. Although it is so far unclear how the centrosome plays a role in that process, it is widely accepted that the process is mediated through interplay of microtubule/ acto-myosin filaments, wherein the centrosome may exert its role to direct the traffic of cytoskeletal complexes. Therefore, a thorough study of the various roles of the centrosome in the zygote will provide a better understanding of how cell polarity is initially established.

1.7.3.3. Elimination of centrioles: A conserved mechanism for proper number of centrioles at fertilization

Canonical mitotic division generates daughter cells that inherit exact copies of DNA from their mother through precise coordination of DNA duplication with cytokinesis. This segregation requires the spindle and its associated centrosomes and it is important that centrosome duplication be tightly coordinated with the DNA replication cycle. Otherwise, the resulting aberrant number of centrosomes may abnormally attach to chromosomes and cause mitotic catastrophe.

In addition to the problem of halving the genetic content of gametes during meiosis,

sexually-reproducing animals must also conserve centrosome number in the zygote. Supernumerary centrosomes would result in multipolar spindles in the zygote control over the number of centrosomes inherited by the zygote is critical. How then, can the centrosome number be achieved and properly maintained at fertilization?

Although the solutions to this problem are manifold, many organisms use differential parental contribution of centrioles to ensure the formation of a bipolar spindle. In a clam species (*Spisula solidissima*) and a brown algae (*Fucus distichus*), each gamete contributes a pair of centrioles at fertilization but maternally-donated centrioles are silenced in the zygote and lose their ability to nucleate microtubules (Nagasato et al., 1999; Wu and Palazzo, 1999). A similar case is found in a starfish (*Asterina pectinifera*), where one half of the maternally-donated centriole pair is lost during polar body exclusion and the other centriole remains silenced in the zygote (Uetake et al., 2002). More commonly, however, centrioles are differentially contributed from each gamete. In *C. elegans*, a pair of centrioles is paternally contributed by entry of the sperm into the oocyte (which loses its centrioles during oogenesis). Thereafter, the centrioles recruit PCM components present in the zygotic cytoplasm and reconstitute a centrosome (Albertson and Thomson, 1993). Although little is understood about these mechanisms, it is thought that the stabilization of centrioles may be involved, or that they are eliminated by an active process in response to developmental signals (Tassin et al., 1985; Connnolly et al., 1986; Ploubidou et al., 2000).

In humans and *Drosophila*, a single centriole is also paternally contributed at fertilization. During spermatogenesis, the mother centriole is lost but the daughter remains. At fertilization, the daughter centriole in the zygote duplicates twice to generate four centrioles which give rise to two centrosomes prior to the first division (Schatten, 1994; Callaini et al, 1999; Manandhar et al., 2000). This elimination process of centrioles occurs more rigorously during spermatogenesis of mice, during which both mother and daughter centrioles are lost, causing fertilization through joining of two acentriolar gametes. As a result, early embryonic divisions of mice occur in a centriole-independent manner until centrioles become visible at the preimplantation stage (Calarco-Gillam et al., 1983;

Abumuslimov et al., 1994). It is still unknown how new centrioles are generated without a preexisting anlage. *De novo* centriole formation is observed when parthenogenesis is artificially induced in sexually-reproducing animals, including *D. melanogaster*, sea urchin, and rabbit (Kallenbach, 1983; Szollosi and Ozil, 1991; Riparbelli and Callaini, 2003). This suggests that centrioles might suppress *de novo* centriole formation during normal sexual reproduction. It is so far unknown whether *de novo* centriole formation occurs using the same machinery involved in canonical centriole synthesis. Considering that this critical process of maintaining the proper number of centrioles is compromised in various cancers, a better understanding of this critical developmental process may allow us to identify novel pathways to intervene at this level of regulation.

1.8. Objectives and Rationales of the Research

A steadily increasing body marks the significance of CKI proteins in the regulation of cell cycle progression from yeast to metazoan animals. In addition to the typical role of CKIs in antagonizing the catalytic function of cyclin/CDK complexes, their previously uncharacterized roles (such as CDK-independent activities) are currently emerging through our greater understanding of these critical cell cycle regulators. Since *C. elegans* is an excellent model organism to study cell cycle progression in various developmental contexts, work on this animal will contribute significantly in our understanding of its role in controlling cell cycle events during development.

Although *cki-2* has been annotated as second CIP/KIP family CKI in *C. elegans*, relatively limited progress has been made in understanding its biological function, while *cki-1* has been well studied in a developmental context. However, it has been suggested that *cki-2* might play a non-redundant role with *cki-1*: *cki-2(RNAi)* causes impenetrant embryonic lethality and *cki-2* shows a distinctive pattern of developmental expression (Feng et al., 1999; Fukuyma et al., 2003). Moreover, its overexpression causes embryonic arrest with obvious cell cycle defects (Fukuyama et al., 2003). These imply that maintaining appropriate levels of CKI-2 is critical to ensure proper embryogenesis. In fact, while a cullin-based degradation mechanism of CKI-1 has been well characterized, it is not clear how CKI-2 levels are appropriately maintained. Therefore, our overall research goal was to investigate the role of CKI-2 during development in *C. elegans*, while also focusing on the regulation of CKI-2 levels and activity.

Since *cki-2 (RNAi)* causes low, impenetrant embryonic lethality that did not allow us to further characterize the embryonic phenotype and in addition, no useful allele of this gene is available, in order to investigate loss of function of *cki-2*, we decided to use a reverse genetic approach called co-suppression, which depends on the use of high copy number transgenes to silence a specific gene activity in the germ line (Ketting and Plasterk, 2000). Using this approach which caused reproducible embryonic lethality, we observed

multiple defects in embryonic cell cycle progression. While the majority of the arrested embryos showed expanded endodermal and mesodermal fields, suggesting a role of *cki-2* as a negative cell cycle regulator, more intriguingly, we observed a low frequency of one-cell arrested embryos with supernumerary centrosomes. We reasoned that it could be due to a problem associated within appropriate cytokinesis during the first mitosis. Several lines of evidence, however suggested that the supernumaerary centrosomes were caused by inappropriate maintenance of centrioles during oogenesis, wherein we observed perduring centrioles in the late stages of oogenesis. Furthermore, based on the typical role of CKI-dependent inhibition of cyclin/CDK complexes, we reasoned that it might be mediated by a cylin E/Cdk2 complex. In fact, reduction of cyclin E or a Cdk2 homologue suppressed the frequency of the supernumerary centrosome phenotype, demonstrating that this critical process during oogenesis might be dependent on the catalytic function of cyclin E/Cdk2 complex. Our finding provides an important step in understanding this critical developmental phenomenon in the cell biological/molecular level. These results were presented in chapter II.

In order to gain further insight on the role of *cki-2*, we performed a yeast two-hybrid screen to identify CKI-2 interacting proteins. In this screen, we identified three interacting partners of CKI-2: orthologue of PCNA (PCN-1) and SUMO (SMO-1), and a RING finger protein called RNF-1. These suggest that CKI-2 may have a similar role as its mammalian counterpart, p21Cip1, during S-phase, since only p21Cip1 among the mammalian CIP/KIP CKIs interacts with both Cyclin/CDK and PCNA (Waga et al., 1994). In fact, like p21Cip1, we found that CKI-2 has two independent domains in its amino- and carboxy-terminus, which are functionally separable. Since SUMO has been well known to affect protein/protein interactions, the subcellular localization, and catalytic activity of a protein (Melchior, 2000), we speculated that *C. elegans* SUMO (SMO-1) might also have similar roles with its mammalian counterpart. In fact, we found that SMO-1 can affect the subcellular localization of CKI-2, which is linked to the rapid destabilization of CKI-2. We found that other CKIs such as p27Kip1 possess a conserved SUMOylation motif in their CDK inhibitory domain and moreover, these same CKIs also contained a similar potential

nucleolar localization signal. Therefore, our findings highlight a potentially evolutionarily-conserved regulatory mechanism that is important to tightly regulate the levels of CKI-2 to ensure appropriate cell cycle progression, while this occurs through SUMO-mediated subcellular localization and degradation. We demonstrated the detailed results in chapter III.

The last part of our study was devoted to understanding the role of the RING finger protein called RNF-1 which was identified as a CKI-2 interacting protein from the two-hybrid screen. As many RING finger proteins act through a multisubunit E3 ubiquitin ligase such as SCF complex, we speculated that RNF-1 might target CKI-2 for degradation, probably in an ubiquitin-dependent manner. We found that RNF-1 interacted with the C-terminus of CKI-2, which seemed to be required for the destabilization of CKI-2 as described in chapter III. In order to investigate the relationship between CKI-2 and RNF-1, we turned to overexpression of RNF-1 as a gain of function strategy since the function of RNF-1 seems redundant with other RING finger proteins. We observed that co-expression of RNF-1 with CKI-2 suppresses the embryonic lethality associated with misexpression of CKI-2, which is correlated with the increased rate of CKI-2 degradation. Moreover, we found that the CKI-2 degradation occurs in an ubiquitin-dependent manner through proteasome- mediated proteolysis. Interestingly, using a yeast-based assay, we found that SMO-1 seems to antagonize the interaction between CKI-2 and RNF-1. Therefore, our study has unraveled a complex mechanism required to ensure that the levels of CKI-2 are appropriately regulated through RNF-1-mediated proteolytic degradation, which may be modulated by SUMO. These results are presented in chapter IV.

1.9. References

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Chapter II

Cell cycle regulators control centrosome elimination during oogenesis in *C. elegans*

2.1. Abstract

In many animals the bipolar spindle of the first zygotic division is established following the contribution of centrioles by the sperm at fertilization. To avoid the formation of a multi-polar spindle in the zygote, centrosomes are eliminated during oogenesis in most organisms, although the mechanism of this selective elimination is poorly understood. Here we show that *cki-2*, a *C. elegans* cyclin-dependent kinase inhibitor, is required for their appropriate elimination during oogenesis. In the absence of *cki-2,* embryos have supernumerary centrosomes and form multi-polar spindles that result in severe aneuploidy following anaphase of the first division. Moreover, we demonstrate that this defect can be suppressed by reducing Cyclin E or CDK2 levels. This implies that the proper regulation of a Cyclin E/CDK complex by *cki-2* is required for the elimination of the centrosome that occurs prior to or during oogenesis in order to ensure the assembly of a bipolar spindle in the *C. elegans* zygote.

2.2. Introduction

Experiments performed by Boveri over a century ago revealed the essential requirement for accurate centrosome inheritance and its role in regulating genome integrity in the developing embryo (Boveri, 1900). In many metazoans, the establishment of the bipolar spindle during the first zygotic cell division is dependent on the paternal contribution of a microtubule organising centre. Following fertilization, this organelle will recruit pericentriolar material present within the oocyte cytoplasm to assemble the two functional centrosomes that will define the first mitotic spindle. In addition to this essential role of the centrosome in organising the spindle, in *C. elegans* this structure is also required to specify the anterior/posterior axis following sperm entry in a microtubule-dependent and-independent manner (Cowan and Hyman, 2004(a); O'Connell et al., 2000; Wallenfang and Seydoux, 2000). Therefore, the appropriate regulation of centrosome number is pivotal since aberrations in these controls result in asymmetrical chromosome segregation and/or severe polarity defects.

Although centrosomes are associated with most nuclei in *C. elegans*, including those in the germ line, they are absent in oocytes, although they are clearly detectable and required for fertility in the sperm (Kemp et al., 2004). The loss of the centrosome from the oocyte is common to many species, while the mechanism responsible for this elimination is currently unknown.

During our characterisation of a *C. elegans* cyclin-dependent kinase inhibitor (*cki-2*) we noticed that compromise of *cki-2* function caused embryos to arrest at the one-cell stage with a multi-polar spindle. We show here that this defect is due to a role of *cki-2* in centrosome elimination and our data provide pioneering evidence on how centrosomes are appropriately eliminated from the developing oocyte.

2.3. Results and Discussion

Recently large-scale screens using RNA-mediated interference (RNAi)-based strategies have provided a framework for understanding many maternally-controlled embryonic processes (Sonnichsen et al., 2005). However, not all genes respond equally to RNAi. Our initial use of RNAi analysis to understand the role of a *C. elegans* CDK inhibitor called *cki-2* was not informative due to the variable penetrance and frequency of the RNAi-related phenotypes, while no *cki-2* alleles are currently available. We therefore turned to an alternative reverse genetic approach called co-suppression, which is a RNAi-related post-transcriptional gene silencing mechanism that is conserved among many phyla (Ketting and Plasterk, 2000).

In wild-type animals *cki-2* mRNA is normally present in the hermaphrodite germ line, but is excluded from the distal mitotic zone (Figure 2.1A). In order to test whether *cki-2* could be compromised through the co-suppression pathway we expressed the 3' portion of the *cki-2* gene (Dernburg et al., 2000), which could not encode a functional protein and shared a very low degree of sequence conservation with *cki-1*, a second *C. elegans* CDK inhibitor (Figure S1.1). The co-suppression transgenic array included a GFP marker facilitating our detection of animals that possessed the transgene. We obtained several transgenic lines in different genetic backgrounds, all of which indicated that reduction of *cki-2* consistently resulted in reproducible embryonic lethality wherein approximately 60% of the GFP transgene-bearing embryos (GFP+) failed to complete embryogenesis (Table 2.1A). The abundance of *cki-2* mRNA was reduced substantially throughout the gonad in these GFP+ animals (Figure 2.1B), while the observed embryonic lethality could be reversed by genetically disrupting this silencing mechanism using mutants in the downstream components of the co-suppression pathway *(mut-7* and *rde-2)*, indicating that the observed lethality was specifically due to the reduction of *cki-2* through co-suppression (Table 2.1A). We therefore refer to these GFP+ animals as *cki-2* co-suppressed (*cki-2cs*).

Although approximately 40% of the *cki-2cs* embryos survive embryogenesis and continue larval development without visible abnormalities, we found that these animals are irradiation-sensitive (Figure 2.1H). This indicates that despite their wild-type appearance, the DNA damage response in *cki-2cs* animals is nonetheless compromised. Therefore, reduction of *cki-2* function results in cell cycle-related abnormalities that reflect the various thresholds of *cki-2* activity required to appropriately execute these cellular processes.

Among the embryonically-arrested embryos, we noticed that 7% of the embryos (n=558) arrested at the one-cell stage with multiple micronuclei (9.1% (n=66)), consistent with abnormal chromosome segregation and/or cytokinesis (Figure 2.1C-E). Examination of the affected zygotes by DIC indicated that early events (contractions of the anterior membrane or ruffling and pseudocleavage) prior to the pronuclear meeting were not significantly different from wild-type (data not shown). Shortly after nuclear envelope breakdown however, the two pronuclei re-formed and several *de novo* micronuclei became apparent. Cleavage furrows appeared occasionally, but would regress and approximately 50% (n=18) of the micronuclei-containing embryos did not form a cleavage furrow. The remaining 50% were defective in cleavage plane orientation, although both classes did undergo multiple rounds of karyokinesis (Figure 2.1C-E).

To better understand the basis of the "one-cell" arrest phenotype, we imaged *cki-2cs* embryos that harboured GFP-histone and GFP-β-tubulin transgenes. In some embryos we observed a second maternal pronucleus (4.5% (n=66)), a meiotic defect that arises due to abnormal polar body exclusion (Figure 2.1F). We also noted that chromosomes failed to align correctly following nuclear envelope breakdown, while the spindle microtubules appeared to be organised around multiple foci, typical of extra microtubule organizing centres or centrosome-like structures (Figure 2.1G).

To confirm that this unique multi-polar spindle phenotype was due to the reduction of *cki-2* and not due to co-suppression-related phenomena or non-specific effects on *cki-1*,

we used an RNAi-sensitive strain (Simmer et al., 2002) to reduce either *cki-1* or *cki-2* levels to reproduce the *cki-2cs*-associated multi-polar spindle phenotype. We did detect one-cell embryos with supernumerary centrosomes following *cki-2(RNAi)* in *rrf-3* (Table 2.1B; Figure 2.3E and F) although the penetrance of the defect was considerably lower than that observed in *cki-2cs* animals. On the other hand, despite causing a high frequency of embryonic arrest in the *rrf-3* background, *cki-1(RNAi)* never caused a one-cell arrest or a multi-polar spindle phenotype (Table 2.1B). Therefore we conclude that the supernumerary centrosomes and the resulting multi-polar spindle defect observed in *cki-2cs* embryos were not due to effects on *cki-1* function or due to co-suppression *per se*, but rather due to a loss or reduction of *cki-2* function.

To address whether *cki-2* affected the centrosome cycle during spermatogenesis, or alternatively during oogenesis, we examined centrosome numbers in early pronuclear stage embryos using an antibody against SPD-2, a coiled-coil protein that associates with the centrosome (Kemp et al., 2004). We noticed that unlike wild-type embryos, strong SPD-2 expression was visible at distinct foci in both the paternal and maternal pronuclei (pronuclear meeting stage) (Figure 2.2A and B). To ascertain whether the presence of the extra centrosomes was indeed due to their contribution from the maternal pronucleus, as opposed to defects associated with failed cytokinesis (Skop et al., 2004), we imaged embryos from meiosis to pronuclear meeting using GFP-γ-tubulin, revealing that GFP-γ-tubulin was associated with the maternal pronucleus in pre-pronuclear migration stage embryos obtained from *cki-2cs* animals (6.7% (n=60); Figure 2.3B and C), while we never observed GFP-γ-tubulin associated with the maternal pronucleus in wild-type embryos (n=80; Figure 2.3A).

Taken together, these results indicate that the supernumerary centrosomes were already associated with the maternal pronucleus at the time of fertilization in *cki-2cs* embryos, possibly because they were not appropriately eliminated in the maternal germ line due to a reduction in *cki-2* function. However, since we could not show definitive live images of an embryonic cell division beginning in the pre-pronuclear stage to the first

mitotic division, we cannot formally rule out that the supernumerary centrosomes may arise from a cytokinesis failure after the first mitotic division.

Therefore, to test whether centrosome elimination is defective in *cki-2cs* oocytes, we stained the gonads of affected (GFP+) and unaffected (GFP-) animals with an anti-SAS-4 antibody to determine if centrioles were abnormally present in the oocytes of *cki-2cs* animals. SAS-4 is associated with all centrioles in *C. elegans* and is required for their duplication (Leidel and Gonzcy, 2003). In wild-type animals SAS-4 is associated with all germ cell nuclei, although SAS-4-staining foci were noticeably absent from oocytes (Figure 2.4A). The absence of the SAS-4/centriole staining in oocytes is consistent with previous observations that the centrosomes are eliminated from the germ cell nuclei at, or around, the stage of oocyte commitment (Albertson and Thomson, 1993).

Anti-SAS-4 staining of the oocytes from the *cki-2cs* hermaphrodite animals revealed that SAS-4 staining structures were present next to the oocyte nuclei at a frequency consistent with the penetrance of the extra centrosome defect caused by the *cki-2cs* transgene (8.9% (n=79)), while no obvious SAS-4 foci were ever observed in oocytes in wild-type animals (Figure 2.4B; data not shown). Although this is the strongest evidence that *cki-2* is required for appropriate centriole elimination during oogenesis, we wanted to further confirm that the anti-SAS-4 staining recognized *bona fide* centrioles and not simply SAS-4 aggregates in the oocyte. We therefore stained the oocytes of wild-type and *cki-2cs* animals using anti-SAS-4 and anti-SAS-6, both of which recognize the centriole (Dammermann et al., 2004; Leidel and Gonczy, 2005). Both antibodies recognized the centrioles of embryos, where they co-localize with γ-tubulin (Figure S1.3 in appendix I). Following double staining we compared the number of overlapping signals between wild-type and *cki-2cs* germ lines (Figure 2.4C-E). Consistent with our previous observation (Figure 2.4B), we noted that significantly more SAS-6 staining oocytes showed overlapping positive signals with anti-SAS-4 in the *cki-2cs* animals (14/55 SAS-6 positive oocytes) compared to wild-type (1/29 SAS-6 positive oocytes-this single overlapping SAS-4 signal may be due to juxtaposition of the signals during the deconvolution process) (Figure 2.4D and E). Therefore, our staining with two independent

centriole-specific antibodies suggests that the observed foci are indeed centrioles, which are not appropriately eliminated in the *cki-2cs* oocytes.

In *C. elegans*, oogenesis occurs in an assembly line-like fashion (Figure 2.5A; Schedl, 1997). We observed that the SAS-4-staining structures persisted into the late stages of oogenesis in *cki-2cs* hermaphrodites (Figure 2.5B-D). These data are consistent with *cki-2* playing a critical role in the timely elimination of the maternal centrioles during oogenesis, and when its activity is reduced below a critical threshold the centrioles persist and eventually will give rise to the supernumerary centrosomes. Although our results strongly argue that *cki-2* is involved in the elimination of maternal centrioles, ultrastructural studies would provide more definitive evidence of centriolar perdurance. Intriguingly, although the maternally-contributed centrosomes are the likely cause of the abnormal division observed in the one-cell arrested *cki-2cs* embryos, we have been unable to show that these supernumerary centrosomes can nucleate microtubules and/or duplicate beyond the first division. We also noticed that the polarity of the affected embryos seems consistently normal based on GFP-PAR-2 (100% (n=17); Figure 2.2C and D) or P-granule staining (Figure 2.2E) (Cowan and Hyman, 2004(b)). Our observation that anterior/posterior polarity does not seem to be affected in *cki-2cs* zygotes suggests that although the maternally-contributed centrosomes appear competent to organise a mitotic spindle, they are seemingly not equivalent to the paternal centrosome in providing the polarity cue in the zygote. The basis of this difference between the centrosome pairs is currently unknown since no difference in centrosomal morphology or molecular composition has been identified between the centrosomes of paternal and maternal origin.

Our observations, although obtained with fixed embryos, suggest that a functional difference may distinguish the maternal and the paternal centrosome in establishing the A/P polarity at fertilization, but because we have been unsuccessful in imaging the maternally-contributed centrosomes into and beyond the first division, while simultaneously monitoring the establishment of the PAR-2 domain, we cannot formally rule out that the polarity is established early by the sperm and the extra centrosomes we

observe in the multinucleate embryos are paternal in origin that have duplicated and appear later due to cytokinesis defects (Figure 2.2A-E).

Because meiotic defects were also observed in *cki-2cs* embryos, we determined whether the abnormal presence of centrosomal components on the meiotic spindle might somehow disrupt the normal mechanism of the acentriolar meiotic division. We found that the morphology of the meiotic spindle in early *cki-2cs* zygotes is disorganised (Figure S1.2C in appendix I), while SPD-2 was detectable as a diffuse haze surrounding the spindle (Figure S1.2A and B in appendix I). We also found that ZYG-1, a protein that is also required for centrosomal duplication (O'Connell et al., 2001), was similarly present on the meiotic spindle in *cki-2cs* zygotes (data not shown), suggesting that the atypical presence of these ectopic centrosomal materials may be responsible for the meiotic spindle abnormalities and the consequent meiotic defects observed in *cki-2cs* embryos.

The loss of *cki-2* could result in misregulated levels of CDK activity within the oocyte, causing a centrosomal anlage to persist and eventually form the tetra-polar spindle that results in a one-cell arrest. To test this scenario, we compromised G1/S CDK activity by performing *cye-1(RNAi)*, which is the only E-type cyclin in *C. elegans* (Fay and Han, 2000).

Loss of cyclin E has no effect on the first cell division in *C. elegans* (Fay and Han, 2000). However, following *cye-1(RNAi)* in *cki-2cs* animals, the characteristic one-cell arrest phenotype was suppressed substantially, which was also reflected in the nearly two-fold reduction in the frequency of the multi-polar spindle defect (Figure 2.2F). A similar degree of suppression was also observed following *K03E5.3(RNAi)*, where *K03E5.3* is the predicted *C. elegans* CDK2 homologue (Liu and Kipreos, 2000; Figure 2.2F). Control animals injected with dsRNA corresponding to cyclin D showed no such effect (data not shown).

That this effect of cyclin E occurs independently of CDK activity (Matsumoto and Maller, 2004) seems unlikely based on the current accepted mechanism of CKI function and our observation that *K03E5.3(RNAi)* suppressed the frequency of the persistence of the

maternal centrosomes to levels comparable to *cye-1(RNAi)*. Our data are thus consistent with the loss of *cki-2* resulting in misregulated cyclin E/CDK2 activity in the germ line that consequently allows centrioles to perdure into the developing oocyte.

That both ZYG-1 and SPD-2 persist during oogenesis and are present on the meiotic spindle in *cki-2cs* embryos suggests that their levels may be regulated by cyclin E/CDK activity, in a manner similar to Mps1 (Fisk and Winey, 2001). The loss of *cki-2* therefore reveals a previously undescribed function of cyclin E/CDK complexes in centrosome stabilisation in the *C. elegans* germ line. Through the timely regulation of this activity, the maternal centrosomes are eliminated as the germ cell acquires its oocyte fate.

This novel function of CDKs and CKIs in centrosome inheritance would probably not have been uncovered through conventional gene targeting in mouse models. Unlike most animals, the sperm does not contribute the centriole(s) in the mouse; instead they arise *de novo* in the fertilised zygote (Schatten, 1994). Why then do most metazoans selectively eliminate the centrosomes within the maternal germline? The answer may come from species that can develop parthenogenetically, where the oocyte is thought to harbour a centriolar anlage (Delattre and Gonczy, 2004.). This would be selected against in species that undergo a biparental mode of development based on sperm-specific centriolar contribution. The elimination of the maternal centrosomes, either through CKI-mediated, or related mechanisms, would block the ability of the oocyte to develop parthenogenetically and strongly favour the union of sperm and egg to trigger the onset of cell division in the zygote.

Because the mode of centrosome inheritance in *C. elegans* shares considerable parallels with that of many animals, identification of the CDK targets in this model may provide invaluable insight pertinent to the mode of centrosome inheritance shared by most metazoans, including humans.

2.4. Materials and Methods

2.4.1. Nematode Strains

The following *C. elegans* strains were used: N2 Bristol was used as the wild-type throughout. MR258 (N2; *rrEx258* [*fem-1*::*cki-2C*; *elt-2*::GFP]), MR306 (N2; *rrEx306* [*fem-1*::GFP; *elt-2*::GFP]), MR294 (*rde-2*; *rrEx294* [*fem-1*::*cki-2C*; *elt-2*::GFP]), MR303 (*mut-7*; *rrEx303* [*fem-1*::*cki-2C*; *elt-2*::GFP]), NL917 (*mut-7* (*pk204*)), WM29 (*rde-2* (*ne221*)), MR446 (*unc-119*; *ruIs32* [*unc-119*(+); *pie-1*::GFP::H2B]; *ojIs1* [*unc-119*(+); *pie-1*::GFP::TBB-2]; *rrEx258* [*fem-1*::*cki-2C*; *elt-2*::GFP]), XA3501 (*unc-119*; *ruIs32* [*unc119*(+); *pie-1*::GFP::H2B]; *ojIs1* [*unc-119*(+); *pie-1*::GFP:: TBB-2]), TH27 (*unc-119*; *ddIs6* [*unc-119*(+); *pie-1*::GFP:: TBG-1]), MR628 (*itIS153* [*rol-6*(+); *pie-1*::PAR-2::GFP]; *rrEx258* [*fem-1*::*cki-2C*; *elt-2*::GFP]), MR824 (*unc119*; *ddIs6* [*unc-119*(+); *pie-1*::GFP::TBG-1]; *rrEx824* [*fem-1*::*cki-2C*; *elt-2*::GFP]), NL2099 (*rrf-3(pk1426)*), KK866 (*itIS153* [*rol-6*(+); *pie-1*:: PAR-2:: GFP]). All *C. elegans* strains were cultured using standard techniques and maintained at 20°C unless stated otherwise (Brenner, 1974).

2.4.2. Constructs

For *cki-2* co-suppression, 3kb of genomic sequence upstream of the *fem-1* translational start site was PCR-amplified from N2 genomic DNA followed by SphI/ Pst1 digestion and insertion into pPD49.26 to yield pMR220. The *cki-2C* fragment (amino acids 116-259-lacking a translational start site (see Fig. S1)) was prepared by PCR and then inserted into pMR220 at the BamHI/XmaI sites to create pMR221. The *fem-1* promoter fragment was inserted into pPD95.77 at SphI/PstI sites to yield pMR266. For RNA interference (RNAi) of *cki-2*: a *cki-2* template for dsRNA synthesis was generated by subcloning the *cki-2* cDNA into the PstI/KpnI sites of pBluescript II to generate pMR215. *cye-1* dsRNA was prepared as described (Fay and Han, 2000). *cki-1* dsRNA was prepared as described (Hong et al., 1998). *K03E5.3* dsRNA template was amplified from a clone of the bacterial feeding RNAi library (I-1D09) using PCR and inserted into the SacI/SacII sites of pBluescript II to generate pMR330.

2.4.3. *cki-2* co-suppression and RNA interference

pMR220 and pMR221 were co-injected (50μg/ml) with 100μg/ml *elt-2*::GFP as a co-injection marker into N2 hermaphrodites as described (Mello et al., 1991). F1 progeny expressing *elt-2*::GFP were singled and their progeny (F2) were scored for transmission of the extra-chromosomal array. Embryonic lethality was scored from each transgenic line. dsRNA was obtained by *in vitro* transcription reactions, annealing, and injection as described (Fire et al., 1998). Injected animals were transferred to new plates every 24 hours and the F1 progeny was examined for visible abnormalities that affected development or cell division.

2.4.4. Antibodies and Immunological methods

The following primary antibodies were used: anti-α-tubulin (Sigma), polyclonal anti-rabbit SPD-2 (a gift from Kevin O'Connell), rabbit polyclonal anti-SAS-4 (a gift from Pierre Gonczy), Cy3-conjugated anti-SAS-6 and Cy5-conjugated anti-SAS-4 (a generous gift from Karen Oegema), rabbit polyclonal anti-P-granule (a gift from Susan Strome). Secondary antibodies were anti-rabbit or anti-mouse Texas-Red or FITC-conjugated secondary antibodies or anti-rabbit Alexa Fluor 594 secondary antibody (all Invitrogen). DAPI (4,6-diamidine-2-phenylindole, Sigma) was used to counterstain slides to reveal DNA. Embryos or hermaphrodite gonads was fixed and stained as described elsewhere. Indirect immunofluorescence microscopy was performed using a 60x oil-immersion objective lens in a Leica DMR compound microscope equipped with a Hamamatsu C4742-95 digital camera, imaging ~0.5 μm-thick optical section. Image analysis, computational deconvolution and pseudocolouring were performed using Openlab 4.0.2 software (Improvision, UK). All images using Cy3-conjugated anti-SAS-4 and Cy-5-conjugated anti-SAS-6 were acquired (using a 60x oil-immersion objective lens) and deconvolved using a DeltaVision Image Restoration System (Applied Precision). Data were collected as a series of 35 optical sections in increments of 0.25 μm under standard parameters using the SoftWoRx 3.0 program (Applied Precision). Images were processed using Adobe Photoshop (version 8.0). All microscopic works were performed at 20°C.

2.4.5. *In situ* hybridisation

Digoxigenin-labelled antisense and sense probes were generated using T7 and T3 kits with digoxigenin-11-UTP (Roche). *In situ* hybridization was performed on the gonads dissected from wild-type or *cki-2cs* (GFP+) adult hermaphrodites as described (Feng et al., 1999).

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2.7. Legends to Figures

Figure 2.1. ***cki-2* co-suppression (*cki-2cs*) causes multiple phenotypes typical of a negative cell cycle regulator.** (A,B) *in situ* RNA hybridisation using an antisense *cki-2* probe on (A) wild-type, or (B) *cki-2cs* gonads extruded from adult hermaphrodites. (C,D) Sequential DIC images of a *cki-2cs* one-cell embryo showing normal pronuclear meeting (C), nuclear divisions without appropriate cytokinesis giving rise to supernumerary nuclei (D, arrowheads) with variable DNA content based on staining with DAPI (E). (F,G) A sequential GFP fluorescence image of *cki-2cs* one-cell arrested embryo that expresses [H2B::GFP; β-tubulin::GFP]. The open arrowhead indicates an extra maternal pronucleus and asterisks (*) mark centrosomes. The arrows indicate polar bodies. (H) Irradiation sensitivity of *cki-2cs* (GFP+) (closed square) or wild-type sibling (GFP-) animals (open circle). The values are presented as the percentage of embryos that hatched from a total population of embryos laid from irradiated, or not, parents that were examined at each point. At point zero (0) in each experiment, the survival (%) was normalized to 100%. The error bars represent the standard deviation (±SD) of two independent experiments ($p<0.05$, 95% confidence). Scale bar is 10μm.

Figure 2.2. **Supernumerary centrosomes observed in *cki-2*cs embryos are contributed by the maternal pronucleus in a cyclin E/CDK2-dependent manner.** (A-B) late pronuclear stage (A) wild-type, or (B) *cki-2cs* one-cell embryo stained with DAPI (blue), anti-SPD-2 (green), and anti-α-tubulin (red). The small arrowheads indicate the pronuclei at different stages. (C-E) (C,D) PAR-2::GFP (red) in the posterior cortex (open arrowhead), of (C) a wild-type, or (D) a *cki-2cs* one-cell embryo. (E) anti-P-granule staining (red spots; closed arrowhead) of a *cki-2cs* one-cell embryo. Arrows indicate polar bodies (anterior) and the white asterisks (*) mark centrosomes. p and m, paternal and maternal pronuclei, respectively. (F) Frequency (%) of *cki-2cs*-associated one-cell arrest and the persistence of maternal centrosome following *cye-1(RNAi)* or *K03E5.3(RNAi)*. Standard deviation (±SD) of at least three independent experiments is shown and asterisks represent significant differences compared to *cki-2cs* controls ($p<0.05$, 95% confidence). n.d, not determined. [1]The one-cell arrest phenotype was presented as the percentage of

unhatched one-cell embryos from total number of progeny (embryos and larvae). [2]The embryos from injected or uninjected (control) animals were labelled with DAPI and anti-SPD-2 antibody 24 hours after dsRNA microinjection and the resulting one-cell embryos were examined for supernumerary centrosomes. The results are presented as the percentage of the total number of embryos examined at the one-cell stage (n). All one-cell embryos examined were at, or prior to the first cell division. [3] The variation observed in the penetrance of the centrosome defect is due to the progressive silencing of the co-suppression transgene over time.

Figure 2.3. ***cki-2(RNAi)* causes defects in the elimination of the maternal centrosome.** (A) Early wild-type one-cell embryo (pre-pronuclear migration stage), or (B-C) *cki-2cs* embryos that express GFP-γ-tubulin to visualize centrosomes. (D-F) Early one-cell embryos (pre-pronuclear migration stage) from (D) *rrf-3*, or (E,F) *rrf-3; cki-2(RNAi)* adult hermaphrodites stained with anti-SPD-2 antibody. The arrow indicates polar bodies stained with DAPI (anterior). White asterisks (*) mark centrosomes (maternal and paternal). p and m, paternal and maternal pronuclei, respectively. The white rectangular box in (A) shows the paternal centrosome that could not be observed in the same focal plane. The rectangular boxed regions in (B,D-F) were magnified to show greater detail.

Figure 2.4. **Centrioles are not appropriately eliminated during oogenesis in *cki-2cs* animals.** (A,B) Extruded gonads from (A) wild-type, or (B) *cki-2cs* adult hermaphrodites stained with DAPI (red) and anti-SAS-4 (green). The bracket in (A) delineates the region that corresponds to oocyte commitment where about 50% of the germ cell nuclei stain positively for SAS-4. The region within the rectangular box is shown in detail and the open arrowheads indicate SAS-4 foci (centrioles), in this inset and throughout. The inset in (B) shows a magnified oocyte (from the white frame) with two SAS-4 staining foci. (C-E) (C) a wild-type meiotic germ cell, or (D) a wild-type oocyte, or (E) an oocyte from a *cki-2cs* adult hermaphrodite; all stained with DAPI (blue), Cy3-conjugated anti-SAS-6 (green), Cy5-conjugated anti-SAS-4 (red). The region within the rectangular box is shown at higher magnification. The scale bar is 10μm (A,B) or 2.5μm (C-E).

Figure 2.5. **Centrioles persist into the later stages of oogenesis in *cki-2cs* animals.** (A) Diagram of late stage oogenesis in the proximal gonad arm. The number indicates the position of the oocyte undergoing meiotic maturation. Oocytes in diakinesis of meiotic prophase I prior to maturation (-3, -2); the oocyte adjacent to the spermatheca is designated as -1. (B-D) A proximal gonad arm from (B) a wild-type animal, or (C,D) *cki-2cs* animals stained with anti-SAS-4 antibody. S, Spermatozoa and/or Spermatids and Sp, Spermatheca. Open arrowheads indicate SAS-4 foci detected in the oocyte nuclei (C,D). The white rectangular boxed region was magnified to provide greater detail. The scale bar equals 10μm.

A

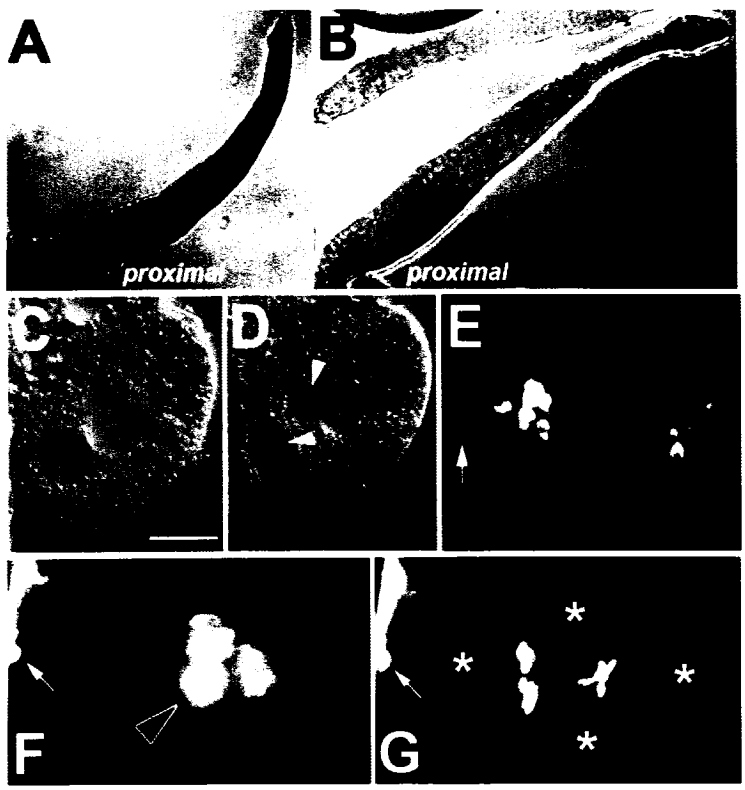
Genotype	% Embryonic Lethality (Emb) (GFP+)	(GFP-)
N2	n.a	0.29 (n=1384)
N2; *cki-2 (RNAi)*	n.a	5.5 (n=710)
N2; [*fem-1*::GFP] (0/4)	0 (n=244)[1]	n.d
N2; [*fem-1::cki-2C*] (3/3)		
line #1	26.9 (n=466)[1]	0.7 (n=280)
line #2	23.3 (n=103)	n.d
line #3	8.1 (n=186)	n.d
rrf-3; [*fem-1::cki-2C*] (2/2)		
line #1	55.3 (n=159)[1]	27.6 (n=116)
line #2	42.2 (n=436)	25.1 (n=231)
TH27 (*pie-1::*γ-tub::GFP); [*fem-1::cki-2C*] (5/5)		
line #1	29.1 (n=1257)[1]	1.7 (n=232)
line #2	21.5 (n=395)	n.d
line #3	19.2 (n=198)	n.d
rde-2; [*fem-1::cki-2C*] (0/2)		
line #1	5.7 (n=357)	7.5 (n=374)
line #2	11.6 (n=404)	17.7 (n=561)
mut-7; [*fem-1::cki-2C*] (0/3)		
line #1	17.9 (n=313)	20.2 (n=325)
line #2	11.4 (n=245)	12.1 (n=440)
line #3	12.7 (n=181)	9.4 (n=276)

B

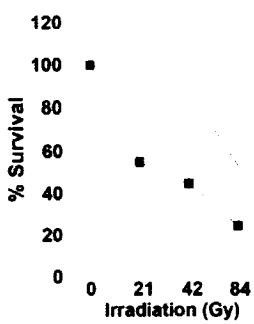
Genotype	% Emb	% Supernumerary centrosome[2]
rrf-3	23.0±1.2 (n=374)	0 (n=76)
rrf-3; cki-1 (RNAi)	94.7 (n=570)	0 (n=40)
rrf-3; cki-2 (RNAi)	27.5±3.7 (n=734)	4.5 (n=111)
N2; [*fem-1::cki-*2C][3]	26.9 (n=466)	13.5 (n=133)
TH27; [*fem-1::cki-*2C][3]	29.1 (n=1257)	6.7 (n=60)

Table 2.1. ***cki-2* co-suppression causes embryonic lethality.** (A) A *C. elegans* strain that harbours an extrachromosomal array containing the [*fem-1::cki-2C*] co-suppression transgene segregates animals that possess the array (GFP+), or not (GFP-), as indicated by the presence of the dominant *elt-2::GFP* co-transformation marker. (B) Similar phenotypes were observed in *cki-2(RNAi); rrf-3* animals, while extra centrosomes were not observed in *cki-1(RNAi)* embryos. The embryonic lethality (Emb) was presented as the percentage of unhatched embryos from total progeny obtained from GFP (+) or (-) young adult animals. The frequency of the Emb phenotype in the various transgenic lines obtained is shown (n/n). n.a, not available and n.d, not determined. The embryonic lethality from GFP (-) animals was determined from only one transgenic line of each tested genotype. [1]The transmission frequency (%) of the transgenic array in these strains was scored as the number of GFP (+) progeny from the total number of progeny, and the transmission rate of the *cki-2cs* strain employed throughout the study was approximately 50%. [2]Embryos were stained with anti-SPD-2 or γ-tubulin::GFP and the results are presented as the percentage of the total number of one-cell stage embryos examined. All one-cell embryos examined were at, or prior to, the first cell division. [3]The frequency of the supernumerary centrosome defect was determined in the most penetrant co-suppressed lines (line #1 of *N2;* [*fem-1::cki*-2C] and TH27; [*fem-1::cki*-2C]) for comparison.

Figure 2.1

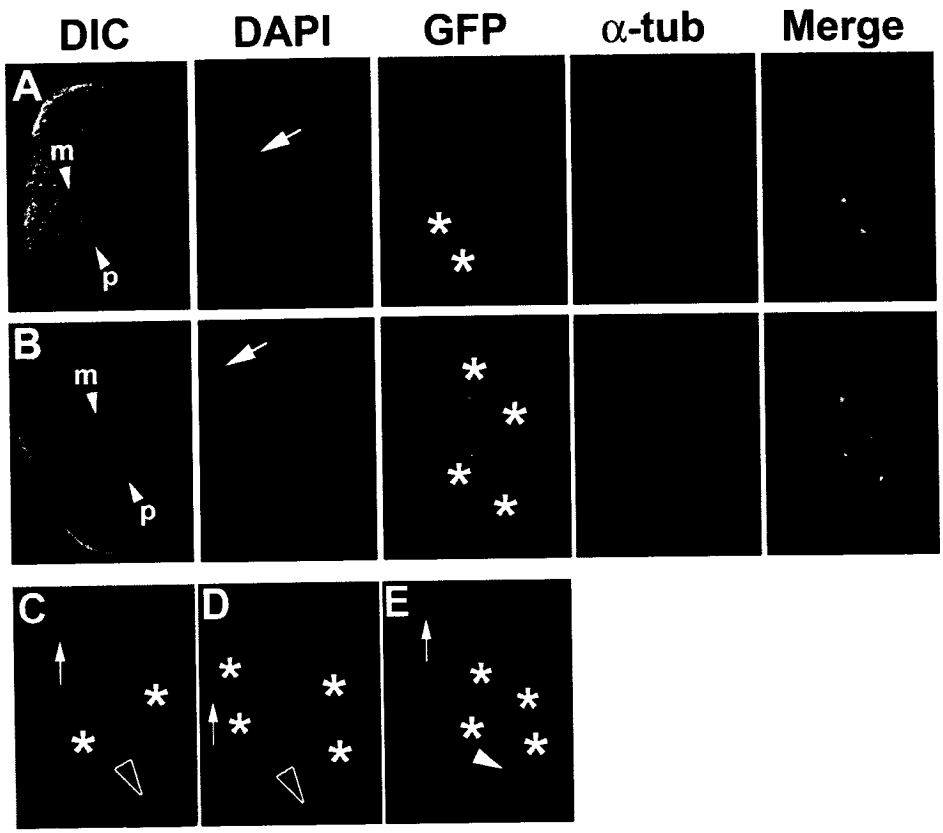


H



Radiation (Gy)	% Survival (GFP-)	% Survival (GFP+)
0	99.3±0.3 (n=766)	69.5±11.3 (n=1409)
21	92.5±1.2 (n=1349)	37.8±4.2 (n=1084)
42	87.1±0.02 (n=1334)	30.8±5.0 (n=527)
84	52.2±10.8 (n=1515)	16.7±4.0 (n=685)

Figure 2.2



F

Genotype	One-cell arrest (%)[1]	Supernumerary centrosome (%)[2]
cki-2cs	1.59±0.45 (n=1860)	14.07±1.85 (n=133)
cki-2cs;cye-1 (RNAi)	0.82±0.22 (n=1720)	5.05±3.99 (n=87)
cki-2cs	n.d	9.48±4.21 (n=102)[3]
cki-2cs; K03E5.3 (RNAi)	n.d	4.58±3.83 (n=55)

Figure 2.3

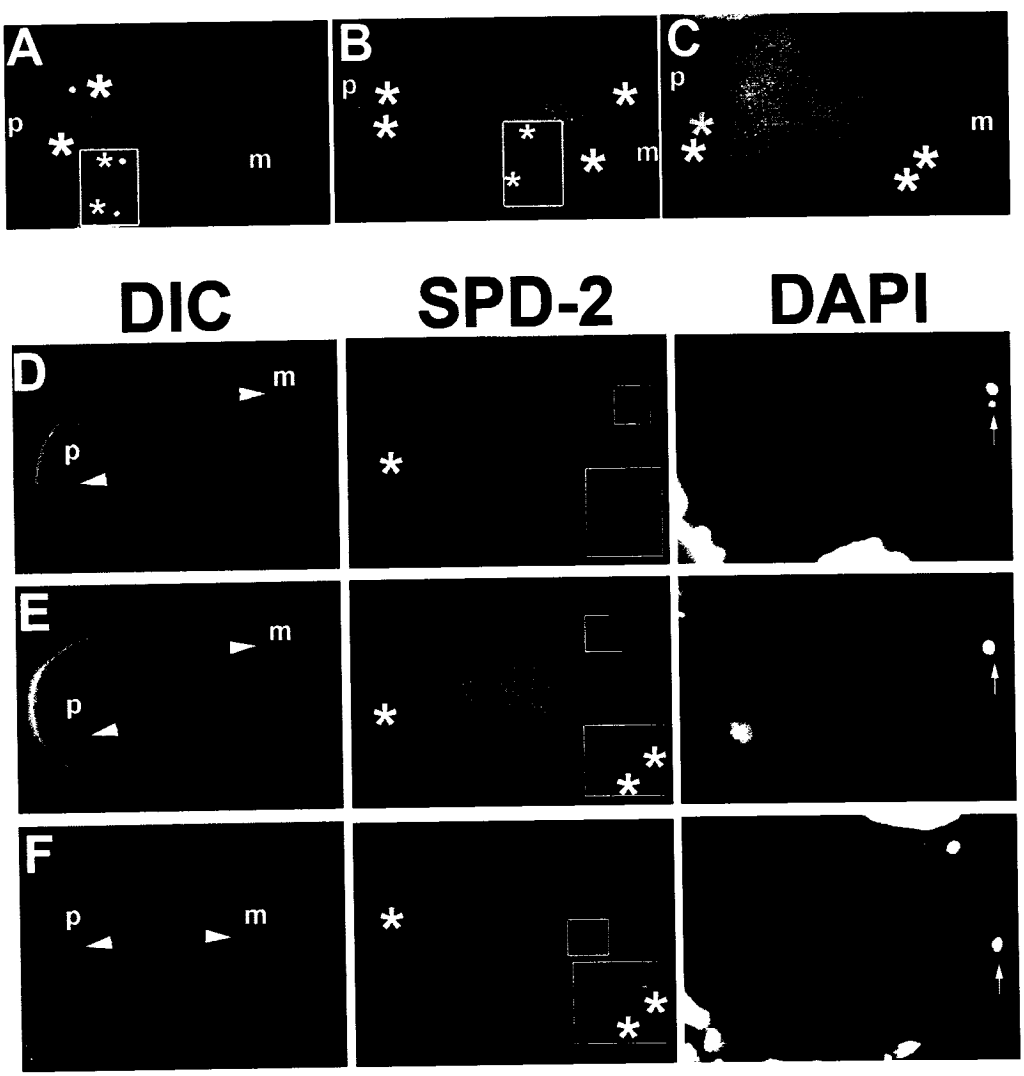


Figure 2.4

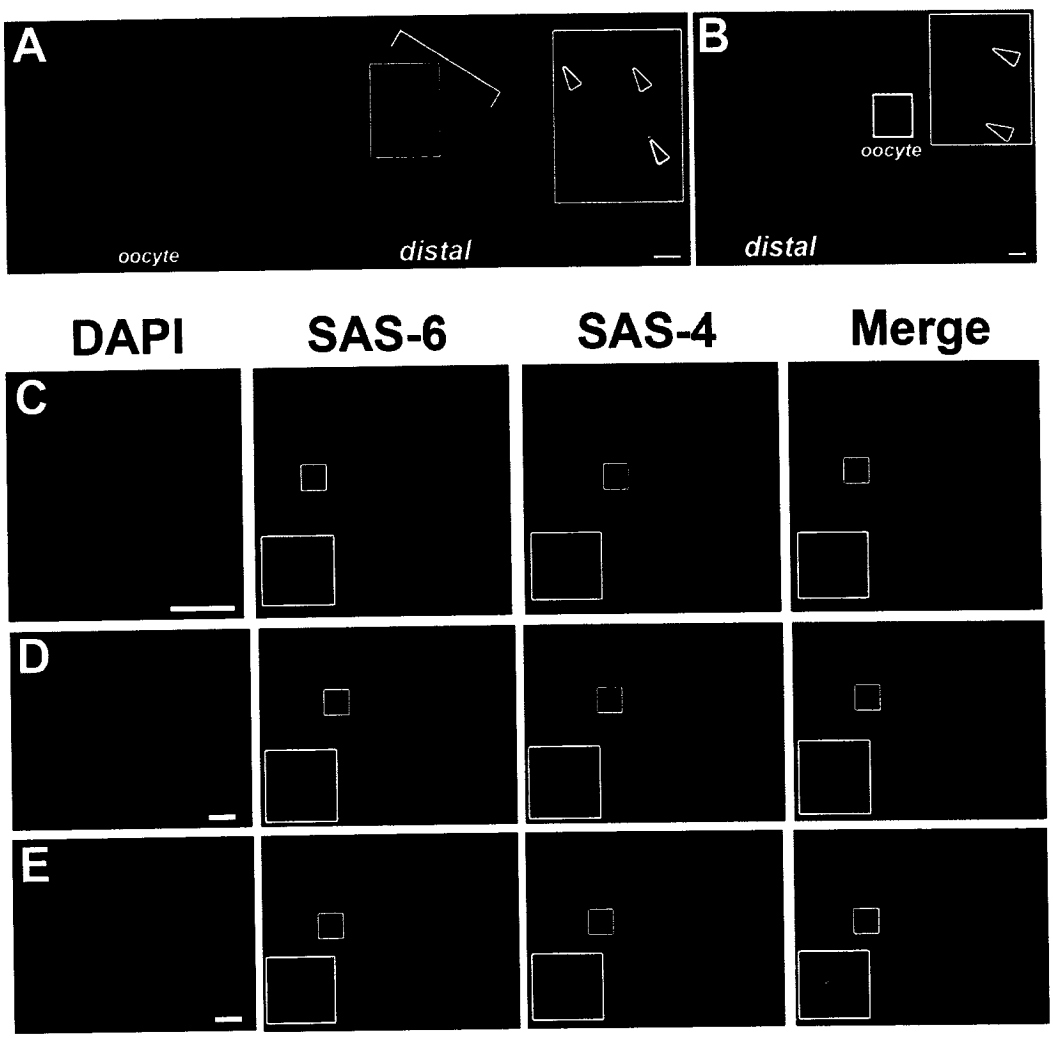
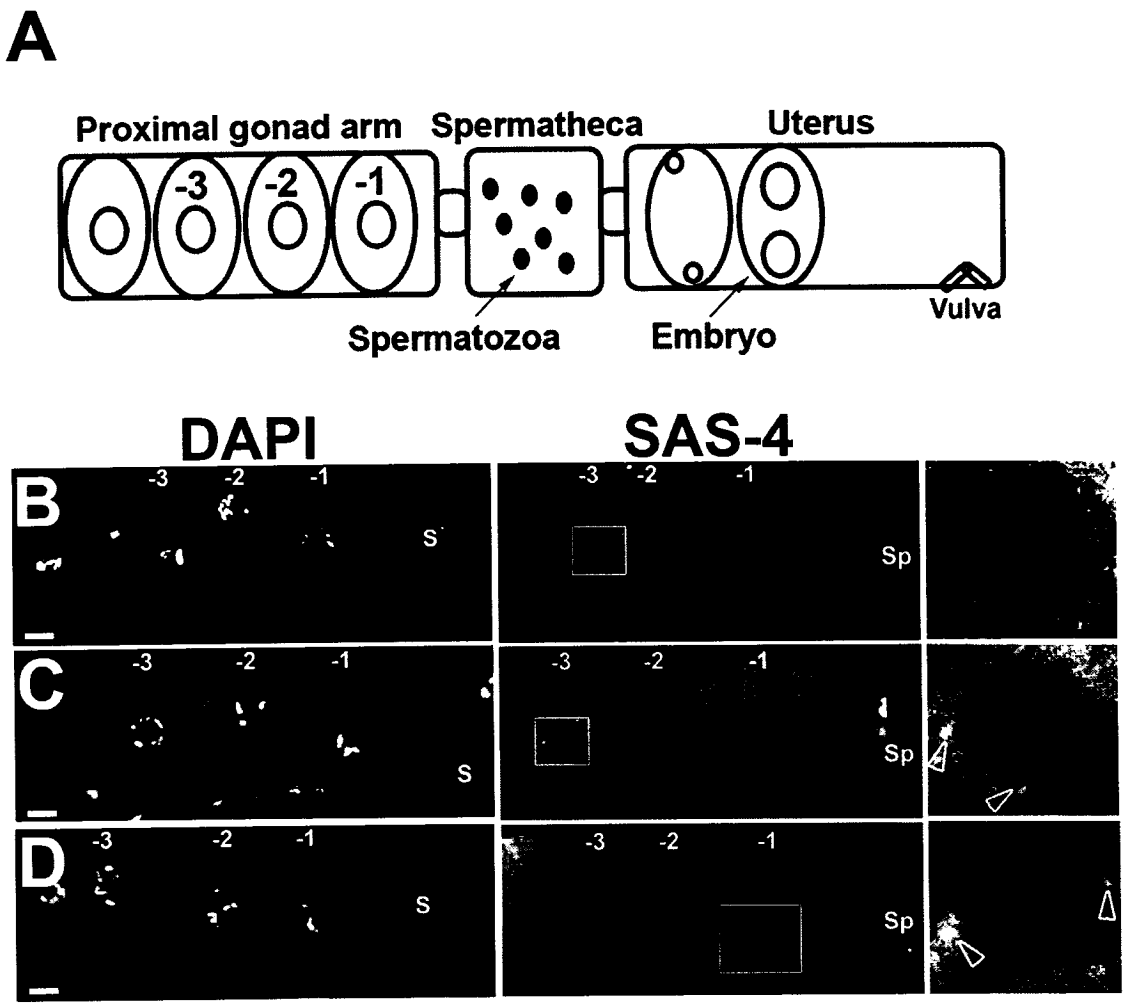


Figure 2.5


A
Proximal gonad arm
Spermatheca
Uterus
-3
-2
-1
Spermatozoa
Embryo
Vulva
DAPI
SAS-4
B
C
D
-3
-2
-1
S
Sp

Connecting text

The data described in chapter II highlight the role of *cki-2*, a CDK inhibitor homologue, in the regulation of centrosome elimination in the germline of hermaphrodite *C. elegans*. We demonstrate that the loss of *cki-2* in the germline results in the formation of a multipolar spindle in the first mitotic division of the embryo due to the presence of extra centrosomes. From detailed characterization of one-cell embryos and the maternal germline using immuno fluorescence and real time imaging, we observed that the centrioles are not appropriately eliminated during oogenesis in the *cki-2* co-suppressed animals, resulting in the maintenance of supernumerary centrosomes into the one-cell embryo. Moreover, we show that *cyclin E (RNAi)* or *K03E5.3 (RNAi)* considerably suppressed the frequency of one-cell embryos carrying the supernumerary centrosomes, suggesting that cyclin E /Cdk2 must be controlled in a timely manner in the germline, presumably by *cki-2* to ensure the proper elimination of centrioles. Overall, we have described a novel role of *cki-2* in the elimination of centrioles during oogenesis, which provides a pioneering step toward understanding how centriole disassembly occurs, in addition to the potential non-cell cycle functions of CDK inhibitors.

Chapter III

CKI-2 regulates embryonic cell divisions and is modulated by SUMO-mediated nucleolar localization and subsequent degradation

3.1. Abstract

In many different cell types, progression through the cell cycle is controlled by regulating the G1-to-S phase transition and this is often achieved by the activity of CDK-inhibitor (CKI) proteins of the CIP/KIP family. During postembryonic development in *C. elegans* diverse developmental and environmental cues impinge on the *cki-1* to confer developmentally-regulated G1 arrest (Hong et al., 1998), however little is known about how cell division may be controlled in the closed environment of the developing embryo. A second *C. elegans* CIP/KIP family homologue, *cki-2,* has been identified but has been less well characterized due to its relative insensitivity to RNAi. We demonstrate here that *cki-2* is an essential regulator of embryonic cell cycle progression and reduction of function leads to extra cells in both the intestinal and pharyngeal fields during embryogenesis. Using a yeast two-hybrid screen we identified two CKI-2 interactors (*C. elegans* PCNA and SUMO). *C. elegans* SUMO covalently modifies CKI-2 resulting in its subsequent localization to the nucleolus followed by rapid degradation of CKI-2, suggestive of a novel mechanism to maintain appropriate cellular CKI-2 levels. Interestingly, evolutionarily divergent CDK inhibitor family proteins carry the consensus SUMOylation sequence at the CDK inhibitory domains and a predicted nucleolar localization signal in the C-terminus. Therefore, we suggest that this novel regulatory mechanism may represent an ancestral method of controlling the activities of these critical cell cycle effectors.

3.2. Introduction

The morphological complexity typical of most animals is generated predominantly during the embryonic stages of development. During this period cell division and cell fate specification must occur synchronously to instruct cell populations to give rise to the tissues necessary for postembryonic life. Despite the importance of these divisions little is understood about how they are regulated, although the importance of localised degradation of key cell cycle effectors has been implicated (Su et al., 1998). Studies in *C. elegans* have indicated that division timing during embryogenesis is dependent on S-phase progression within the individual blastomeres (Edgar and McGee, 1988; Lambie, 2002). How these rapid cycles can occur in an environment where positive cell cycle effectors remain non-limiting, and remain regulated in a robust, invariant, oscillatory manner is also unclear. This becomes particularly important since any reduction in replication efficiency that may occur during these cleavage divisions could result in cell fate alterations, suggesting that the integrity of the DNA replication complex, or its timely function, is required to appropriately specify cell fates during these crucial divisions (Encalada et al., 2000).

In some organisms cyclin-dependent kinase inhibitors (CKIs) provide this control through their canonical function in S-phase regulation (Sherr and Roberts, 1999). p21Cip1/Waf1, a mammalian CIP/KIP family protein, negatively regulates the cell cycle through binding a variety of CDKs in addition to its ability to associate with the DNA replication processivity factor PCNA (proliferating cell nuclear antigen) (Waga et al., 1994). In *Drosophila* and *C. elegans* G1/S progression during development is controlled, at least in part, by the CKI family members Dacapo and *cki-1*, respectively (Lane et al., 1996; Hong et al., 1998). Both of these genes have roles in cell cycle regulation during embryogenesis (Lane et al., 1996; Kostic and Roy, 2002), therefore it is conceivable that the regulation of S-phase progression which governs cell division during embryogenesis, could be regulated through the action of maternally-deposited CKI proteins or mRNA.

The role of *cki-1* has been characterised in numerous developmental contexts in *C. elegans* (Hong et al., 1998; Kostic and Roy, 2002; Fukuyama et al., 2003; Baugh and Sternberg, 2006), but its role appears to be critical for later embryonic events and larval development. A second CKI present on chromosome II adjacent to *cki-1* is under different promoter control and is not functionally redundant with *cki-1* (Feng et al., 1999; Fukuyama et al., 2003). This CKI, referred to as *cki-2,* has been difficult to characterise due to its refractoriness to standard RNAi analysis.

Here we show that *cki-2* is an essential cell cycle regulator during early embryonic cell divisions. Its endogenous levels are very low and appear to be under strict control mediated through site-specific SUMOylation of lysine residues present in the N-terminal domain of the protein. This modification results in subsequent localization to the nucleolar compartment after which CKI-2 is rapidly degraded. This novel means of regulation may be a conserved mechanism of cell cycle control as other conserved CKI-2 orthologues share similar putative C-terminal nucleolar localization sequences in combination with consensus SUMOylation sites in their respective N-terminal domains.

3.3. Results

3.3.1. CKI-2 interacts with PCNA and SUMO

Although characterization of *cki-1* has shown that this CKI plays a critical role in the timing and specification of postembryonic cell divisions the role of *cki-2* has remained somewhat enigmatic. Initial studies showed that *cki-2(RNAi)* caused embryonic lethality although the precise role of *cki-2* in this embryonic arrest was not characterized in detail probably because of the associated invariable penetrance of the phenotype (Feng, et al., 1999). Using a related post-transcriptional gene silencing strategy called co-suppression (Plasterk and Ketting, 2000) we showed that specific loss of *cki-2* resulted in reproducible embryonic lethality, whereby small subset of embryos arrested at one-cell stage with multipolar spindles, due to extra centrioles derived from maternal germline (Kim and Roy, 2006). Our study suggested that CKI-2 might be required for timely elimination of centrioles during oogenesis, likely through its activity to inhibit the catalytic function of cyclin E or cyclin E/Cdk2 complex (Kim and Roy, 2006). Thus, in order to gain further functional insight concerning the role of *cki-2* during development, we screened a *C. elegans* cDNA library for interacting partners using three different CKI-2 bait variants (full length (CKI-2(1-259)), N-terminal domain (CKI-2N(1-115)) and C-terminal domain (CKI-2C(116-259)) (Figure 3.1A; Figure S2.1A and B). We isolated two interacting proteins from independent screens: PCN-1, the *C. elegans* PCNA orthologue, was isolated with both the full length and the C-terminal variant; SMO-1, the SUMO orthologue, was identified from screens with the N-terminal variant only (Figure 3.1A, top).

Because of the similarity shared between CKI-1 and CKI-2 we tested whether the CKI-2 interacting proteins could also associate with CKI-1. Our data indicated that PCN-1 interacted with both CKI bait targets in the directional yeast-two hybrid assay (Figure 3.1A, bottom), while SMO-1 seems specific for CKI-2 (Figure 3.1A, bottom). We mapped the PCN-1-binding region of CKI-2 using a series of deletion constructs (LexA-DBD fused baits of CKI-2) (Figure 3.1B), which indicated that the PCN-1 interaction domain mapped to regions that shared considerable conservation with previously described PCNA-binding

motifs (Figure 3.1C) (Warbrick, 1998). These data imply that CKI-2 might act in a similar manner to mammalian p21Cip1 which comprises two separate domains (cyclin/CDK inhibitory domain and the PCNA binding domain) that are functionally independent and may be regulated by different mechanisms (Lio et al., 1995).

3.3.2. Heat shock expression of CKI-2 and its variants show distinct phenotypic effects on developmental cell cycle progression

Previous studies have revealed that the ability of p21Cip1 to inhibit CDK and PCNA is due to two separate domains and are functionally independent (Figure 3.2, top) (Luo et al., 1995). Since CKI-2 possesses a conserved N-terminal domain that is critical for binding cyclin/CDK and a C-terminal PCN-1 binding domain, we wondered whether each CKI-2 domain might also possess distinct functional properties, whereby heat shock expression of truncated CKI-2 variants may have distinguishable effects on cell division or development consistent with their ability to interact with different binding partners through their various domains.

Heat shock expression of the full-length CKI-2 (CKI-2::GFP) or the N-terminal variant of CKI-2 (CKI-2N::GFP) caused substantial embryonic lethality, while expression of the C-terminal variant (CKI-2C::GFP), which contained the PCNA interaction domain, but not the CDK-inhibitory domain, showed little to no embryonic lethality (Table 3.1). Both the full length CKI-2 and both of the CKI-2 variants were expressed in the nucleus at comparable levels (Figure 3.2, bottom). Therefore, presumed titration of PCNA following heat-shock expression of the C-terminal fragment of CKI-2 had little or no obvious effect on viability, while the contrary was true for the CDK-inhibitory domain-containing N-terminal portion of CKI-2 which caused embryonic lethality at a level similar as the full length protein, suggesting that the embryonic lethality caused by heat shock expression of CKI-2 may derive from elements present in the N-terminal domain of the protein, and not due to its ability to titrate the replication factor PCNA.

Using a transcriptional fusion GFP reporter driven by 5 kb of *cki-2* genomic sequence upstream of the translational start site, we found that CKI-2::GFP is zygotically expressed only in a restricted number of cells, namely in the vulval cells suggesting a role during the later stages of vulva formation (data not shown). Since we did not detect any visible postembryonic phenotypes following *cki-2 (RNAi),* we misexpressed CKI-2::GFP and its two variants (CKI-2N::GFP and CKI-2C::GFP) postembryonically and monitored the effects on vulval patterning or other aspects of vulval formation. Our data revealed that heat shock expression of CKI-2N::GFP, or CKI-2::GFP during early larval stages (L2 and L3) disrupted vulval morphology, resulting in an apparent protruding vulva (Pvl) phenotype, typical of factors that perturb cell cycle timing or division integrity in this epithelium (Table 3.2) (Ambros, 1999; Fay and Han, 2000; Wang and Sternberg, 2001). On the other hand, heat shock expression of CKI-2 and the variants at the L1 stage had no such effect (Table 3.2). However, the frequency (%) of the Pvl phenotype was dramatically increased when CKI-2::GFP and CKI-2N::GFP were induced at the L2 stage, just prior to the initiation of vulval specification, while almost all the animals expressing CKI-2N::GFP were Pvl following induction during the L3 stage; the period when the characteristic cell divisions and specification events occur in the vulval lineage (Table 3.2). Our data showed that the full length CKI-2 was much less effective in disturbing vulva patterning than the N-terminal variant. This could reflect an antagonism between the CKI-2 C-terminal domain and the cyclin/CDK inhibitory domain in the full length protein.

Taken together, our findings indicate that the maintenance of appropriately low CKI-2 levels is important for correct embryonic and postembryonic development. Furthermore, this activity itself may be modulated through interactions between the N-terminal inhibitory domain with the C-terminal region of the protein.

3.3.3. CKI-2 is SUMOylated in vivo

Most protein targets that have been identified as SUMO-interacting partners from the yeast two-hybrid screen have also proven to be *bona fide* SUMO substrates (Gostissa et al., 1999; Minty et al., 2000). By scanning the N-terminal domain of CKI-2 (1-115) we

identified two consensus SUMOylation target sites (ψKxD/E) (Melchior, 2000), K20 and K40, where K20 (lysine 20) is strongly predicted (www.abgent.com/sumoplot.html) (Figure 3.1B and S2.1A). Our analysis did not identify a SUMOylation target site in CKI-1, consistent with the yeast two-hybrid result (Figure 3.1B). We therefore postulated that CKI-2 might be a substrate for SUMOylation *in vivo*. To verify whether CKI-2 is indeed SUMOylated in *C. elegans*, we used the transgenic strain that carried a heat-shock inducible *cki-2* transgene (*hs*::CKI-2) (because endogenous levels of CKI-2 are below the detectable levels of our antibody) to see if overexpression of CKI-2 (~28 KDa) could give rise to higher molecular weight entities that contained CKI-2, typical of posttranslational modifications mediated by ubiquitin and its family members. In a western blot analysis performed with *C. elegans* extracts prepared from heat-shocked animals, two distinct proteins (~38 and ~64 KDa) were detected by anti-CKI-2 antibody (Figure 3.3A, lane 1). These molecular weights correspond to the predicted size of CKI-2 if one or both potential lysines present in the N-terminal domain (K20 and K40) were modified by SMO-1. We did not detect any such signal in control (non-heat shocked) embryo extracts (Figure 3.3A, lane 3).

To test whether the presence of these modified CKI-2 variants required SUMOylation we repeated the heat shock experiment, but only this time we removed endogenous SMO-1 by feeding animals bacteria that expressed *smo-1* dsRNA. Immunoblot analysis of animals that overexpressed CKI-2, but were subjected to *smo-1(RNAi)* by feeding (Kamath et al., 2001), indicated that the higher molecular weight CKI-2 containing bands disappeared, while the lower band was still detected by CKI-2 antiserum (Figure 3.3A, lane 2), indicating that the upper band requires SMO-1. This therefore suggested that the higher molecular weight band we observed in the extracts made from animals that overexpress CKI-2 likely corresponds to CKI-2 modified by endogenous SMO-1.

Although this implicates SUMO in the appearance of the higher molecular weight entities present following the ectopic expression of CKI-2, we wanted to show that SUMO was also present in these modified CKI-2-containing bands. To do this we generated a heat-shock inducible HA-tagged version of SMO-1 (HA::SMO-1) and crossed it into the *hs*::CKI-2 background. We then performed immunoblot analyses using embryo extracts prepared from heat-shock induced transgenic embryos where we found that the anti-CKI-2 antibody recognized two bands (~38 and ~64kD), consistent with SUMOylation on one or both potential lysines (Figure 3.3B, left panel, column 3). Furthermore, when the anti-HA antibody was used for detection, the two bands recognized by the anti-CKI-2 antibody were also predominantly labeled with the anti-HA (Figure 3.3B, right panel, column 3), indicating that the HA::SMO-1 and CKI-2 were present in the same bands, consistent with CKI-2 being SUMOylated following heat shock induction of the two proteins. The presence of the two bands suggests that CKI-2 could be SUMOylated on the predicted lysines (K20 and K40), and in support of this, when both lysines are mutated to alanine (CKI-2(Δsmo)), no higher molecular weight species are detectable consistent with the CKI-2 protein being modified at either one or two of the predicted SUMOylation sites in the N-terminal domain (data not shown). To show that this SUMOylated band is indeed associated with CKI-2 we performed an immunoprecipitation experiment to see if the HA::SUMO signal could be detected in the anti-CKI-2 immunoprecipitates. Our CKI-2 antibody efficiently immunoprecipitates CKI-2 (Figure 3.3B, left panel, column 1 and 2) while in these fractions the CKI-2-specific band was also recognized by the anti-HA antibody indicating that both CKI-2 and HA::SUMO are present in the same band (Figure 3.3B, right panel, column 1 and 2). Taken together, these data suggest that our anti-CKI-2 antibody recognizes the overexpressed CKI-2, although endogenous levels are below the threshold of detection, and these increased levels of CKI-2 are SUMOylated *in vivo*, likely on lysine residues present in the N-terminus of CKI-2.

3.3.4. CKI-2 is a nuclear protein that co-localizes with chromosomal DNA

Since the expression levels of each of the CKI-2 variants were comparable (Figure 3.2), yet they showed distinguishable effects at different developmental stages, we wondered

whether SUMOylation could have a differential effect on the function of the CKI-2 variants. SUMOylation has been implicated in regulating sub-cellular localization therefore the SUMOylation of CKI-2 could result in changes in its ability to interact with its targets. Therefore, to gain further insight of the CKI-2 localization, we raised anti-CKI-2 antisera to detect the endogenous CKI-2 protein. Although the antibodies clearly recognize CKI-2 protein produced in *E. coli* (data not shown) no signal could be detected in gonads, embryos, or larvae. However, when CKI-2::GFP expression is driven from a transgenic array using a heat shock-inducible promoter, the anti-CKI-2 antibodies recognized a single polypeptide band in extracts that corresponded to the expected size of the CKI-2::GFP fusion, but was not present in control animal extracts (Figure S2.2A, left panel). That this same band was detected using an anti-GFP antibody suggests that the anti-CKI-2 recognizes its target (Figure S2.2A, right panel, top). The anti-CKI-2 which was generated using the N-terminus of CKI-2 (CKI-2N) did not recognize CKI-2C::GFP, while anti-GFP antibody detected the same band (Figure S2.2A, right panel, bottom). When transgenic animals were stained following heat shock, CKI-2 could be detected on or near the chromatin, which overlapped precisely with GFP (Figure 3.4A), while pre-immune serum did not stain the CKI-2::GFP-expressing embryo (Figure S2.2B). Moreover, the anti-CKI-2 antibody did not detect CKI-2C::GFP (Figure S2.2C). Our data therefore suggest that CKI-2 is normally maintained at very low levels which are below the limit of detection of our antibody and that when these levels are increased the protein accumulates in the nucleus, most likely in association with chromatin.

Because we could not detect endogenous CKI-2 *in situ* we expressed CKI-2::CFP using a *pie-1* promoter in order to visualize the sub-cellular localization and expression dynamics of maternally-contributed CKI-2 during the early stages of embryogenesis and in the germ line. CKI-2::CFP was reproducibly detected in all germ cell nuclei including the oocytes. In prophase nuclei CKI-2::CFP appears to be associated with chromatin based on the overlap with the DAPI stained entity. Very little or no signal above background could be detected in non-transformed siblings (Figure 3.4B).

3.3.5. The C-terminal domain of CKI-2 possesses signals important for nucleolar localization

We next examined the localization of GFP-tagged CKI-2 and the variants following heat shock induction and in order to facilitate our imaging we focussed our attention on the largest individual cells in the growing larva; the intestinal cells. Heat shock induction of the various CKI-2 variants was sufficient to allow us to detect the fusion proteins within these cells. Consistent with our antibody staining results in the embryo, CKI-2::GFP and CKI-2N::GFP were present predominantly in the nucleoplasm, (Figure 3.5A-C), while quite surprisingly, CKI-2C::GFP was exclusively nucleolar in most somatic cells including the intestinal cells, which was confirmed by staining with an anti-fibrillarin antibody that specifically recognizes this organelle (Figure 3.5D).

Intriguingly, using a SMO-1::GFP translational fusion driven by 3kb of endogenous *smo-1* genomic sequence (*smo-1*::SMO-1::GFP) we found that SMO-1 localized primarily to the nucleoplasm (Figure 3.5E), but was also present in nucleoli (Figure 3.5E). This was further corroborated by anti-HA antibody staining following heat shock induction of HA::SMO-1. HA::SMO-1 is seen predominantly within the nucleolus of the intestinal cells following heat shock, although relatively lower levels of the HA signal continues to be visible in patches within the nucleoplasm (Figure 3.5F). These observations suggest that the localization of both CKI-2 and SMO-1 may be dynamic and may depend either on its various targets or specific signals received.

3.3.6. Co-expression of CKI-2 and SMO-1 results in nucleolar localization of CKI-2

Because the C-terminal domain of CKI-2 constitutively localizes to the nucleolus, we concluded that this domain must contain important information for determining sub-nuclear localization. Furthermore, because the overexpression of the N-terminal domain of CKI-2 produced a significantly greater frequency of Pvl than the full-length protein, we propose that an interaction between these two domains may modulate CKI-2 function. Interestingly, the canonical SUMOylation sites are also located in the N-terminal domain of CKI-2 indicating that SUMO may be a key regulator of CKI-2 activity via these

sites in the N-terminus adjacent to the cyclin/CDK inhibitory domain. Consistent with this possibility, co-expression of SMO-1 suppressed the embryonic lethality caused by ectopic expression of CKI-2::GFP or CKI-2N::GFP (Table 3.2), Similarly, co-expression of HA::SMO-1 was also able to suppress the frequency (%) of the Pvl phenotype caused by heat shock expression of CKI-2N::GFP following induction at both the L2 and L3 stages (Table 3.2), yet no suppression in the embryonic lethality, or in the frequency of Pvl could be detected when a CKI-2::GFP construct (CKI-2(Δsmo)::GFP) that lacked the consensus SUMOylation sites in the N-terminus was used (Table 3.1 and 2), confirming that the SUMO-dependent suppression is indeed mediated through the N-terminal SUMOylation sites.

SUMOylation has been recently implicated in vulval development and *smo-1 (RNAi)* animals that survive embryogenesis exhibit Pvl phenotpes (Broday et al., 2004). Therefore, the SUMO-mediated suppression we observe in the vulva may arise from the ability of overexpressed CKI-2 or CKI-2N to titrate limiting amounts of SUMO from its normal physiological targets. This suppression could also be due to SMO-1-dependent regulation of sub-nuclear localization by modifying CKI-2 such that the SUMO moiety is recognized as a signal for nucleolar localization. Alternatively, SUMO- dependent conformational changes that permit accessibility to C-terminal regions that mediate nucleolar shuttling factors could also be involved.

To investigate the possibility that SMO-1 may affect the sub-cellular localization of CKI-2 either directly or indirectly, we imaged the intestinal cells of animals that co-expressed both CKI-2::GFP and HA::SMO-1 after heat shock in order to monitor their localization patterns. Surprisingly, the GFP signal (CKI-2) was present predominantly in the nucleolar compartment wherein HA::SMO-1 co-localized with the GFP in the intestinal cells and in other cell types (Figure 3.5G). This change in sub-cellular localization is dependent on SUMO conjugation mediated through the consensus N-terminal SUMOylation sites since CKI-2(Δsmo)::GFP, although expressed at somewhat lower levels than the wild-type CKI-2::GFP, is clearly nuclear and upon co-expression

with SMO-1 and does not localize to the nucleolus (Figure 3.5H), attesting to the importance of SUMOylation of CKI-2 in triggering this sub-nuclear localization switch. These results strongly suggest that SMO-1 can modify the sub-nuclear localization of CKI-2 and the conjugation of SMO-1 precedes the localization of CKI-2 to the nucleolar compartment where its sequestration may counterbalance the negative effects of increased levels of CKI-2. However our data cannot distinguish whether indeed SUMOylation is directly associated with the observed sub-nuclear localization of CKI-2 ie...SUMOylation is sufficient; or whether this modification results in a conformational change in the protein that is recognized by another factor(s) that shuttles to the nucleolus.

Previous studies have shown that an in frame SUMO fusion can mimic the constitutively SUMOylated protein (Ross et al., 2002; Taylor and LaBonne, 2005). Therefore, if SUMOylation of CKI-2 were sufficient for the nucleolar localization of CKI-2, an in frame SMO-1 fusion to the N-terminal variant of CKI-2, which normally remains within the nucleoplasm when overexpressed, would be sufficient to instruct the CKI-2N::GFP to localize to the nucleolar compartment. We therefore generated an in frame HA::SMO-1 fusion to the N-terminus of CKI-2N::GFP (HA::SMO-1:: CKI-2N::GFP) and expressed it using heat shock induction. Surprisingly, in most cells examined, and most obviously in the intestinal cells, the addition of a SUMO domain to CKI-2N::GFP was sufficient to localize the protein from the nucleoplasm where CKI-2 normally resides, to the nucleolus, albeit not as efficiently as that observed for the CKI-2C::GFP fusions which are constitutively nucleolar (Figure 3.6A).

Furthermore, we observed that even though the level of expression was comparable to the CKI-2N::GFP alone, the resultant embryonic lethality or the Pvl frequency was reduced in strains overexpressing HA::SMO-1::CKI-2N::GFP and was comparable to strains that coexpressed both CKI-2N::GFP and HA::SMO-1 (Table 3.2). However, we cannot rule out that the SMO-1 modification of, or in proximity to, the N-terminal inhibitory domain of CKI-2 may inadvertently compromise its inhibitory function in addition to its role in altering its capacity to localize to the nucleolus. Taken together, our

results indicate that SMO-1 modifies the N-terminus of CKI-2 causing a change in the sub-nuclear localization of CKI-2, which correlates with its ability to suppress the embryonic lethality and the vulval patterning defects associated with increased levels of CKI-2.

3.3.7. Nucleolar localization of CKI-2 coincides with its reduced stability.

While examining protein expression levels following heat shock induction of transgenic lines that coexpressed the CKI-2 variants and HA::SMO-1 we noticed that CKI-2::GFP peaks in expression approximately 8-12h post heat-shock (Figure 3.6B, top panel). However, when coexpressed with HA::SMO-1 this peak is shifted substantially with a maximum between 2-5h, decreasing to baseline levels very quickly thereafter (Figure 3.6B, top panel, lane 1 and 2). Similarly, when the levels of CKI-2C::GFP (the variant that localizes to the nucleolus consitutively) were examined post heat-shock, we found that its kinetics were comparable to those observed when CKI-2::GFP is coexpressed with HA::SMO-1, that is it reached maximum levels between 2-5h (Figure 3.6B, top panel, lane 3). In contrast, the levels of the nucleolar protein Fibrillarin remain unchanged during our experiments (Figure 3.6B, top panel, lane 4). Surprisingly, when we performed the same time course following induction of HA::SMO-1::CKI-2N::GFP expression, the levels of HA::SMO-1::CKI-2N::GFP showed a similar pattern as the CKI-2N::GFP alone (Figure 3.6B, bottom panel). This suggests that the nucleolar localization is not sufficient to destabilise CKI-2, but information present in the C-terminus of CKI-2 may have an important role to instruct the destabilisation of CKI-2 when sequestered within the nucleolar compartment.

Taken altogether, these data show that SUMOylation of the N-terminus of CKI-2 is sufficient to trigger its nucleolar localization, which is independent of information present within the C-terminus. The C-terminus is also sufficient to take CKI-2 to the nucleolus independently of any SUMO modification, however its nucleolar localization results in the rapid destabilisation of the CKI-2 variant. These results suggest a novel, active mechanism

that includes a nucleolar-associated pathway that leads to degradation in order to maintain steady state levels of this essential cell cycle regulator.

3.3.8. Elements in the C-terminus contribute to subnuclear localization of CKI-2

Since the C-terminus of CKI-2 exclusively localizes to nucleolus as previously shown (Figure 3.5C), we speculated that the C-terminus might include a sequence motif involved in the nucleolar localization. Using several C-terminal variants, we determined a region that is required for the nucleolar localization (Figure 3.7A), which shares sequence conservation with known nucleolar-localizing proteins (Figure 3.7B, bottom panel). From protein sequence analysis of other cell cycle regulators that possess consensus SUMO conjugation sites (ΨKxD/E), we found that mammalian p21Cip1 and p57Kip2 lack such motifs, while p27Kip1 and Dacapo, a *Drosophila* CDK inhibitor protein, carry putative SUMOylation sites (Figure 3.7B, top panel). Interestingly, like CKI-2, these CKIs also possess putative nucleolar localization sequences in addition to the putative SUMOylation sites suggesting that the combination of SUMOylation and nucleolar localization may be conserved as a cassette that is required for controlling these cell cycle effectors in specific developmental contexts (Figure 3.7B, bottom panel).

3.4. Discussion

Ensuring the correct integrity of embryonic cell divisions is critical in generating all the tissues required for postembryonic development. In addition to the role of *cki-2* in eliminating the maternal centrioles during oogenesis (Kim and Roy, 2006), we also noticed that reduction of *cki-2* function results in a Pie-like (pharynx, intestine in excess) embryonic arrest associated with increased numbers of pharyngeal precursors and intestinal cells, which were largely disorganized and apparently ungastrulated as an essential negative cell cycle regulator that controls cell divisions during embryogenesis. (Figure S2.3) (Mello et al., 1992).

Based on the accepted mechanism of CKI function *cki-2* likely exerts S-phase regulation by blocking CDK2-like activity during embryonic cell divisions. Interestingly, mutants in components of the replication machinery that impede cell cycle progress do not make pharynx or gut, and demonstrate a *skn-1*-like phenotype (Bowerman et al., 1992; Encalada et al., 2000). It is possible that the reciprocal scenario may occur in the absence of *cki-2*, causing embryonic divisions to occur prematurely prior to the establishment of appropriate specification cues, thereby resulting in an opposite phenotype. This role in timing fate specification and competence is true of *cki-1* during postembryonic development, where it is involved in coordinating G1 arrest with fate determination (Hong et al., 1998; Kostic and Roy, 2002; Baugh and Sternberg, 2006).

CKI-2 is not abundant and these low levels are likely regulated at the local level during cell cycle progression. Its interaction with PCNA infers that CKI-2 may have some role in DNA replication, while its association with SUMO reflects a novel regulatory mechanism that may govern the effective levels of CKI-2 within the cell. Because the SMO-1/CKI-2 interaction occurs via the N-terminal domain of CKI-2 adjacent to the CDK inhibitory domain we presumed that SUMO was involved in antagonising the CKI-2/CDK interaction, thereby promoting cell cycle progression. CKI-2 variants that lack this domain have little cell cycle inhibitory effect based on the observed embryonic lethality or Pvl

frequency following their heat shock expression. However, we have found that these inactive variants localize to the nucleolus constitutively and therefore might not have access to key cell cycle regulators such as PCNA or the cyclin/CDK complexes. Nucleolar localization is triggered by N-terminal SUMOylation in the full-length protein, while CKI-2 is subsequently degraded.

This degradation that occurs following nucleolar localization seems specific since levels of nucleolar structural protein (Fibrillarin) remains unaffected (Figure 3.6B). Our data do not clarify how this degradation occurs or even whether it occurs in the nucleolus. A recent proteomic survey of the nucleolus did not identify components of the proteasome in this organelle (Andersen et al., 2002), although data from numerous laboratories have shown that inhibition of the proteasome results in accumulation of specific proteins within the nucleolus (Arabi et al., 2003). This suggests that this organelle may be a bottleneck that precedes the degradation of certain protein targets. Furthermore, recent experiments demonstrated that disruption of the nucleolus causes increased stability of p53 suggesting an important function of this organelle in maintaining steady state levels of important cellular effectors (Rubbi and Milner, 2003). Taken together these data suggest that the degradation of specific proteins may require a transient association of the proteasome with this nuclear compartment, or that it may occur through a novel proteasome-independent pathway preceded by nucleolar localization.

Our results obtained with the CKI-2(Δsmo) mutant however suggest that sub-cellular localization may not be the only role of SUMO. Although this mutation disrupts nucleolar localization in response to SMO-1 expression causing CKI-2(Δsmo) to remain nuclear, this mutated variant does not confer a strong embryonic lethality when induced in the embryo (Table 3.1). Therefore altering these lysines has a negative effect on the inhibitory function of CKI-2, presumably by disrupting its ability to interact with the cyclin/CDK complex (Chen et al., 1996). Since a modification of these lysines had significant effects on CKI-2 function, SUMOylation of these residues could have a

bipartite function in modulating the inhibitory capacity of CKI-2, while also targeting it for sub-nuclear re-allocation, thereby triggering its rapid degradation.

At least two models might account for this translocation. A SUMO-mediated conformational change in the CKI-2 protein could expose signals within its C-terminus to efficiently direct the protein to the nucleolus. (Figure 3.7A). Since SUMO-fused N-terminus of CKI-2 goes to nucleolus, however, at present it is unclear how the signal within the C-terminus of CKI-2 may function and remains to be further characterized. More plausibly, SUMO modification of the N-terminus could disrupt a tether that retains CKI-2 in the nucleoplasm, perhaps bound to chromatin, or on proteins such as CDK2-containing complexes associated with origins of replication (Jackson et al., 1995; Furstenthal et al., 2001). Our finding that another CKI-2 interactor, RNF-1, a RING finger domain protein (Joazeiro and Weissman, 2000), binds specifically to the C-terminal domain of CKI-2 (unpublished data) suggests that this factor might well fulfil this function, a possibility that is currently being investigated.

Our study showed that an in frame fusion of SUMO to the CKI-2 N-terminal variant resulted in nucleolar translocation, but without the associated degradation (Figure 3.6A and B). The importance of the nucleolus in sequestering important cell cycle regulators has been clearly demonstrated in several aspects of cell cycle regulation (Visintin and Amon, 2000). One striking example involves mitotic exit in *S. cerevisiae,* which is tightly controlled by the timely release of Cdc14p from the nucleolus (Visintin et al., 1999). It is tempting to speculate that the progressive nucleolar localization and destabilisation of CKI-2 might confer the S-phase regulation that is typical of these early embryonic divisions.

Is SUMOylation an evolutionarily conserved mechanism for cell cycle regulation? Dacapo, p27KIP play similar roles during development and these SUMOylation sites may indeed be relevant to their function and/or their regulation. It is therefore quite possible that this modification has been conserved as a means of initiating rapid change within the cell

in response to developmental cues such as during embryogenesis, when transcription is silent. Analysis of additional CKI proteins from diverse organisms may confirm this while searches for other proteins that might use this cassette may provide invaluable insight about the evolution of this novel SUMO-associated function.

3.5. Materials and Methods

3.5.1. Nematode Strains

The following *C. elegans* strains were used: N2 Bristol was used as the wild-type throughout. MR251 (*unc-119*; rrEx251 [*hs*::CKI-2::GFP; *unc-119*(+)]), MR253 (*unc-119*; rrEx253 [*smo-1*::GFP::SMO-1; *unc-119*(+)]), MR353 (*unc-119*; rrEx353 [*hs*::CKI-2N::GFP; *unc-119*(+)]), MR354 (*unc-119*; rrEx354 [*hs*::CKI-2C::GFP; *unc-119*(+)]), MR377 (*unc-119*; rrEx377 [*hs*::CKI-2(Δsmo)::GFP; *unc-119*(+)]), MR378 (*unc-119*; rrEx378 [*hs*::HA::SMO-1; *hs*::CKI-2::GFP; *unc-119*(+)]), MR390 (*unc-119*; rrEx390 [*hs*::HA::SMO-1; *unc-119*(+)]), MR397 (*unc-119*; rrEx397 [*hs*::HA::SMO-1; *hs*::CKI-2(Δsmo)::GFP; *unc-119*(+)]), MR408 (*unc-119*; rrEx408 [*hs*::HA::SMO-1; *hs*::CKI-2; *unc-119*(+)]), MR772 (*unc-119*; rrEx772 [*hs*::HA::SMO-1::CKI-2N::GFP; *unc-119*(+)]). All *C. elegans* strains were cultured using standard techniques and maintained at 20°C unless stated otherwise (Brenner, 1974).

3.5.2. Yeast two-hybrid screen

Saccharomyces cerevisiae strain *W303 Y1003* (*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 and PCR was performed to generate each inserts (CKI-2 and CKI-2N) using *cki-2* cDNA as a template. The PCR prepared inserts were then inserted into BamHI/SalI sites of pEG202-NLS. Yeast was transformed with one of each bait construct (LexA-DBD::CKI-2 or LexA-DBD::CKI-2N) and grown on selective media deficient in histidine. The resulting bait strains were then transformed with 60μg of cDNA library expressing the GAL4-AD (transcriptional aqctivation domain) fused to mixed stage *C. elegans* cDNAs (a gift from A. La Volpe) and screened as described (Gietz et al., 1997).

Directional two-hybrid assay. To generate LexA-DBD::CKI-1, *cki-1* cDNA was used as a template for PCR, which was then inserted into BamHI/SalI sites of pEG202-NLS. The

yeast strain containing LexA-DBD::CKI-1 were transformed with GAL4-AD::PCN-1 or GAL4-AD::SMO-1 independently 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.

3.5.3. Mapping protein-protein interaction domains

To map the CKI-2 interaction domains of PCN-1 (W03D2.4), constructs encoding CKI-2 variants were generated using PCR and were inserted into the BamHI/SalI sites of pEG202-NLS (9.8-kb) to yield the LexA-DBD fusion constructs pMR203 (LexA-DBD::CKI-2 (amino acids 1-150)), pMR204 (LexA-DBD::CKI-2 (amino acids 1-157)), pMR205 (LexA-DBD::CKI-2 (amino acids 1-163)), pMR206 (LexA-DBD:: CKI-2 (amino acids 164-259)), pMR207 (LexA-DBD::CKI-2 (amino acids 167-259)).

3.5.4. *C. elegans* transgenes and heat shock experiments

The following constructs were used for the heat shock-related 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), and both promoter containing constructs were co-injected to generate heat shock-related transgenic animals): *hs*::GFP::SMO-1, *hs*::CKI-2, *hs*::CKI-2(Δsmo), *hs*::CKI-2::GFP, *hs*::CKI-2N::GFP, *hs*::CKI-2C::GFP, *hs*::CKI-2(Δsmo)::GFP. For the SMO-1 translational GFP fusion construct, *smo-1* genomic upstream sequence was amplified and inserted into BamHI/XmaI sites of pPD49.26 to make pMR218. GFP::SMO-1 was prepared by PCR using a [*hs*::GFP::SMO-1] as a template and then inserted into NheI/SacI of pMR218 to generate pMR219. The constructs were microinjected into the *C. elegans* gonad to generate transgenic animals as described (Mello et al., 1991).

Heat shock-induced expression was performed by floating parafilm-sealed culture plates in a 33°C water bath for 1 hour followed by a 4 hour-recovery period at 20°C. To check the embryonic lethality after heat shock, embryos laid from gravid adults were heat

shocked for up to 30 minute and the result was presented as the percentage of the unhatched embryos in a total population.

3.5.5. Antibodies and Immunological methods

Generation of recombinant proteins and antiserum. CKI-2N was prepared by PCR using *cki-2* cDNA as a template, which was inserted into BamHI/SalI sites of PGEX-5X-1 (Amersham Pharmacia) GST fusion vector. GST::CKI-2N was over-expressed in *E. coli XL1-Blue* and purified as described (Amersham Pharmacia). GST::CKI-2N was further purified by electroelution (Bio-Rad) and rabbits were immunized using a standard protocol.

Immunoblotting. Worms were picked into SDS sample buffer and were freezed/thawed twice at -80°C and 100°C. 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. Primary antibodies were rabbit polyclonal anti-CKI-2, monoclonal MCA-38F3 (anti-Fibrillarin antibody, Encore), and rabbit polyclonal anti-GFP (Clonetech). Secondary antibodies were HRP (Horse raddish peroxidase)-conjugated anti-rabbit or mouse antibodies. Protein bands were detected using a chemifluoresence (ECL Plus, Amersham Pharmacia) and imaged with a STORM™ (Amersham Pharmacia).

Immunofluorescence. The following primary antibodies were used: rabbit polyclonal anti-CKI-2, monoclonal MCA-38F3 (anti-Fibrillarin antibody, Encore). Secondary antibodies were anti-rabbit Texas-Red (Invitrogen). Embryos was 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. Indirect immunofluorescence microscopy was performed using a Leica DMR compound microscope (x60) equipped with a Hamamatsu C4742-95 digital camera, imaging ~0.5μm-thick optical section (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).

3.6. Acknowledgments

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3.8. Legends to Figures

Figure 3.1. **PCN-1 and SMO-1 interact with CKI-2.** (A) Summary of the interaction between LexA-DBD fused CKI-2 (top) or CKI-1 (bottom) and the GAL4-AD fused CKI-2 interacting proteins (PCN-1 and SMO-1) using a directional yeast two-hybrid analysis. DBD or AD indicates DNA binding domain or transcriptional activation domain, respectively. The control (-control) carries LexA-DBD fused CKI-2 or CKI-1 bait plasmid and empty GAL4-AD fusion plasmid (prey plasmid). (+) or (-) in the table (right) indicates "interaction" or "no-interaction", respectively. (B) Mapping of the PCN-1 binding region in CKI-2. The yeast strain containing GAL4-AD::PCN-1 was transformed separately with the individual CKI-2 bait variants (LexA-DBD fused) followed by determination of *lacZ* expression from each transformant. (+) or (-) indicates "expression" or "no expression" of *lacZ*, respectively. The number indicates the position of the amino acid residue in the primary sequnce. The arrows indicate the minimal region that is necessary for PCN-1 binding in CKI-2. CDI, CDK inhibitory domain. K20 and K40, lysine residue 20 and 40, respectively. The PCNA binding region in CKI-2 is shown by a black closed oval with arrow, while the predicted PCNA binding region in CKI-1 is indicated by bold underline. (C) Protein sequence alignment between known PCNA interactors and CKI-1/CKI-2. Rectangular boxes mark amino acid residues conserved for the PCNA binding. In consensus, x, any amino acid residue; h, moderately hydrophobic residues (leucine, isoleucine, methionine); a, highly hydrophobic residues with aromatic side chains (phenylalanine, tyrosine) (Warbrick, 2001).

Figure. 3.2. **CKI-2 comprises of two functionally distinct domains.** Diagram of p21Cip1 depicting the CDK and PCNA inhibitory domains (arrow), and CKI-2 (full-length) and the CKI-2 variants (CKI-2N and CKI-2C) based on the conserved cyclin/CDK inhibitory domain at the N-terminus (See Figure S2.1A). The number indicates the position of the amino acid residue in the protein sequence. Arrows indicate binding domains for cyclin/CDK and PCNA at the N- and C-terminus, respectively. Embryos were observed to

compare the levels or the localization of GFP after heat-shock (bottom). The arrowheads mark nuclei.

Figure 3.3. **CKI-2 is modified by SMO-1 *in vivo*.** (A) *smo-1(RNAi)* eliminates high molecular weight entities. Extracts obtained from [*hs*::CKI-2] or [*hs*::CKI-2; *smo-1(RNAi)*] embryo were analyzed by western blotting using anti-CKI-2 antibody. Embryo extracts prepared from non-induced animals were used as a control (-). (B) CKI-2 is covalently modified by HA::SMO-1 in transgenic strains co-expressing HA-tagged SMO-1 (HA::SMO-1) and CKI-2. [*hs*::HA::SMO-1; *hs*::CKI-2] bearing animals were heat shocked and extracts prepared from embryos were analyzed using western blot (WB) and immunoprecipitation (IP). P.I, pre-immune serum. Black arrowheads indicate bands recognized by anti-CKI-2 (α-CKI-2), or anti-HA (α-HA) antibody. *embryos*, embryo extracts. The arrows indicate the position of 64 KDa and 38 KDa size markers.

Figure. 3.4. **CKI-2 is a nuclear protein that co-localizes with chromosomal DNA.** (A) Embryos carrying the *hs*::CKI-2::GFP transgenic array labelled with anti-CKI-2 antibody (A, red) and DAPI (B, blue) after heat-shock induction. (B) Germ line expression of CKI-2::CFP using the *pie-1*::CKI-2::CFP transgenic array. Insets correspond to detailed view of germ cell nuclei from the region outlined by the white rectangular frames.

Figure. 3.5. **CKI-2 localizes to the nucleolus following co-expression of SMO-1.** (A-C) (A) CKI-2::GFP ([*hs*::CKI-2::GFP]), or (B) CKI-2N::GFP ([*hs*::CKI-2N::GFP]), or (C) CKI-2C::GFP ([*hs*::CKI-2C::GFP]) were induced by heat shock and imaged in the intestinal cell nuclei by monitoring GFP expression (green) and DAPI (blue). (D) Intestinal nuclei in (A) were counterstained with anti-Fibrillarin (α-FBR) antibody (MCA-38F3; red). (E) Sub-nuclear localization of GFP-SMO-1 expressed under an endogenous *smo-1* upstream promoter region ([*smo-1*::GFP::SMO-1]). (F) HA::SMO-1 was induced by heat shock and imaged in the intestinal cell nuclei by immunostaining with anti-HA antibody (α-HA) and DAPI (blue). (G,H) (G) [*hs*::CKI-2::GFP; *hs*::HA::SMO-1], or (H) [*hs*::CKI-2(Δsmo)::GFP; *hs*::HA::SMO-1] co-expressing intestinal nuclei labelled with

anti-HA antibody (α-HA; red). The open and closed arrowhead mark nucleolus and nucleoplasm, respectively. The white rectangular boxed region was magnified to show more detail of the nuclei.

Figure. 3.6. **SMO-1-dependent changes in CKI-2 subnuclear localization and subsequent degradation.** (A) HA::SMO-1::CKI-2N::GFP expressed in intestinal cells with a schematic drawing of the HA::SMO-1::CKI-2N::GFP transgene. Western blot analysis (WB) shows the expression of HA::SMO-1::CKI-2N::GFP in heat shock induced (+) and uninduced (-) animals. The open and closed arrowheads indicate the nucleolus and the nucleoplasm, respectively. (B) Time course analysis of CKI-2 levels by immunoblotting with anti-CKI-2 (α-CKI-2), or anti-GFP (α-GFP; used for detection of CKI-2C::GFP), or anti-Fibrillarin antibody. Protein extracts were prepared from a mixed population of the transgenic animals at various times after heat-shock (0.5 to 12 hours). Fibrillarin (asterisk) was used as a nucleolar control. The arrows indicate the position of 64 KDa and 48 KDa standard size markers.

Figure. 3.7. **Mapping of a nucleolar localization signal in CKI-2.** (A) Animals with heat shock inducible transgenes that included full length CKI-2 and its truncated variants (CKI-2x::GFP) were expressed as GFP fusion proteins afterwhich subnuclear localization was determined. (++) indicates strong; (+) moderately strong specific nucleolar localization "NoL", while (+/-) indicates weak or dispersed NoL. The number indicates the position of amino acid residue in the protein sequence. The arrows indicate the minimal region that is necessary for the nucleolar localization. (B) Diagram depicting the consensus SUMOylation target sites present among diverse CIP/KIP family of CDK inhibitors. The nucleolar localization signals of p14ARF (Rizos et al., 2000) and human MDM2 (Lohrum et al., 2000), two known nucleolar localizing proteins, were aligned with the putative nucleolar localization signal of CKI-2 and other SUMOylation motif-containing CDK inhibitors (p27Kip1 and Dacapo) (Lane et al., 1996; Sherr and Roberts, 1999). Conserved amino acid residues are marked by rectangular boxes. The number indicates the position of amino acid residue in the protein sequence.

Genotype	Embryonic lethality (%)	
	No heat-shock	Heat-shock
hs::GFP	0 (n=239)	1.76±1.8 (n=199)
hs::CKI-2::GFP	2.43 (n=525)	29.63±3.4 (n=481)
hs::CKI-2N::GFP	0.9 (n=202)	18.26±4.7 (n=628)
hs::CKI-2C::GFP	1.13 (n=177)	5.99±2.8 (n=770)
hs::CKI-2(Δsmo)::GFP	n.d	9.02±5.0 (n=1075)
hs::HA::SMO-1	n.d	1.70±1.02 (n=549)
hs::CKI-2::GFP; *hs*::HA::SMO-1	n.d	8.17±1.40 (n=903)
hs::CKI-2N::GFP; *hs*::HA::SMO-1	n.d	9.99±1.38 (n=557)
hs::CKI-2(Δsmo)::GFP; *hs*::HA::SMO-1	n.d	8.40±3.01 (n=787)
hs::HA::SMO-1::CKI-2N::GFP	n.d	7.18±1.87 (n=488)

Table 3.1. **CKI-2 heat shock expression causes distinct developmental abnormalities typical of cell cycle perturbation.** Heat shock induction of CKI-2 or its truncated variants result defects in embryonic development. For embryonic lethality (%), embryos from young adult animals carrying each heat shock transgenes were heat-shocked and examined 30 hours later for embryonic lethality determined by the number of L1 larvae present on the plate. Non-heat shocked embryos were used as a control. The values represent the percentage of unhatched embryos that arise from the initial population of embryos (n). n.d, not determined. The standard deviation (±SD) was obtained from at least two independent experiments.

Genotype	Protruding vulva (Pvl) (%)		
	L1	L2	L3
hs::CKI-2::GFP	0 (n=1130)	36.29±1.6 (n=1427)	16.41±2.7 (n=758)
hs::CKI-2N::GFP	2.02±0.3 (n=911)	67.41±6.4 (n=1423)	94.72±3.3 (n=1328)
hs::CKI-2C::GFP	0 (n=907)	0 (n=417)	0 (n=410)
hs::CKI-2(Δsmo)::GFP	n.d	0 (n=702)	0.2 (n=500)
hs::CKI-2::GFP; *hs*::HA::SMO-1	n.d	17.18±3.91 (n=392)	13.50±1.88 (n=524)
hs::CKI-2N::GFP; *hs*::HA::SMO-1	n.d	19.79±0.21 (n=692)	58.17±2.17 (n=424)
hs::CKI-2(Δsmo)::GFP; *hs*::HA::SMO-1	n.d	0 (n=315)	0.2 (n=430)
hs::HA::SMO-1::CKI-2N::GFP	n.d	37.18±0.08 (n=495)	53.99±2.77 (n=505)

Table 3.2. **SMO-1 suppresses the developmental defects caused by misexpression of CKI-2 and CKI-2N.** For the frequency (%) of Pvl, developmentally synchronized animals were induced at different laval stages (L1, L2, L3) and the frequency (%) of Pvl was determined by scoring protruding vulvae in the animals. The values represent the percentage of Pvl animals in a total population of animals (n). The standard deviation (±SD) was obtained from at least two independent experiments. n.d, not determined.

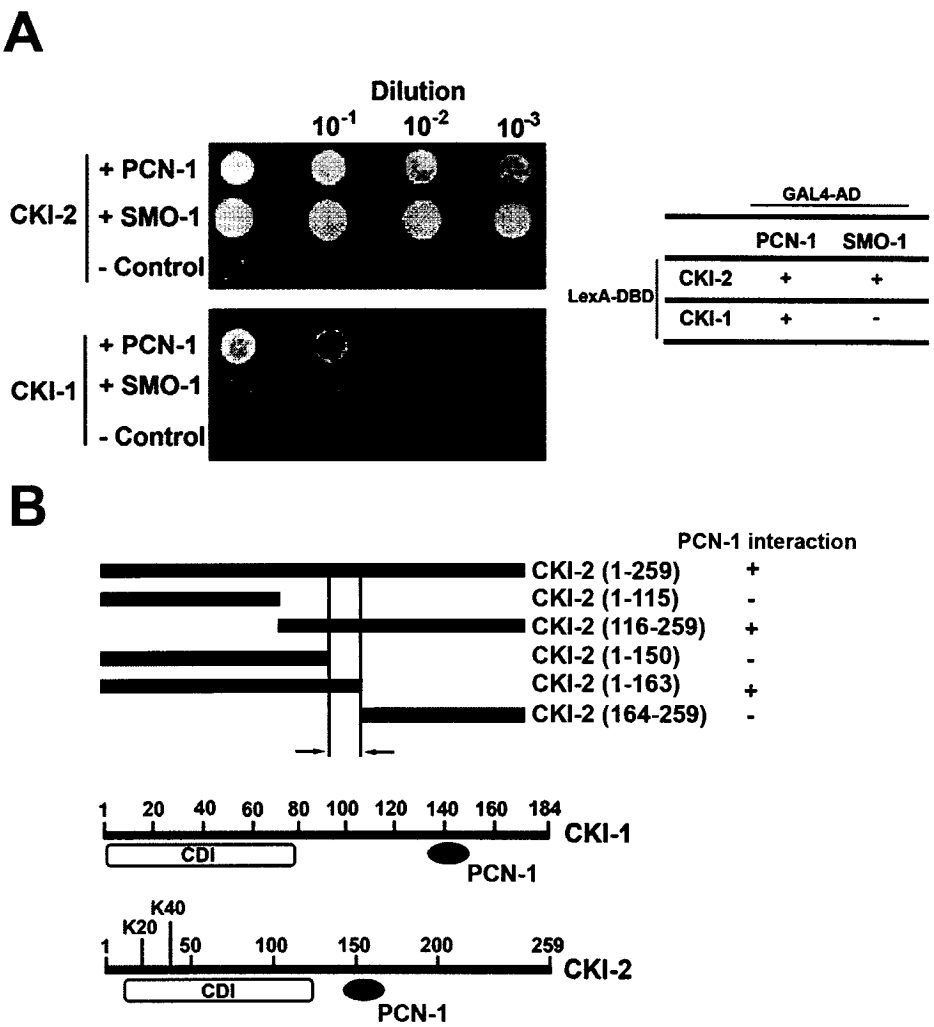

A
Dilution
10-1
10-2
10-3
CKI-2
+ PCN-1
+ SMO-1
- Control
CKI-1
+ PCN-1
+ SMO-1
- Control
GAL4-AD
PCN-1
SMO-1
LexA-DBD
CKI-2
+
+
CKI-1
+
-
B
PCN-1 interaction
CKI-2 (1-259) +
CKI-2 (1-115) -
CKI-2 (116-259) +
CKI-2 (1-150) -
CKI-2 (1-163) +
CKI-2 (164-259) -
1 20 40 60 80 100 120 140 160 184
CKI-1
CDI
PCN-1
K40
K20
1 50 100 150 200 259
CKI-2
CDI
PCN-1
C
XPG (981-1009) LKQLDAQQTQLRIDSFFRLAQQEKEDAKR
FEN-1 (328-355) SRQGSTQGRLDDFFKVTGSL
p21Cip1 (139-160) KRRQTSMTDFYHSKRRLIFS
MCMT (162-174) TRQTTITSHFAQG
CKI-1 (136-150) KRQQKMTDFMAVSR
CKI-2 (151-163) LKQTKLTNYMPVR
Consensus Qxx(h)xx(a)

Figure 3.2

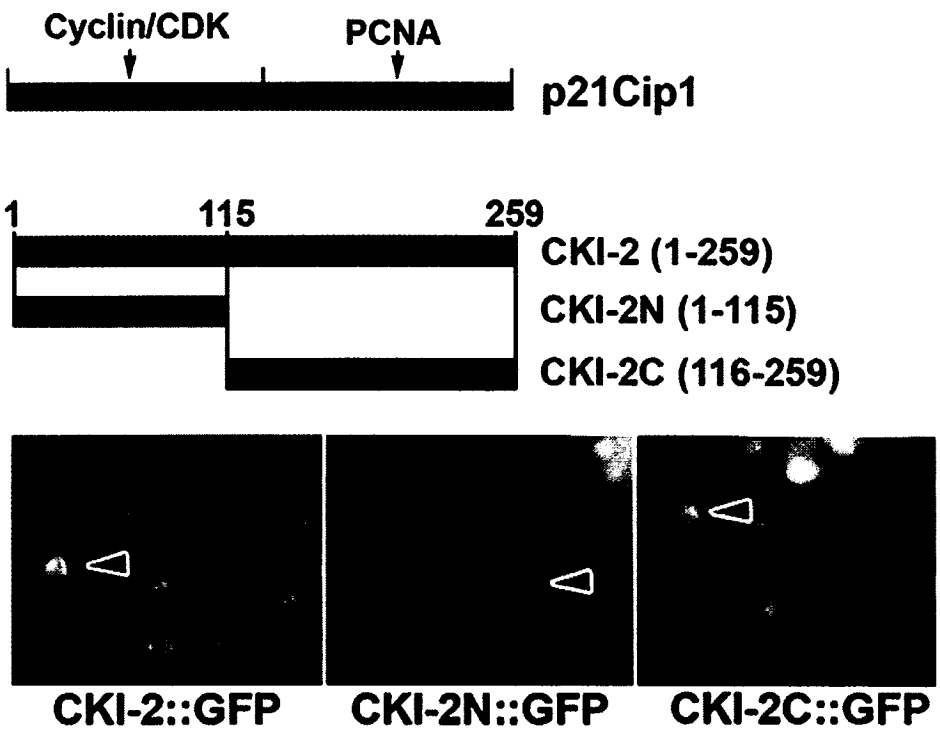

Cyclin/CDK
PCNA
p21Cip1
1
115
259
CKI-2 (1-259)
CKI-2N (1-115)
CKI-2C (116-259)
CKI-2::GFP
CKI-2N::GFP
CKI-2C::GFP

Figure 3.3

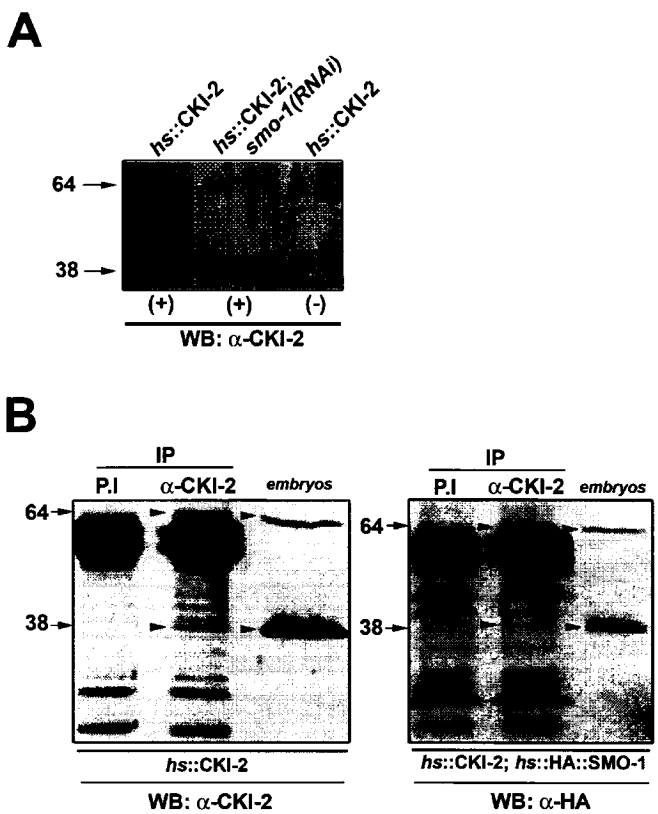

A
hs::CKI-2
hs::CKI-2; smo-1(RNAi)
hs::CKI-2
64
38
(+)
(+)
(-)
WB: α-CKI-2
B
IP
P.I
α-CKI-2
embryos
64
38
hs::CKI-2
WB: α-CKI-2
IP
P.I
α-CKI-2
embryos
64
38
hs::CKI-2; hs::HA::SMO-1
WB: α-HA

Figure 3.4

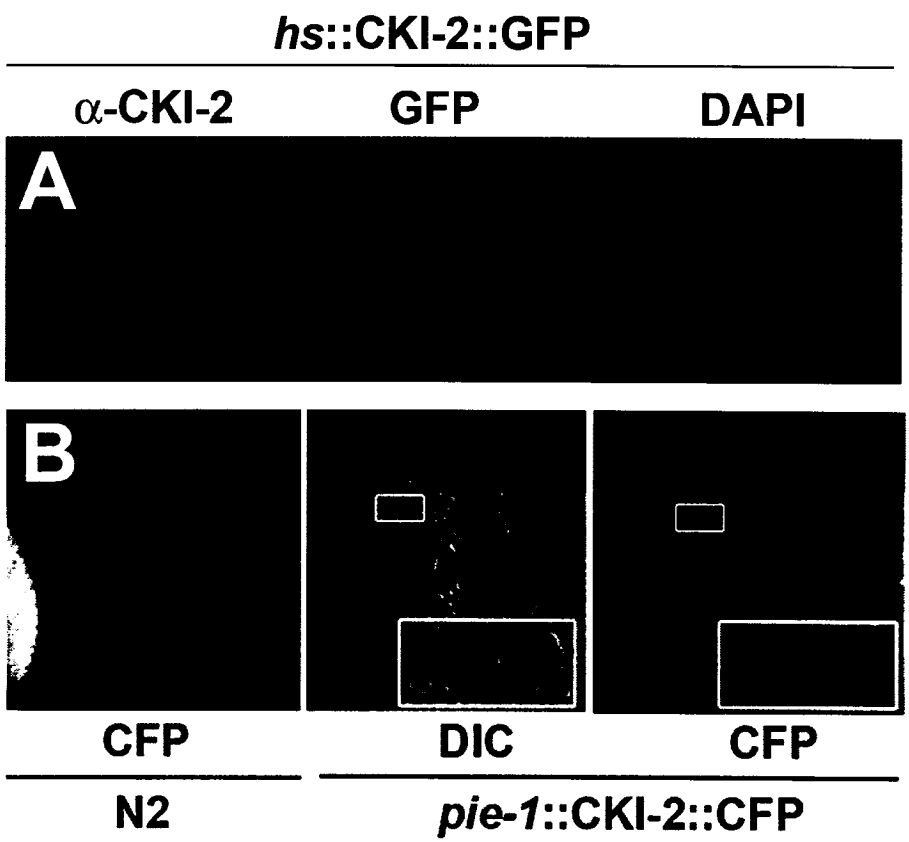


Figure 3.5

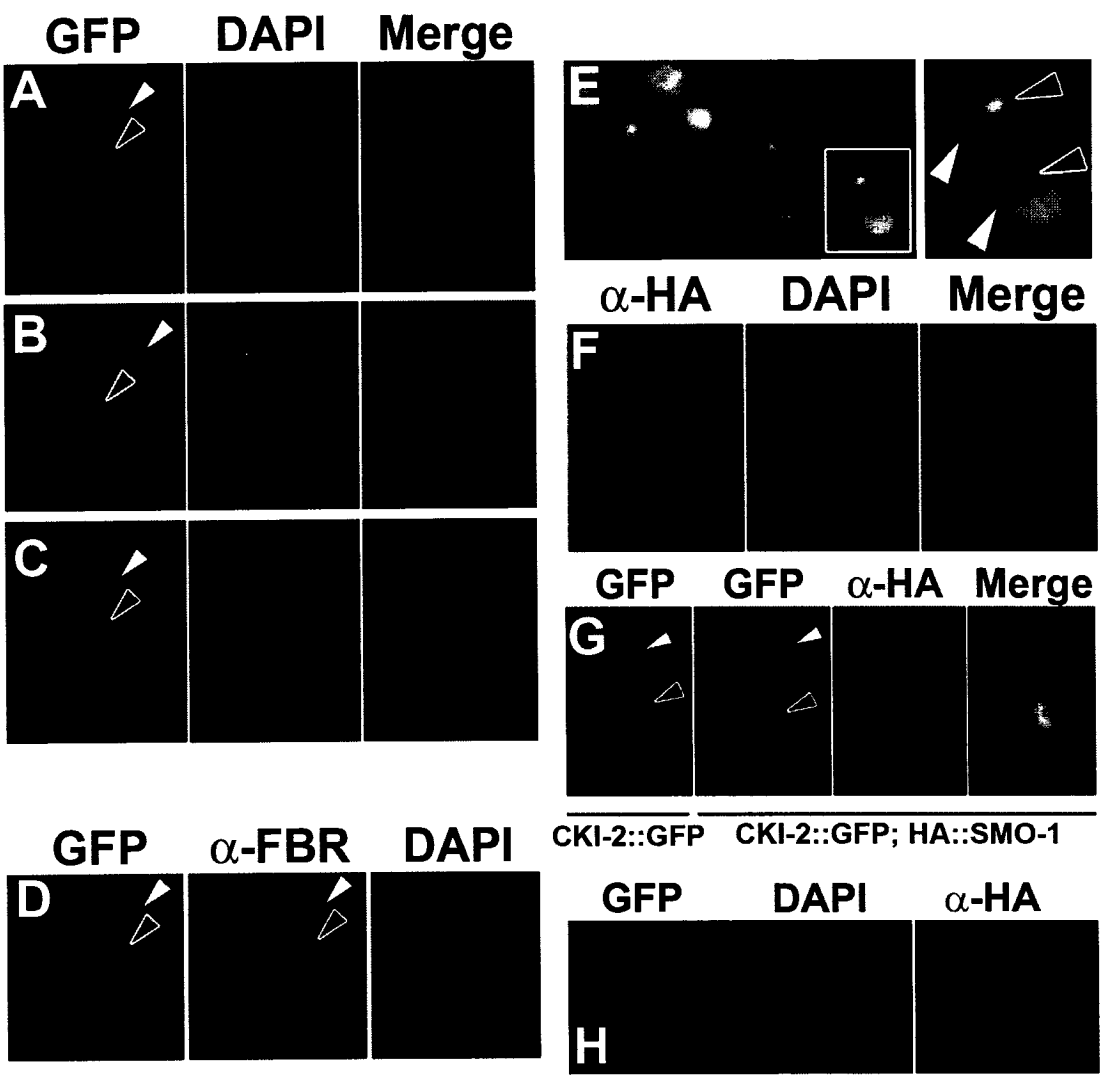

GFP
DAPI
Merge
A
B
C
E
α-HA
DAPI
Merge
F
GFP
GFP
α-HA
Merge
G
CKI-2::GFP
CKI-2::GFP; HA::SMO-1
GFP
α-FBR
DAPI
D
GFP
DAPI
α-HA
H

Figure 3.6

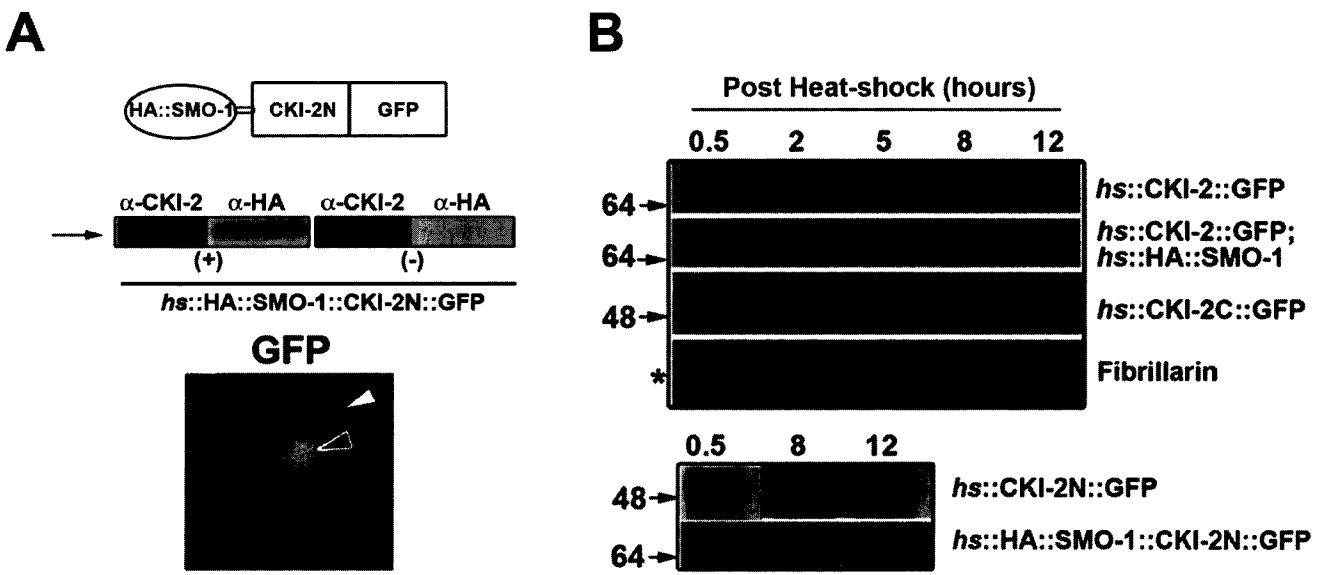
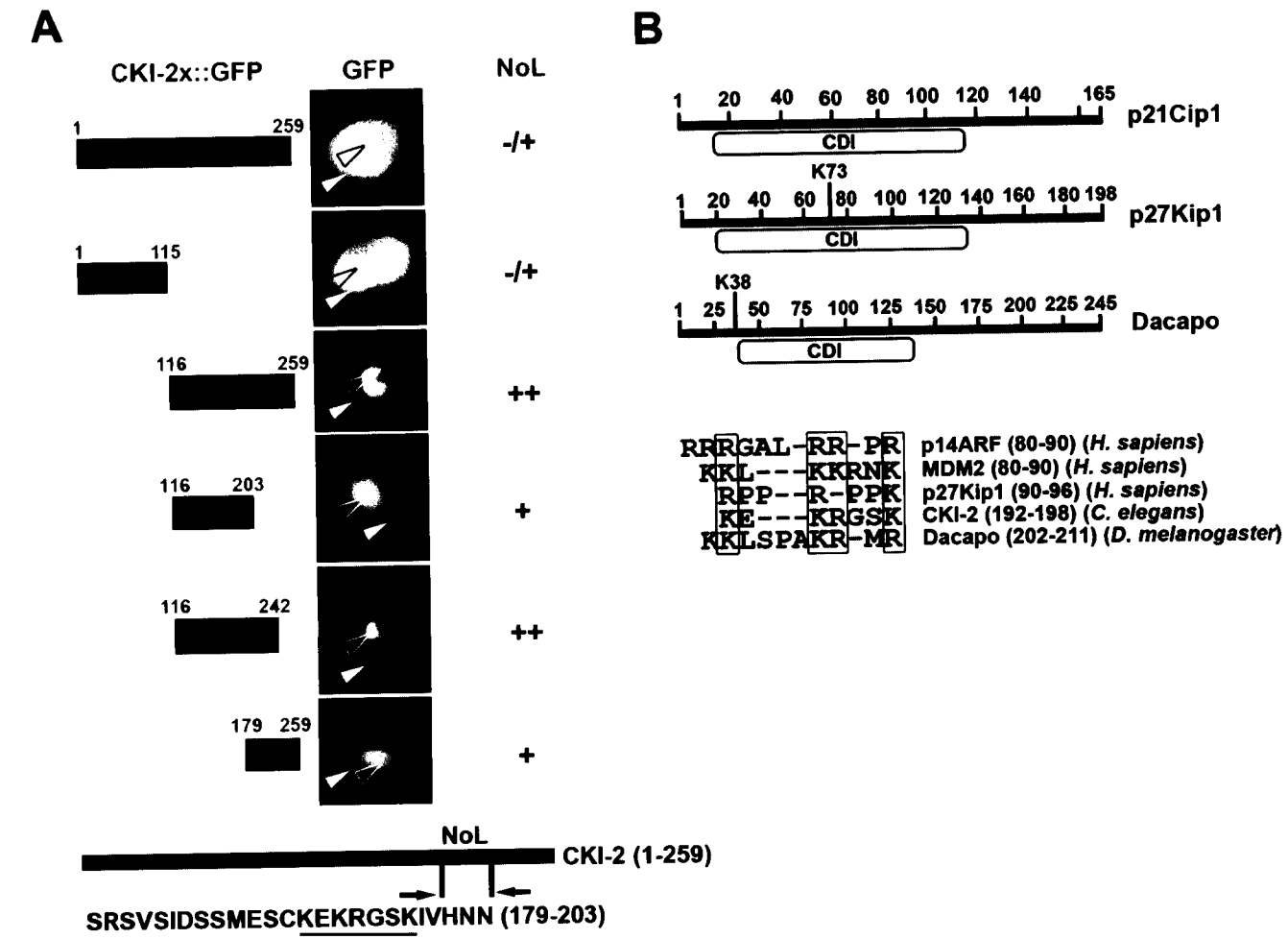

A
HA::SMO-1
CKI-2N
GFP
α-CKI-2
α-HA
α-CKI-2
α-HA
(+)
(-)
hs::HA::SMO-1::CKI-2N::GFP
GFP
B
Post Heat-shock (hours)
0.5
2
5
8
12
64
64
48
*
hs::CKI-2::GFP
hs::CKI-2::GFP;
hs::HA::SMO-1
hs::CKI-2C::GFP
Fibrillarin
0.5
8
12
48
64
hs::CKI-2N::GFP
hs::HA::SMO-1::CKI-2N::GFP

Figure 3.7


A
CKI-2x::GFP
GFP
NoL
1 259
-/+
1 115
-/+
116 259
++
116 203
+
116 242
++
179 259
+
NoL
CKI-2 (1-259)
SRSVSIDSSMESCKEKRGSKIVHNN (179-203)
B
1 20 40 60 80 100 120 140 165
p21Cip1
CDI
K73
1 20 40 60 80 100 120 140 160 180 198
p27Kip1
CDI
K38
1 25 50 75 100 125 150 175 200 225 245
Dacapo
CDI
RRRGAL-RR-PR p14ARF (80-90) (H. sapiens)
KKL---KKRNK MDM2 (80-90) (H. sapiens)
RPP--R-PPK p27Kip1 (90-96) (H. sapiens)
KE---KRGSK CKI-2 (192-198) (C. elegans)
KKLSPAKR-MR Dacapo (202-211) (D. melanogaster)

Connecting text

In the chapter III, we describe the identification of two CKI-2 interactors that we uncovered using a yeast two hybrid-screen. Our data indicate that SMO-1 and RNF-1 might be unique for CKI-2 function and that CKI-2, like p21Cip1, is composed of two domains, which may be functionally independent. Misexpression of CKI-2 variants that contain these different domains causes defects in embryogenesis and vulva morphogenesis, where the N-terminus was consistently more potent in each context. Our data suggest that the levels of CKI-2 must be appropriately maintained to ensure proper embryonic or post-embryonic development. We showed that CKI-2 was covalently modified by SUMOylation which caused CKI-2 to localize where it was subsequently degraded. Since many RING domain proteins are intrinsic E3 ubiquitin ligases, we speculated that RNF-1, a third interactor identified by a yeast two-hybrid screening, could be involved in this degradation.

Chapter IV

RNF-1, a *Caenorhabditis elegans* RING domain protein, modulates CKI-2 through ubiquitin-dependent proteolytic pathway

4.1. Abstract

In many cell types, cell cycle progression is controlled at the point of entry into S-phase where afterward the cycle is committed to termination following mitosis. This control point is referred to as the restriction point in mammalian cells or START as it is referred to in yeast (Sherr and Roberts, 2004). During *C. elegans* development, the regulation of this transition is largely controlled by a CIP/KIP family Cyclin-dependent kinase inhibitor (CKI) homologue called *cki-1*, which responds to both developmental and environmental signals to mediate timely cell cycle arrest from late embryogenesis to adulthood (Hong et al., 1998; Kipreos, 2005). Although a second CIP/KIP family CKI, *cki-2*, has been suggested to play a non-redundant role with *cki-1* during embryogenesis, *cki-2* has not been well characterized mainly due to its refractoriness to RNAi (Feng et al., 1999; Fukuyama et al., 2003). While the maintenance of appropriate levels of CKI-1 has been characterized quite well in a developmental context, little is known about how CKI-2 levels are maintained at the post-transcriptional level. Unlike CKI-1, CKI-2 does not seem to be degraded by a canonical cullin-based SCF (Skp1/Cullin/ F-box) pathway (Feng et al., 1999). Here we show that a RING domain protein (RNF-1) interacts strongly with CKI-2 and co-expression of RNF-1 with CKI-2 suppresses the embryonic lethality caused by increased levels of CKI-2 in the embryo. This suppression is mediated by the increased rate of CKI-2 degradation in a proteasome-dependent manner. In addition, we show that RNF-1 is involved in the poly-ubiquitination of CKI-2. Furthermore, we have found that SMO-1, the *C. elegans* SUMO orthologue, can block the association between CKI-2 and RNF-1 in a yeast-based competition assay. Therefore, our data suggest that RNF-1 functions in a novel regulatory mechanism to maintain the appropriate levels of CKI-2 through the differential regulation of a RING domain protein with SUMO.

4.2. Introduction

Eukaryotic cell cycle progression is driven predominantly by the cyclical fluctuation of cyclin-dependent kinase (CDK) activities. These enzymes are regulated in a timely manner by an interplay of positive and negative regulators in response to diverse environmental and developmental cues that may exert their effects at different stages of cell cycle (Morgan, 1997; Sherr and Roberts, 1999).

Progression through the cell cycle is irreversibly controlled by the proteolytic degradation of the major regulatory proteins (Krek, 1998; Cardozo and Pagano, 2004). In budding yeast, p40Sic1 controls the G1/S transition by blocking G1 Cyclins/Cdk function until it is degraded at START, while progression through mitosis and eventual mitotic exit occurs through inactivation of the mitotic kinase (Cdk1) by the targeted degradation of mitotic cyclins, in addition to association with increasing levels of p40Sic1 (Verma et al., 1997; Deshaies, 1997).

In mammalian cells that receive mitogenic signals, p27Kip1, a mammalian CDK inhibitor protein, is eliminated at the G1/S transition to allow S phase entry (Bloom and Pagano, 2003). The levels of this CKI then accumulate to finally reach a peak in the subsequent G1 phase. This oscillatory cycle of elimination is mediated by the 26S proteasomal complex, which is catalyzed by the covalent attachment of ubiquitin to a lysine residue on the targeted proteins (Pickart and Cohen, 2004).

Ubiquitin is a highly conserved small polypeptide composed of 76 amino acids, which is transferred to target proteins by a cascade that includes separate enzymatic activities referred to as E1, E2, and E3 (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Initially, ubiquitin is activated by an ubiquitin-activating enzyme (UBA or E1) in an ATP-dependent manner. Secondly, the activated ubiquitin is transferred to an ubquitin-conjugating enzyme (UBC or E2). Finally, E3 ubiquitin ligases recruit E2 conjugating enzymes and mediate the ubiquitination of a lysine residue with target proteins.

Most E3 ligases act as multiprotein complexes, such as the SCF (Skp/Cullin/F-box) complex which is required for the G1/S and the G2/M transition, or the APC/C (anaphase promoting complex/cyclosome), which is required for the metaphase-to-anaphase transition and subsequent mitotic exit (Peter, 1998; Vodermaier, 2004; Nakayama and Nakayama, 2006).

The cullin proteins are critical SCF components and this protein family plays an important role during diverse eukaryotic developmental processes, including cell cycle progression, cell fate determination, and cytoskeletal function (Petroski and Deshaies, 2005). Cullins link the E2 enzyme to the E3 ubiquitin ligase complex together with the RING domain protein, which binds E2 enzymes through their RING domains (Zheng et al., 2000). In higher eukaryotic cells, the RING domain protein Rbx1 associates with different cullin proteins (Cul1 to Cul5) and an F-box protein to constitute an active SCF complex (Petroski and Deshaies, 2005).

In *C. elegans*, cullin-based multi-subunit E3 ligases are required for the regulation of cell cycle progression during embryogenesis (Bowerman and Kurtz, 2006). *cul-2* promotes the G1/S transition by targeting key regulators where it also plays an important role in mitosis and in meiosis (Feng et al., 1999; Liu et al., 2004; Sonneville and Gonczy, 2004). In *cul-2* mutants, germ cells undergo G1 arrest which correlates with an increased level of CKI-1 in their nuclei, while depletion of CKI-1 restores S phase entry in *cul-2* mutants. However, no nuclear accumulation of CKI-2 (a second CKI in *C. elegans*) was observed. These data suggest that *cul-2* regulates CKI-1 levels, but not CKI-2, probably through timely degradation during G1 progression (Feng et al., 1999). Since most ubiquitin-mediated proteolysis occurs through a phosphorylation- dependent manner, it has been presumed that the degradation of CKI-1 might be phosphorylation-dependent. Recent data showed that the inactivation of *C. elegans cdc-14* phosphatase causes extra divisions in multiple tissues (Saito et al., 2004). Genetic studies have demonstrated that *cdc-14* acts upstream of *cki-1* (Saito et al., 2004), where CDC-14 seems to maintain CKI-1 in a hypophosphorylated state thus protecting it from ubiquitin-dependent proteolysis,

while consequently causing a nuclear accumulation of CKI-1. In budding yeast, Cdc14p dephosphorylates p40Sic1, which stabilises p40Sic1 (Visintin et al., 1998). In mammalian cells, Cdc14A is also known to dephosphorylate p27Kip1 *in vitro* (Kaiser et al., 2002). These data are consistent with an evolutionarily conserved role of CDC-14 in the maintenance of more stable hypophosphorylated forms of CKIs.

While the regulation of CKI-1 levels through cullin-mediated mechanisms has been well characterized, little has been demonstrated concerning the role of CKI-2, which is located in tandem to CKI-1 on chromosome II. Since overexpression of CKI-2 causes embryonic arrest and morphological defects and, *cki-2* shows a distinctive embryonic expression profile compared to that of *cki-1* (Fukuyama et al., 2003), it has been suggested that *cki-2* might have a non-redundant role with *cki-1* during embryogenesis. Because reverse genetic analysis of *cki-2* has not proven to be very informative due to RNAi refractoriness and the difficulties in obtaining mutations in this gene, we performed a yeast two-hybrid screen to identify interacting partners of CKI-2 in order to better understand the processes that are controlled by this CKI.

Here we report a novel RING finger protein (RNF-1) interacts strongly and specifically with CKI-2. RNF-1 appears to antagonize CKI-2 function since co-expression of RNF-1 with CKI-2 suppresses the embryonic lethality associated with misexpression of CKI-2 in the embryo which is mediated by the increased rate of CKI-2 degradation. In addition, we show that RNF-1 is involved in the ubiquitination of CKI-2. Moreover, a novel yeast-based competition assay developed to study the relationships between these factors indicates that *C. elegans* SUMO (SMO-1) antagonizes the interaction between CKI-2 and RNF-1. These data suggest that a novel regulatory mechanism may exist to maintain appropriate levels of CKI-2 through an interaction between the RING domain protein, RNF-1, and SUMO.

4.3. Results

4.3.1. RNF-1 interacts with CKI-2

Unlike *cki-1*, RNAi has not been useful in characterizing the function of *cki-2*. Therefore, to gain further insight as to how *cki-2* exerts its function during embryogenesis, we used a yeast two-hybrid analysis using the C-terminus of CKI-2 as bait. From this screen we isolated two candidates, one of which corresponded to a novel RING finger protein that contained a conserved RING domain motif in its N-terminus, and as such we refer to this protein as RNF-1 (Figure 4.1A and D).

Since there are two predicted CIP/KIP family CDK inhibitors (CKI-1 and CKI-2) located in tandem on *C. elegans* chromosome II, we wanted to know whether RNF-1 could interact with CKI-1 or if it bound exclusively to CKI-2. By doing a directional two-hybrid analysis, we found that CKI-1 did not interact with RNF-1 (Figure 4.1A, bottom), suggesting that the RNF-1 interaction may be specific to CKI-2 and not a general interaction with all CKIs.

To better understand the interaction between CKI-2 and RNF-1, we mapped the CKI-2 interaction domain on RNF-1 using a series of deletion constructs (LexA-DBD fused baits of CKI-2) (Figure 4.1B). Consistent with the yeast two-hybrid screen performed with the C-terminus of CKI-2 as bait (Figure S3.1 in appendix III), this analysis showed that RNF-1 bound to a specific region in the C-terminus of CKI-2 (amino acid residues 201-212 (HNNKGAPKRPLR)). This domain is in close proximity with PCNA binding region (amino acid residues151-163 and unpublished data) (Figure 4.1C, top) and a putative nucleolar localization signal (amino acid residues 192-198 and unpublished data) (Figure 4.1C, bottom), suggesting that the RNF-1 binding domain may cooperate with the other domains on the C-terminus of CKI-2. In addition to the RING finger motif, we found that RNF-1 also has a conserved peroxisomal targeting sequence 2 (PTS2) in its C-terminus (Figure 4.1D), although the role of the PTS2 remains to be sudied.

4.3.2. RNF-1 is involved in the degradation of CKI-2

Previous studies have shown that overexpression of CKI-2 causes embryonic lethality (Fukuyama et al., 2003), suggesting that it is important to maintain appropriate levels of CKI-2 to ensure proper embryonic development. Since many RING domain proteins act as components of multi-subunit E3 ubiquitin ligases (Joazeiro and Weissman, 2000), we speculated that RNF-1 might target CKI-2 for subsequent degradation. If it is the case, co-expression of RNF-1 should suppress the embryonic lethality associated with the misexpression of CKI-2, probably through its ability to increase the rate of CKI-2 degradation. Thus, to test this possibility, RNF-1 was co-expressed with CKI-2 in *C. elegans* and both embryonic lethality and CKI-2 levels were examined. Misexpression of RNF-1 itself did not cause any embryonic defect (Table 4.1), although the embryonic lethality associated with the misexpressed CKI-2 was considerably reduced by the co-expression of RNF-1. This suggests that RNF-1 antagonizes CKI-2 function. In addition, co-expression of RNF-1 with the N-terminus of CKI-2, which does not bind to RNF-1 (Figure 4.1B), did not suppress the embryonic lethality caused by misexpression of the CKI-2 variant (Table 4.1), suggesting that suppression of the embryonic lethality may require a direct interaction between RNF-1 and CKI-2.

Based on the typical role of RING domain proteins, we reasoned that the suppression of the embryonic lethality might be due to a role of RNF-1 in increasing the rate of CKI-2 degradation. To determine whether this was the case, we performed western blot analyses on whole *C. elegans* extracts prepared from transgenic lines co-expressing CKI-2 and RNF-1 which were collected at various time points post heat-shock (Figure 4.2A, top). The CKI-2::GFP peaks in expression approximately 5h post heat-shock, while when co-expressed with RNF-1 this peak shifts substantially with a maximum 2-3h post heat-shock, decreasing to baseline levels at 6 h post heat-shock while it remains thereafter (Figure 4.2A, top), suggesting that RNF-1 genetically interacts with CKI-2 and may enhance the degradation of CKI-2 thereby alleviating some of the negative effects caused by CKI-2 overexpression.

Since RNF-1 seems to play a role in the degradation of CKI-2, we point out again that there is degradation but past work indicates that it is not mediated by CUL-2-based SCF. We wanted to know whether the degradation of CKI-2 was mediated by the proteasomal complex. To test this possibility, we performed western blot analyses using protein extracts prepared from the transgenic animals co-expressing CKI-2 and RNF-1 in a *pas-4 (RNAi)* background, where PAS-4 is an essential component of the proteasomal complex (Davy et al., 2001). Interestingly, we found that the CKI-2 levels remained stable even 5 hour post heat shock, indicating that CKI-2 most likely degraded in a proteasome-dependent manner although not by a cullin-based SCF E3 ligase system (Figure 4.2A, bottom). Taken together, RNF-1 is involved in the degradation of CKI-2 through the proteasome-mediated proteoysis.

4.3.3. RNF-1 is involved in the ubiquitination of CKI-2

Since multi-subunit E3 ubiquitin ligase complexes target the proteins mostly in an ubiquitin-dependent manner, we reasoned that RNF-1 may be involved in the ubquitination of CKI-2 and consequently its degradation. To test this possibility, western blot analyses were performed using protein extracts prepared from transgenic lines co-expressing CKI-2 and RNF-1. We found that in addition to a CKI-2 band (~64 KDa), an extra band with higher molecular weight (~84 KDa) was recognized by an anti-CKI-2 serum, where a similar band was not observed in controls (non-heat shocked or misexpression of CKI-2 without RNF-1) (Figure 4.2B, top). The higher molecular weight band is detected beginning at abut 30 minute post heat shock and peaks at 1 h post heat shock, and thereafter high molecular weight entities become apparent in addition to the 84 KDa band (Figure 4.2C). The higher molecular weight band was approximately 20 kDa greater than the molecular weight of CKI-2, which was consistent with the possibility that CKI-2 was modified by ubiquitin. In recent two-hybrid studies, RNF-1 was found to interact with two E2 ubiquitin-conjugating enzymes (UBC-8 and UBC-20) (Jones et al., 2002; Gudgen et al., 2004). Thus, if RNF-1 is involved in the ubiquitination of CKI-2 through the E2 enzyme(s), removal of UBC-8 or UBC-20 activity may result in the loss of the higher molecular weight band observed following heat shock induced accumulation of

CKI-2. We tested this possibility by performing western blot analyses. Protein extracts were prepared 1 hour post heat shock from *ubc-20 (RNAi)*-treated transgenic animals which co-express CKI-2 and RNF-1 followed by a western blot analysis with anti-CKI-2 serum. We found that the intensity of the higher molecular weight band was significantly reduced by removing *ubc-20*, indicating that the higher molecular weight band is very likely to be due to ubquitination (Figure 4.2B, top). Since no useful antibody that detects *C. elegans* ubiquitin is currently available, to further confirm that CKI-2 is ubiquitinated, we performed *ubq-1 (RNAi)*, which removes UBQ-1, a *C. elegans* ubiquitin orthologue (Stringham et al., 1992). If the higher molecular weight band contained ubiquitin, *ubq-1 (RNAi)* should remove or reduce the apparent molecular weight of the band. We performed a western blot analysis in a similar manner as those performed following *ubc-20 (RNAi)* to show the greater molecular weight band eventually disappeared in animals treated with *ubq-1 (RNAi)* (Figure 4.2B, bottom). This confirms that RNF-1 is involved in the ubiquitination of CKI-2. Because CKI-2 has been found to be a target of SUMOylation (Figure 4.1C and unpublished data), we confirmed that the greater molecular weight band was not due to a modification of CKI-2 by SUMO, which is a small ubiquitin-related protein family and causes a similar molecular weight shift as in ubiquitination (Melchior, 2000). We found that depletion of UBC-9 (E2 SUMO conjugating enzyme) or SMO-1 (*C. elegans* SUMO orthologue) by RNAi did not affect the presence or intensity of the higher molecular weight band, indicating that the extra band was not due to SUMOylation and most likely ubquitination (Figure 4.2B, bottom) (Jones et al., 2002).

Interestingly, a similar extra band or high molecular weight entities were not observed in the co-expression of RNF-1 with N-terminus of CKI-2 (Figure 4.2C). Because RNF-1 interacts uniquely with the C-terminus of CKI-2 (Figure 4.1B), these data suggest that the higher molecular weight band(s) may be mediated by direct interaction of CKI-2 with RNF-1. This is consistent with the data that the embryonic lethality associated with the CKI-2 N-terminal variant is not suppressed by the co-expression of RNF-1 (Table 4.1).

Taken together, our data strongly argue that RNF-1 is involved in the degradation of CKI-2 through an ubiquitin-dependent proteolysis, which seems to be mediated by direct interaction between CKI-2 and RNF-1.

4.3.4. SMO-1 may modulate the RNF-1 function

Previously we identified the *C. elegans* orthologue of PCNA (proliferating cell nuclear antigen) (PCN-1) (Warbrick, 1998) and SUMO (SMO-1) as CKI-2 interacting partners using a similar yeast two-hybrid strategy (unpublished data). Since SUMO has been demonstrated to modify protein/protein interactions (Melchior, 2000), we reasoned that SMO-1 might antagonize these interactions.

To investigate this possibility we developed a yeast-based competition assay using a galactose-inducible SMO-1 expression system (Ronicke et al., 1997) (Figure 4.3). Using this approach, protein-protein interactions that can be antagonized by the binding of SMO-1 result in reduced growth on galactose plates. To demonstrate that this system is an efficient means to test this idea, a yeast strain containing LexA-DBD (DNA binding domain)::CKI-2, GAL4-AD (transcriptional activation domain):: SMO-1 and *Gal1*::SMO-1 was used as a control wherein the CKI-2 binding domain of SMO-1 driven by *Gal1* promoter overlaps with that of the SMO-1 fused to GAL4-AD (GAL4-AD::SMO-1). The induced SMO-1 successfully competed the CKI-2/SMO-1 interaction and thereby reduced the reporter gene (*ADE2*) expression (Figure 4.3, lane 5).

We applied this system to test whether SMO-1 could antagonize the association of CKI-2 with its known interacting partners. Co-expression of SMO-1 blocked the CKI-2/RNF-1 interaction, while the CKI-2/PCN-1 interaction although slightly affected after galactose induction was not significantly blocked by SMO-1 (Figure 4.3, lane 1-4). Since SUMO interacts with the N-terminus of CKI-2 (unpublished data), while RNF-1 binds to the C-terminus of CKI-2, we postulated that interaction of SMO-1 with the N-terminus of CKI-2 might play a major role in disrupting the CKI-2/RNF-1 interaction

through likely conformation but not due to a steric hindrance. No yeast growth defect was observed when a CKI-2C variant that lacks the consensus SUMO conjugation site was used (Figure S3.2 in appendix III), thus confirming that the interaction of the N-terminus of CKI-2 with SMO-1 is required to antagonize the CKI-2/RNF-1 interaction. Taken together, our results highlight that SUMO may play a role in disrupting the interaction between CKI-2 and RNF-1 by acting as an N-terminal switch that probably changes the conformation of CKI-2.

4.4. Discussion

Considering the significance of the post-transcriptional levels of CKI proteins in the regulation of cell cycle progression, it is not surprising that the CKI levels are frequently misregulated in many cancer cells (Nakayama and Nakayama, 2006). Although it has been suggested that appropriate levels of CKI-2 are critical to ensure proper embryonic development (Feng et al., 1999; Fukuyama et al., 2003), little has been characterized concerning a mechanism to maintain appropriate levels of CKI-2. While CKI-1 has been known to be targeted by a canonical cullin-based SCF E3 ligase complex, it seems not the case for CKI-2 (Feng et al., 1999).

No functional allele of *rnf-1* has been available. Moreover, *rnf-1 (RNAi)* did not give rise to an apparent defect during development in *C. elegans*, although we have confirmed that the *rnf-1 (RNAi)* significantly removes endogenous RNF-1 (data not shown). Since a number of RING finger family proteins have been predicted in the *C. elegans* genome database, we speculated that the loss of RNF-1 could be tolerated by a redundant activity from other RING domain protein(s). Thus, we turned to a different strategy to study the role of CKI-2. Using a yeast two-hybrid screen, we identified RNF-1 as a CKI-2 interacting partner, which is a C3HC4 type RING domain protein (Figure 4.1D). This RING finger domain is conserved among proteins involved in ubiquitination (Joazeiro and Weissman, 2000), suggesting that RNF-1 may be implicated in proteolytic degradation. Although, to date, we have not been able to demonstrate an E3 ubiquitin ligase activity for RNF-1, our data provide a promising link that CKI-2 may be targeted by RNF-1 in an ubiquitin-dependent manner. Moreover, this relationship seems specific for the CKI-2 function since CKI-1 did not interact with RNF-1 in our analysis. This would not therefore be entirely unexpected since CKI-1 levels are regulated by a SCF^{CUL-2} complex, which has presumably no role in regulating CKI-2 levels (Feng et al., 1999).

A recent study showed that RNF-1 interacts with at least two *C. elegans* E2 ubiquitin-conjugating enzymes (Gudgen et al., 2004). Our data suggest a possible link

since we observed that the high molecular weight form of CKI-2 associated with the co-expression of RNF-1 disappeared following *ubc-20 (RNAi)* (Figure 4.2B). Moreover, the co-expression of RNF-1 is associated with the ubiquitination of CKI-2 (Figure 4.2B). Therefore, establishing whether these E2 enzymes genetically interact with CKI-2, or whether the reduction of function of these E2 enzymes affects CKI-2 abundance in *C. elegans* will be critical. In addition, it would be interesting to characterize whether RNF-1 acts as a component of multi-protein E3 complex or through an unknown novel pathway.

Using a directional yeast two-hybrid analysis, we determined that the RNF-1 binding region of CKI-2 is in close proximity to a putative nucleolar localization signal (amino acid residues 192-198) that we have characterized (Figure 4.1C and unpublished data). Since the nucleolar localization of CKI-2 seems to be linked to its degradation (unpublished data), it is possible that RNF-1 might cooperate with the nucleolar targeting signal in an unknown manner. Because we observed that the C-terminus of CKI-2 localizes constitutively to the nucleolus (unpublished data), it would be of interest to test whether RNF-1 affects the localization of the CKI-2 variant.

Interestingly, we noticed that in addition to RING finger motif, RNF-1 possesses a PTS2 peroxisomal-matrix targeting sequence (Figure 4.1D) in its C-terminus, although the role of this motif remains unclear at present. Since it has been shown that peroxisome signals can affect nuclear gene expression in response to cellular stress (Corpas et al., 2001), it is possible that RNF-1 may link cell cycle control to the stress response pathway, although this remains to be further characterized.

Two previously identified CKI-2 interactors included the *C. elegans* orthologues of PCNA and SUMO. PCNA plays an essential role as a DNA replication factor and also has a role in DNA repair (Warbrick, 1998), while SUMO is a small ubiquitin-related modifier which modulates protein/protein interactions and/or sub-cellular localization of targeted polypeptides (Melchior, 2000). The fact that CKI-2 interacts with these partners suggests that it might be involved in the regulation of cell cycle progression controlling S-phase

entry, reminiscent of p21Cip1 in mammalian cells (Waga et al., 1994; Jackson et al., 1995). Furthermore, this function may be modulated by SMO-1 through timely modification of CKI-2. Using a yeast-based competition assay we demonstrated that SMO-1 specifically antagonizes the interaction between CKI-2 and RNF-1, while the effect in the CKI-2/PCN-1 interaction is minimal (Figure 4.3). These data suggest an intriguing model wherein SMO-1 may modulate RNF-1 function and as a result affect the levels of CKI-2.

Taken together, our data show that RNF-1 may play a role in regulating the levels of CKI-2 by controlling its rate of degradation, particularly through an uncharacterized E3 ubiquitin ligase activity or more intriguingly, that the RNF-1 function may be modulated by the CKI-2/SMO-1 interaction. Given that nothing has been reported concerning a mechanism of the CKI-2 degradation to date, we believe that our study provides an avenue for further expansion of the understanding of CKI biology.

4.5. Materials and Methods

4.5.1. Nematode Strains

The following *C. elegans* strains were used: N2 Bristol was used as the wild-type throughout. MR251 (*unc-119*; *rr*Ex251 [*hs*::CKI-2::GFP; *unc-119*(+)]), MR353 (*unc-119*; *rr*Ex251 [*hs*::CKI-2N::GFP; *unc-119*(+)]), MR664 (*unc-119*; *rr*Ex664 [*hs*::GFP::RNF-1; *unc-119*(+)]), MR665 (*unc-119*; (*rr*Ex251 [*hs*::CKI-2::GFP; *unc-119*(+)]; *rr*Ex664 [*hs*::GFP::RNF-1; *unc-119*(+)])), MR666 (*unc-119*; (*rr*Ex353 [*hs*::CKI-2N::GFP; *unc-119*(+)]; *rr*Ex664 [*hs*::GFP::RNF-1; *unc-119*(+)])). All *C. elegans* strains were cultured using standard techniques and maintained at 20°C unless stated otherwise (Brenner, 1974).

4.5.2. Yeast two-hybrid screen

Saccharomyces cerevisiae strain *W303 Y1003* (*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. The yeast strains were 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 60μg of cDNA library expressing the GAL4-AD (transcriptional aqctivation domain) fused to mixed stage *C. elegans* cDNAs (a gift from A. La Volpe) and screened as described (Gietz et al., 1997).

Directional two-hybrid assay. The yeast strain containing LexA-DBD::CKI-1 was 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.

4.5.3. *In vivo* competition studies

The yeast strains containing LexA-DBD::CKI-2 and GAL4-AD::RNF-1, or LexA-DBD::CKI-2 and GAL4-AD::PCN-1 were transformed with *Gal1*::SMO-1 independently and then grown on selective media deficient in tryptophan, leucine, and histidine. Selected colonies were cultured in liquid to exponential phase ($O.D_{600} = 0.5$) and after 10-fold serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}), the diluted cells were spotted onto Adenine (+); galactose (+) or Adenine (-); galactose (+) plates deficient in tryptophan, leucine, histidine, and incubated for 5 days at 30°C. Yeast strains containing (LexA-DBD::CKI-2; GAL4-AD::SMO-1; *Gal1*::SMO-1) were used as controls.

4.5.4. Mapping protein-protein interaction domains

To map the CKI-2 interaction domains of C06A5.9 (RNF-1), constructs encoding CKI-2 variants were generated using PCR and were inserted into the BamHI/SalI sites of pEG202-NLS (9.8-kb) to yield the LexA-DBD fusion constructs LexA-DBD::CKI-2 (amino acids 1-115), LexA-DBD::CKI-2 (amino acids 116-259), LexA-DBD::CKI-2 (amino acids 164-259), LexA-DBD::CKI-2 (amino acids 1-200), LexA-DBD::CKI-2 (amino acids 1-212).

4.5.5. Heat-shock experiments

The following constructs were used for the heat shock-related 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), and both promoter containing constructs were co-injected to generate heat shock-related transgenic animals): *hs*::GFP::RNF-1, *hs*::CKI-2::GFP, and *hs*::CKI-2N::GFP. Transgenes (10μg/ml) were microinjected with UNC-119(+) rescuing plasmid (100μg/ml) as a co-injection marker into *unc-119 (ed-4)* hermaphrodites as described (Mello et al., 1991). Heat shock-induced expression was performed by floating parafilm-sealed culture plates in a 33°C water bath for 1 hour followed by a 4 hour-recovery period at 20°C. To check the embryonic lethality after heat shock, embryos laid from gravid adults were heat shocked for up to 30 minute and the result was presented as the percentage of the unhatched embryos in a total population.

4.5.6. RNA mediated interference (RNAi)

RNAi was performed by a feeding method as described (Fire et al., 1998; Kamath et al., 2001). Briefly, L1 or L2 transgenic larvae were transferred onto the plate containing IPTG-induced dsRNA producing bacteria and placed at 20°C. The transferred animals allowed to grow until adult stage were heat shocked to induce the transgenes followed by a preparation of protein extracts for western blot analyses.

4.5.7. Antibodies and Western blot analysis

Worms were picked into SDS sample buffer and were freezed/thawed twice at -80°C and 100°C. The supernatant was subjected to 10% SDS-PAGE and proteins were transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Pharmacia) and blotted as described elsewhere. Primary antibodies were rabbit polyclonal anti-CKI-2 (generated in our laboratory), monoclonal α-tubulin (Sigma), and anti-GFP (Clonetech). 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™ (Amersham Pharmacia).

4.6. Acknowledgments

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4.8. Legends to Figures

Figure 4.1. **CKI-2 interacts with RNF-1.** (A) Summary of the interaction between LexA-DBD fused CKI-2 (top) or CKI-1 (bottom) and the GAL4-AD fused RNF-1 using a directional yeast two-hybrid analysis. DBD or AD indicates DNA binding domain or transcriptional activation domain, respectively. The control (-control) carries LexA-DBD fused CKI-2 or CKI-1 bait plasmid and empty GAL4-AD fusion plasmid (prey plasmid). (+) or (-) in the table (right) indicates "interaction" or "no-interaction", respectively. (B) Mapping of the RNF-1 binding region on CKI-2. The yeast strain containing GAL4-AD::RNF-1 was transformed separately with the individual CKI-2 bait variants (LexA-DBD fused) followed by determination of *lacZ* expression from each transformant. (+) or (-) indicates "expression" or "no expression" of *lacZ*, respectively. (C) Summary of interaction domains (top) and organelle targeting signals (bottom) on CKI-2. CDI, CDK inhibitory domain; PBD (black closed oval), PCNA binding domain; RBD (gray closed oval), RNF-1 binding domain; SBD (black bar), SMO-1 binding domain (K20 and K40 are predicted SUMOylation target sites); NoL, Nucleolar localization signal. (D) Conserved motives on RNF-1. (D, top) The primary sequence of the RING domain (amino acid residues 22-67) on RNF-1 aligned with the consensus RING finger motif (C3HC4 type). Dots mark the conserved amino acid residues (Cysteine (C) and Histidine (H)). (D, bottom) A peroxisomal targeting sequence 2 (PTS2) was identified in the C-terminus of RNF-1 (amino acid residues 340-347). R, arginine; K, lysine; L, leucine; I, isoleucine; x, any amino acid; Q, glutamine; H, histidine. The number indicates the position of amino acid residues in the primary sequence. The arrows indicate the minimal region that is necessary for RNF-1 binding on CKI-2. The RNF-1 binding region on CKI-2 is shown by a gray closed oval with arrow.

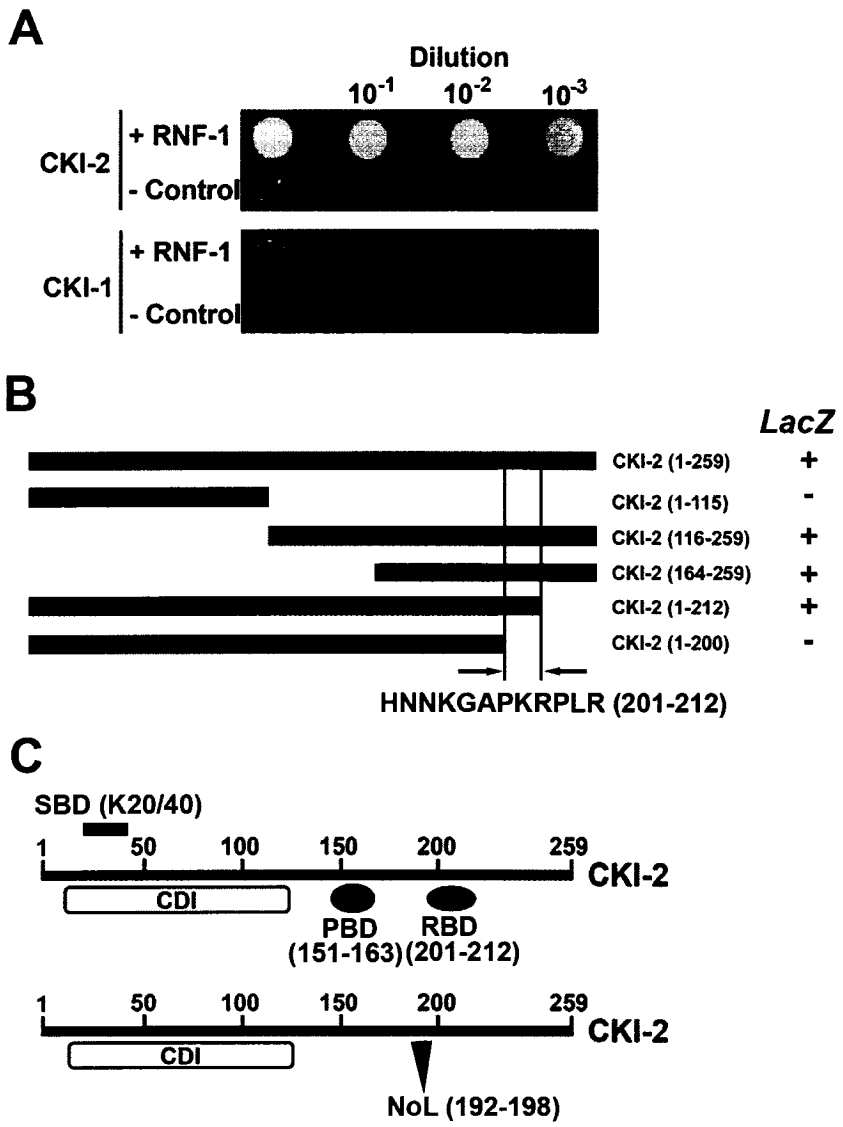
Figure 4.2. **RNF-1 mediates CKI-2 degradation through ubiquitin-dependent proteolysis.** (A, top) Time course analysis of CKI-2 levels using western blotting with anti-CKI-2 (α-CKI-2), or anti-tubulin (α-tubulin) antibody. Protein extracts were prepared from a mixed population of the transgenic animals 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). (A, bottom) A western blot analysis performed using protein extracts prepared from *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. (B, top) 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, bottom) Western blot analyses performed using protein extracts prepared from the *ubc-9 (RNAi)*, or *smo-1 (RNAi),* or *ubq-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. (C) 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. The arrows indicate the position of 64 KDa, or 48 KDa, or 84 KDa standard size markers, respectively.

Figure 4.3. **SMO-1 antagonizes the interaction between CKI-2 and RNF-1.** An *in vivo* competition assay using a galactose-inducible system in yeast. *Gal1*, *Gal1* promoter. Ade and Gal, Adenine and Galactose, respectively. (+) or (-) indicates possession (+) or deficiency (-) of the component on the media.

	Embryonic lethality (%)	
Genotype	No heat-shock	Heat-shock
hs::GFP	0 (n=471)	3.2 (n=472)
hs::GFP::RNF-1	0.6 (n=498)	5.4 (n=463)
hs::CKI-2::GFP	1.4 (n=439)	20.6 (n=402)
hs::CKI-2::GFP; *hs*::GFP::RNF-1	1.2 (n=365)	6.6 (n=426)
hs::CKI-2::GFP; *hs*::GFP	1.1 (n=437)	19.4 (n=458)
hs::CKI-2N::GFP	1.2 (n=410)	18.3 (n=437)
hs::CKI-2N::GFP; *hs*::GFP::RNF-1	1.1 (n=365)	19.8 (n=414)
hs::CKI-2N::GFP; *hs*::GFP	1.0 (n=396)	18.4 (n=425)

Table 4.1. **Co-expression of RNF-1 suppresses the embryonic lethality associated with misexpression of CKI-2 but not the N-terminal variant.** For the embryonic lethality (%), embryos from young adult animals that carry heat shock constructs were heat-shocked and examined 30 hours later for embryonic lethality determined by the number of L1 larvae present on the plate. Non-heat shocked embryos were used as a control. The values represent the percentage of unhatched embryos that arise from the initial population of embryos (n).


A
Dilution
10-1
10-2
10-3
CKI-2
+ RNF-1
- Control
CKI-1
+ RNF-1
- Control
B
LacZ
CKI-2 (1-259) +
CKI-2 (1-115) -
CKI-2 (116-259) +
CKI-2 (164-259) +
CKI-2 (1-212) +
CKI-2 (1-200) -
HNNKGAPKRPLR (201-212)
C
SBD (K20/40)
1 50 100 150 200 259
CKI-2
CDI
PBD (151-163)
RBD (201-212)
1 50 100 150 200 259
CKI-2
CDI
NoL (192-198)
D
RNF-1 (22-67) CQVCYQPFNETTKLARSLHCGHTFCTECIRNVQNYGNSPHLECPTC
Consensus CPICLEEYLKDPVV---LPCGHTFCRSCIRKWLESSNSNT--CPIC
1 340 347 408
RNF-1 (340-347) RLFFKLKQL
Consensus (PTS2) (R/K)(L/I)XXXXX(Q/H)L

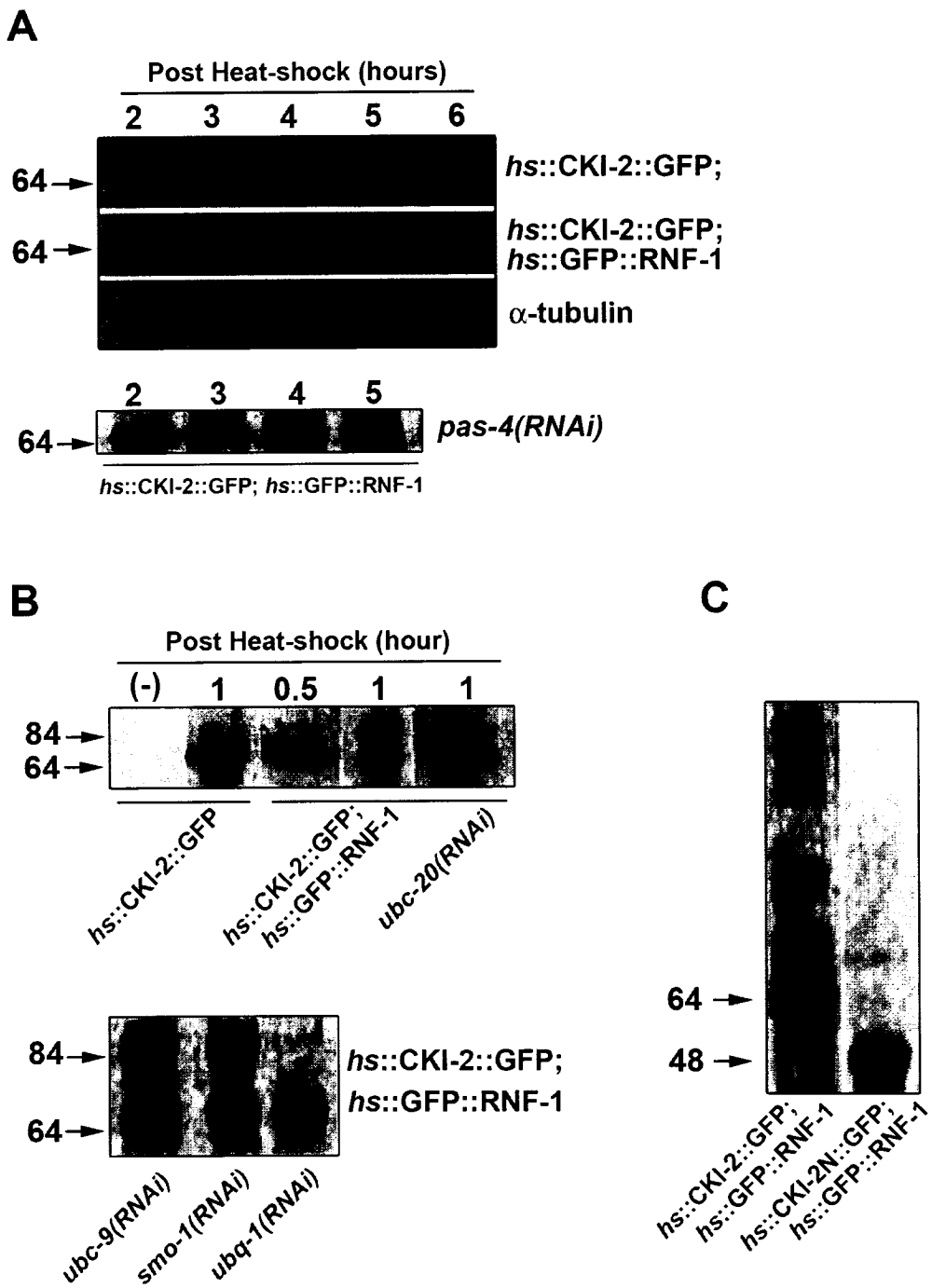
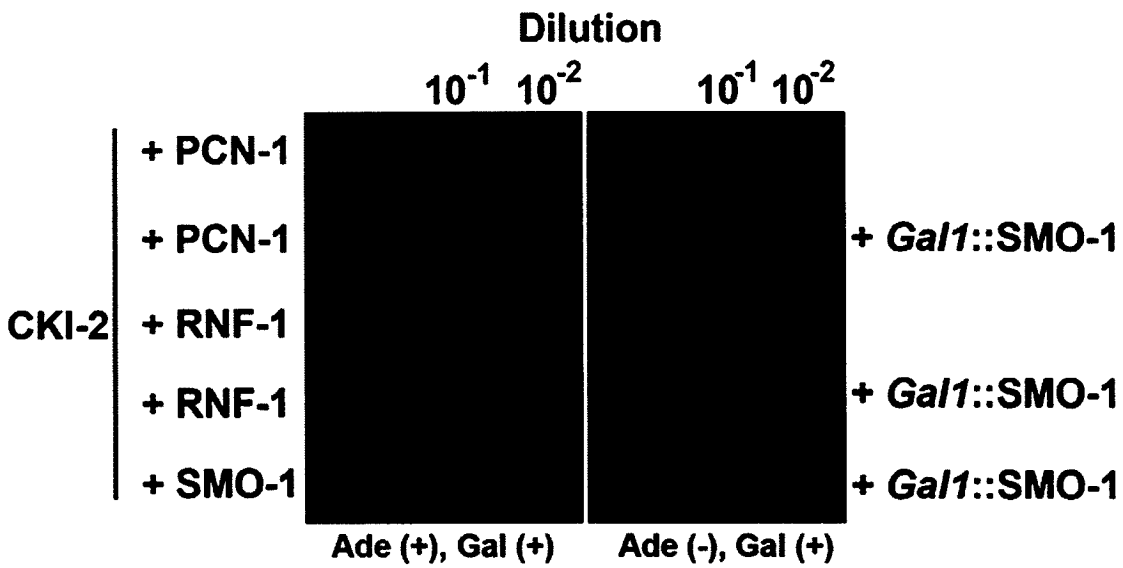

A
Post Heat-shock (hours)
2 3 4 5 6
64
64
hs::CKI-2::GFP;
hs::CKI-2::GFP;
hs::GFP::RNF-1
α-tubulin
2 3 4 5
64
pas-4(RNAi)
hs::CKI-2::GFP; hs::GFP::RNF-1
B
Post Heat-shock (hour)
(-) 1 0.5 1 1
84
64
hs::CKI-2::GFP
hs::CKI-2::GFP; hs::GFP::RNF-1
ubc-20(RNAi)
84
64
hs::CKI-2::GFP;
hs::GFP::RNF-1
ubc-9(RNAi)
smo-1(RNAi)
ubq-1(RNAi)
C
64
48
hs::CKI-2::GFP; hs::GFP::RNF-1
hs::CKI-2N::GFP; hs::GFP::RNF-1

Figure 4.3


Dilution
10-1 10-2
10-1 10-2
CKI-2
+ PCN-1
+ PCN-1
+ RNF-1
+ RNF-1
+ SMO-1
+ Gal1::SMO-1
+ Gal1::SMO-1
+ Gal1::SMO-1
Ade (+), Gal (+)
Ade (-), Gal (+)

Chapter V

General Discussion

5.1. Summary and Conclusions

The research in this thesis has described the role of *cki-2* in multiple aspects of development in *C. elegans*. Using a reverse genetic approach called co-suppression, we found that loss of *cki-2* in the germ line causes the perdurance of centrioles until late stages of oocyte development, giving rise to supernumerary centrosomes in a fertilized embryo thereby resulting in embryonic arrest caused by severe aneuploidy. We also noticed that a catalytic function of cyclin E/Cdk2 complex is involved in this critical developmental process. Therefore, our results reveal that *cki-2* is required for the appropriate elimination of centrioles during oogenesis presumably by blocking the catalytic function of a cyclin E/Cdk2 complex.

The chracterization of CKI-2 interacting proteins identified by a yeast two-hybrid screen provided a novel point of entry to describe how the post-translational levels of CKI-2 are regulated. We presented that CKI-2 is covalently modified by SMO-1 and that this causes re-localization of CKI-2 to the nucleolar compartment followed by a rapid degradation of CKI-2. Furthermore, we found that RNF-1 has the ability to increase the rate of CKI-2 degradation in an ubiquitin-dependent manner, which is correlated with the suppression of embryonic lethality associated with CKI-2 overexpression. We also noticed that SMO-1 antagonizes the interaction between CKI-2 and RNF-1. Therefore, we conclude that the levels of CKI-2 are regulated by SUMO-mediated nucleolar localization where the degradation of CKI-2 occurs through an ubiquitin-dependent proteolysis mediated by RNF-1.

Although our results have expanded our understanding of CKI in a developmental context, there are still a number of questions that remain to be answered. Therefore, in this chapter, I will discuss some of the major questions concerning the role of *cki-2* that could not be discussed in detail in the previous chapters.

5.2. Mechanisms involved in the Centriole Destabilization

Although it has been observed that centrioles disappear during specific developmental processes, such as during spermatogenesis in humans (the mother centriole is destabilized, while the daughter centriole is remains), in mice (both the mother centriole and the daughter centriole are destabilized), and in oogenesis in a number of organisms including *C. elegans* (both the mother centriole and the daughter centriole disappear), it is still unclear how this critical developmental event is driven (Schatten, 1994; Delattre and Gonczy, 2004). However, an intriguing finding that a viral infection induces disappearance of centrioles in HeLa cells suggested that there might be an active process to eliminate centrioles (Ploubidou et al., 2000). Our study of *cki-2* in *C. elegans* has revealed a novel mechanism to ensure the timely elimination of centrioles during oogenesis. The results presented in chapter II of this thesis have provided a breakthrough on which to further expand our understanding of centriole assembly, maintenance, and disassembly at the molecular level.

5.2.1. Molecules involved in the centriole destabilization

In Chapter II, we showed that cyclin E/Cdk2 is somehow involved in the centriole stabilization/elimination decision. A series of studies performed using different cellular systems have revealed that a catalytic function of cyclin E/Cdk2 complex is required for centrosome duplication during S-phase and have suggested a mechanism indicating that centrosome duplication is coupled to DNA synthesis and mitotic division. Several studies have suggested that this occurs through the timely control of cell cycle regulators, namely Cdk2. In mammalian cells, degradation of p27Kip1 is required for S phase progression prior to the intitation of DNA replication and this is mediated by Cdk2-dependent phosphorylation (Slingerland and Pagano, 2000). Ectopic expression of p21Cip1 or p27Kip1 represses centrosome duplication as well as DNA synthesis (Waga et al., 1994; Lacey et al., 1999), while loss of p21Cip1 causes overduplication of centrioles (Mantel et al., 1999). Cdk2 also is involved in centrosome duplication through its role in stabilizing a Mps1 kinase, a key protein implicated in this process. Like yeast Mps1 kinase (Lauze et al., 1995), mouse Mps1 kinase (mMps1p) is required for centrosome duplication and its protein stability is controlled by Cdk2-mediated phosphorylation (Fisk and Winey, 2001).

Inhibition of Cdk2 activity causes the destabilization of mMps1p, resulting in the loss of mMps1p in centrosomes. This indicates that Cdk2 may promote centrosome duplication through its ability to stabilize Mps1 kinase. These results highlight the significant role of Cdk2 in regulating protein stability, which must be regulated to ensure appropriate maintenance of centrosome numbers during cell cycle progression. If it was also true in the germ line in *C. elegans*, the altered activity of Cdk2 caused by the loss of CKI-2 might affect the stability of the centriolar proteins and/or other Cdk2 target proteins.

In *C. elegans*, genetic studies have identified a number of proteins that are involved in centriole duplication. While ZYG-1 is a protein kinase which localizes to centrioles only during mitosis (O'Connell, 2001), SAS-4/5/6 and SPD-2 are coiled-coil scaffold proteins associated with the centrioles throughout the cell cycle (Leidel and Gonczy, 2005). It has been shown that ZYG-1 is involved in the centriolar localization of SAS-5 and the SAS-4/SAS-5 association is required for the centriolar recruitment of SAS-6. Since the loss of *zyg-1* causes a monopolar spindle (MPS) in the zygote (due to paternal defect) or in the two-cell stage embryo (due to maternal defect) quite similar to Mps1 kinase mutants in yeast, it has been postulated that ZYG-1 might take its place in *C. elegans*, although there seems to be no apparent homologue of ZYG-1. In this thesis, we have presented that the loss of *cki-2* causes accumulation of ZYG-1 and SPD-2 on meiotic spindles (Appendix I). This observation suggested that the catalytic function of Cdk2 may be involved in the regulation of ZYG-1, probably through its ability to stabilize ZYG-1. Since ZYG-1 is present in centrioles only during mitosis, stabilization of ZYG-1 might affect the centrosome cycle. It would be interesting to test the possibility using ZYG-1 variants, which were mutated to affect its stability, with genetic and biochemical approaches. One very plausible scenario for such a regulatory mechanism would include the phosphorylation of key centriolar components via cyclin E/Cdk2. Such phosphorylation would be stabilizing and thus would be analogous to the Cdk- dependent phosphorylation of Mps1 kinase

We have scanned primary sequences of the centriolar proteins for putative Cdk2-mediated phosphorylation sites using a bioinformatic algorithm (Xue et al., 2005) to find that a number of highly predicted Cdk2-mediated phosphorylation sites are present in many of the centriolar proteins (data not shown), suggesting that this stabilization may occur through multiple centriolar targets. Since it has been shown that centriole duplication can be studied using GFP-fused centriolar proteins (Leidel and Gonczy, 2005), it may be informative to examine the effects of variants of these centriolar proteins which have been mutated in their putative Cdk-phosphorylation sites. Through the molecular characterization of these centriolar proteins and the resulting centriolar behavior that arises from such modifications, it may be possible to elucidate the mechanisms involved in the stabilization/elimination of centrioles in the context of oogenesis.

5.2.2. Why do centrioles disappear during a specific stage?

Our observation, presented in chapter II, showed that centrioles disappear at late pachytene stage during oogenesis. Thus, a challenging question to be answered is why centrioles disappear in this specific stage of oogenesis. During female germ cell development in *C. elegans* hermaphrodites, two major transitions occur: the mitotic/meiotic transition and pachytene exit (oocyte differentiation). The mitotic exit to the meiotic state occurs through downregulation of GLP-1 signalling (see chapter I). On the other hand, the meiotic switch to oogenesis occurs through exit from the pachytene stage, where LET-60 RAS/MPK-1 MAP kinase pathway has been known to be required for progress through this stage (Church et al., 1995). In addition, it has been shown that GLD-1 is also critically involved in oocyte differentiation. Whereas GLD-1 is present in low levels in the mitotic region, consistent with a non-functional role of GLD-1 in germ cell proliferation, entry into the meiotic state is accompanied by increased levels of GLD-1. Moreover, GLD-1 is present in the germ line throughout the pachytene stage, however, at the point of pachytene exit prior to oocyte differentiation, GLD-1 levels dramatically decrease and remain absent until the completion of oogenesis (Jones et al., 1996). These findings suggest that GLD-1 may be involved in oocyte differentiation through its ability to repress the translation of maternal mRNAs that are synthesized during the early meiotic stages until pachytene exit when they

all release from this inhibition. Since this coincides with the time that the centrioles disappear, it would be interesting to characterize whether there is a genetic interaction between these two different developmental events and more intriguingly, whether translational repression mediated by GLD-1 is required for centriole maintenance.

Unlike canonical cell cycles in which cell growth is coupled with cell division, variations of the cell cycle, which are normal and highly regulated, must also somehow confront the problem of regulating centrosome numbers. During endoreplication in *Drosophila* larvae and in the *C. elegans* intestine and hypodermis, the centrosome cycle is also uncoupled from DNA synthesis (see Chapter I in this thesis). However, little is understood as to whether the centrioles do indeed duplicate and are subsequently eliminated, or whether their duplication is uncoupled from the activity of cyclin E/Cdk2 during S-phase. Thus, it will be of particular interest to examine each of these possibilities and test whether there is a novel link between cell cycle variation and the centriole destabilization/disassembly.

5.3. SUMO-mediated Nucleolar Localization and CKI-2 Degradation

In chapter III, we showed that CKI-2 is degraded following SUMO-mediated nucleolar localization. Moreover, in chapter IV, we showed that this degradation is mediated by a RING finger protein, RNF-1, in an ubiquitin-dependent manner. Since our study deals with a degradation of previously uncharacterized CKI through a novel mechanism, we believe that this will contribute to further understanding of the significance of post-translational modifications involved in maintaining appropriate CKI levels.

5.3.1. Does CKI-2 shuttle between two compartments?

The importance of the nucleolus in sequestering important cell cycle regulators has recently been brought to the forefront (Visintin and Amon, 2000). Mitotic exit in *S. cerevisiae* is tightly controlled by the timely release of Cdc14p from the nucleolus (Vistinin et al., 1999). Although Cdc14p controls late stages of the cell cycle, one could envisage

that progressive nucleolar localisation and destabilisation of CKI-2 during the period in which daughter cells are reforming following a division might confer the S-phase regulation that is typical of these early embryonic divisions. Once levels of CKI-2 fall below a critical baseline threshold due to SUMOylation of an active CKI-2 population on or around origins of replication, the origins become active and S-phase proceeds. At mitosis the nucleolus disappears and then reforms in the daughters and the cycle resumes with the re-establishment of a functional nucleolus. This would allow for the cell cycle oscillations typical of embryogenesis without the necessity of cyclic bursts of transcription/translation to renew the levels of positive and negative regulators that drive mitotic cell cycle progression under zygotic control late in embryogenesis and during postembryonic development. Recent data have shown that treatment with a proteasome inhibitor causes a nucleolar accumulation of proteins (Mattsson et al., 2001; Pokrovskaja et al., 2001; Le Goff et al., 2004), suggesting that the nucleolus is involved in regulating some aspect of protein sorting associated with proteasome-mediated protein degradation.

How can this possibility be tested? It has been shown that *cki-2* begins to express at an early stage of embryogenesis (approximately 64 cell stage) and remains high throughout the course of embryonic development (Fukuyama et al., 2003). Thus, it would be informative to image CKI-2 localization in real time using GFP-fused CKI-2 in embryonic cells during the early cell divisions prior to the onset of global zygotic transcription. In addition, a nucleolar marker such as Fibrillarin could be adopted to mark the nucleolar compartment and to use as a reference to monitor changes in CKI-2 localization during specific times during the embryonic cell division cycles. Evidence of cyclical change in CKI-2::GFP localization to the nucleolar compartment would be consistent with this hypothesis.

5.3.2. Mechanisms mediating the nucleolar localization of CKI-2

Our results in chapter III argued that SUMO is sufficient for the nucleolar localization of CKI-2. Indeed, recent data have shown that nucleotide binding in the N-terminal domain of MDM2 induces a similar nucleolar localization event, therefore modification of the

N-terminus of CKI-2 by SMO-1 may play a similar function (Poyurovsky et al., 2003). However, our data demonstrated that a nucleolar signal in the C-terminus of CKI-2 seems to be conflicting with this model. In fact, the C-terminal domain of CKI-2, when expressed in intestinal cells, is strictly localized to the nucleolus, indicating that the C-terminus is also sufficient for the nucleolar localization. How can these findings be accounted for? In the SUMO-fusion experiment presented in chapter III, we noticed that although SUMO was sufficient for the nucleolar localization of the CKI-2 N-terminal variant, a considerable portion of the fusion protein was still present in the nucleoplasm. This observation suggests that the C-terminus of CKI-2 may have a major role in promoting efficient nucleolar localization. Because the C-terminus of CKI-2 localises constitutively to the nucleolus, a genetic screen using this fragment may be useful to identify the proteins that are involved in mediating this nucleolar translocation in *C. elegans*.

5.3.3. Relationship between CKI-2 and PCNA

The yeast two-hybrid screen that we conducted identified PCNA as a C-terminal interacting protein of CKI-2. Studies in mammalian cells suggest that proliferating cell nuclear antigen (PCNA) may play an important role during DNA replication and repair since it recruits DNA polymerase δ and ε to replication origins after formation of pre-replication complexes, thereby stabilizing the enzyme on the chromatin. In addition to its role as a DNA polymerase accessory protein, PCNA also serves as a platform for a number of proteins involved in DNA replication/repair, cell cycle control, and other post-replicative processing (Warbrick, 2000). Therefore, PCNA plays a central role as a recruiting factor for a multitude of proteins required to coordinate DNA replication and repair with the cell division cycle.

The data presented in chapter IV highlighted the possibility that SUMO might act to antagonize protein/protein interactions. Although SUMO did not seem to antagonize the CKI-2/PCNA interaction, we cannot formally exclude that the association of SUMO with CKI-2 could play a role in modulating the interaction of CKI-2 with the DNA replication factors. Moreover, a growing body of studies has demonstrated a critical role of SUMO in

the regulation of the function of PCNA during DNA replication and repair (Ulrich, 2005). Recent data in yeast showed that SUMO modifies PCNA and this modification promotes recruitment of Srs2, a helicase that blocks the recombinational repair through the disruption of Rad51 filaments, thereby preventing any unwanted recombination from occurring during DNA polymerization (Papouli et al., 2005; Pfander et al., 2005). Although it is so far unclear whether it is also the case for higher eukaryotic organisms, it would be interesting to investigate how CKI-2 interacts with PCNA and the proteins composing the DNA replication machinery to gain a more profound understanding of the role of PCNA in S-phase regulation, particularly during the early embryonic divisions.

5.4. Mechanisms mediating the Degradation of CKI-2

As presented in chapter IV, a yeast two hybrid screen identified a RING finger protein called RNF-1 as a CKI-2 C-terminal interactor. We showed that the degradation of CKI-2 is associated with RNF-1 and this degradation seems to be mediated by ubiquitin-dependent proteolysis. Our data, therefore, provide further understanding of the appropriate maintenance of the regulation of CKI-2 levels in *C. elegans*.

5.4.1. Does RNF-1 act as a component in a multi-subunit E3 ligase?

At the moment, our foremost interest is whether RNF-1 acts as a novel component of a multi-subunit E3 ligase. The canonical SCF complexes are composed of Skp1, Cullin, F box protein, and a RING domain protein such as Rbx1/Roc1. However, recent data have shown that during synaptic formation in *C. elegans* neuron, FSN-1 (a novel F-box protein) associates with SKP1, CUL-1, and RPM-1 (a RING finger protein) to form a new type of SCF-like complex (Liao et al., 2004). This study suggests that a novel SCF can be formed in a tissue-specific manner. It has been previously demonstrated that RING domain proteins recruit E2 ubiquitin conjugating enzymes through their RING fingers that will also interact with cullin proteins, thereby linking the E2 enzyme to the E3 ligase complex (Petroski and Deshaies, 2005). Although CKI-2 degradation does not seem to be mediated by CUL-2 (Feng et al., 1999), it cannot be formally excluded that other cullin members

may be associated with the CKI-2 degradation. In addition, since F-box proteins physically interact with RING domain proteins, it would be important to study whether there are any predicted F-box proteins in the *C. elegans* genome database that interacts with RNF-1. These possibilities may be tested by a biochemical approach such as co-immunoprecipitation using an anti-RNF-1 antibody generated in our laboratory, or by a directional yeast two-hybrid analysis. These studies may reveal a new type of SCF-like E3 ligase that includes RNF-1. We believe that these studies will allow us to gain more insight concerning mechanisms involved in the maintenance of the appropriate levels of CKI-2 and the degradation of CKI-2.

5.4.2. SUMOylation and Ubiquitination: exclusive or sequential?

In chapter IV, we presented that co-expression of RNF-1 with CKI-2 results in an increased degree of ubiquitination of CKI-2. Interestingly, right after the induction, mono-ubiquitination of CKI-2 begins to occur and this is preceded before high molecular weight CKI-2 entities are generated. Since co-expression of RNF-1 with the N-terminus of CKI-2 did not give rise to mono-ubiquitinated CKI-2 or the high molecular weight CKI-2 ladders, it strongly supports that the C-terminus of CKI-2 is important, and that it is consistent with the ubiquitination of CKI-2 which may be mediated by a direct interaction between CKI-2 and RNF-1.

It has been suggested that the function of many proteins is modulated through a crosstalk between mono-ubiquitination and SUMOylation (Ulrich, 2005). This is quite often an antagonistic or mutually exclusive relationship, as is the case for IKBα, or alternatively, these steps can also occur in a sequential or successive manner, as is the case for NEMO where its ubiquitination requires an initial SUMOylation step. Since CKI-2 seems modified through its two conserved SUMOylation motifs in the N-terminal inhibitory domain (see Chapter IIII), it would be informative to precisely characterize where the ubiquitination occurs.

Although our observations are by no means unequivocal, based on our current results, the SUMOylation and the ubiquitination do not seem to be mutually exclusive, but rather they seem more sequential. However, a puzzling issue arises from our results shown in chapter III wherein SUMO antagonizes the interaction between CKI-2 and RNF-1 in yeast, suggesting that the SUMOyaltion and the ubiquitination must also be antagonistic. Interestingly, however, a recent study showed that a SUMO-specific isopeptidase localizes in the nucleolus during interphase (Nishida et al., 2000). Thus, opening the door for speculation that SUMOylated CKI-2 may be targeted by a nucleolar-specific SUMO protease during interphase and which allows RNF-1 to interact with CKI-2 to trigger its degradation. However, this is speculative and remains to be further characterized.

5.5. Synopsis

Through our study of the role of *cki-2* during development in *C. elegans*, we have provided some of the first results as to how centrioles can be appropriately destabilized during oogenesis. Future experiments will be focused on finding the target molecules which are involved in this and other contexts of centriole destabilization. Since many types of cancer show abnormal numbers of centrosomes (although it is unclear whether inappropriate maintenance of centrosome number is a cause or a consequence in tumorigenesis), our characterization of the target proteins involved in this process may contribute to a better understanding of the role of centrosomes in tumorigenesis. Characterization of the CKI-2 interacting proteins has uncovered a novel mechanism through which the levels of CKI-2 may be appropriately maintained through SUMO-mediated nucleolar localization. Further investigation will be targeted toward understanding the mechanism in a more developmental context. In addition, more effort will be concentrated on identifying the players and their functions in this novel pathway at the molecular level. Through these studies, we believe that our work will contribute considerable insight to our current knowledge of how CKIs function during development in animals.

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Original contributions to knowledge

1. As presented in chapter II, in a process to study the loss of function of *cki*-2 using a reverse genetic approach called co-suppression in *C. elegans*, we have identified a novel mechanism that *cki-2* is required for the specific elimination of centrioles during oogenesis. We have demonstrated that this critical developmental process is likely mediated through the catalytic activity of cyclin E/Cdk2 complex. Intriguingly, we found that the maternal centrosomes, originating from the perduring centrioles, do not seem to affect the determining process of A/P polarity at fertilization, although supernumerary centrosomes in the zygote give rise to a severe aneuploidy. This suggests that although the maternally-derived centrosome retains the potential to nucleate and to associate with the kinetochore complex on the chromosomes, such abilities are short of determining the initial embryonic polarity, probably due to its inability to correctly contact with the embryonic cortex. Moreover, we have shown that the loss of *cki-2* causes ectopic accumulation of centriolar proteins such as ZYG-1 and SPD-2 on the meiotic spindles in the zygote. Therefore, our results indicate that cyclin E/Cdk2 complex may be involved in centriole stabilization through its ability to phosphorylate key target proteins, likely centriole-localizing proteins. Our work thus provides pioneering observations that will allow further study of this critical developmental process at the molecular level.

2. In chapter III, we presented that a yeast two-hybrid screen identified orthologues of PCNA and SUMO as CKI-2 interacting proteins. We mapped the PCNA binding region on CKI-2 and demonstrated that the region located in the C-terminus of CKI-2 is highly conserved among diverse PCNA interacting proteins. Moreover, through the overexpression of CKI-2 and its N- or C-terminal variant (CKI-2N or CKI-2C, respectively), we found that CKI-2 has two functionally separable domains, reminiscent of p21Cip1 in mammalian cells. We demonstrated that CKI-2 is covalently modified by SUMO through which CKI-2 localizes to the nucleolar compartment. Intriguingly, we have shown that this nucleolar localization is linked to the degradation of CKI-2. Using serial deletion constructs of CKI-2, we mapped a sequence element required for the

nucleolar localization of CKI-2. Since we noticed similar nucleolar localizing sequences on other CKIs (Dacapo and p27Kip1) containing conserved SUMOylation target sites, these data imply that this degradation through the SUMO-mediated nucleolar localization may be an evolutionarily conserved mechanism to maintain the appropriate levels of CKIs or other cell cycle regulators. Therefore, through the characterization of CKI-2 interacting partners, our work demonstrated a novel mechanism for the CKI-2 degradation and thus our results should help to further expand our understanding of the mechanisms implicated in the maintenance of the CKI levels

3. As we presented in chapter IV, a RING finger protein named RNF-1 was identified as a CKI-2 interactor in a yeast two-hybrid screen. We mapped the RNF-1 binding region on the CKI-2 C-terminus, where we found that the RNF-1 binding region is in close proximity to the PCNA binding region and the nucleolar localization element, suggesting that RNF-1 may somehow interact with these sequence elements. Through genetic and biochemical studies, we have shown that RNF-1 negatively interacts with CKI-2 through its ability to mediate an ubiquitin-dependent proteolysis of CKI-2 and that this degradation of CKI-2 is correlated with the suppression of embryonic lethality associated with CKI-2 overexpression. Therefore, our work provided a molecular mechanism that may undertake the CKI-2 degradation. Moreover, using a yeast-based assay, we showed that SUMO antagonizes the CKI-2/RNF-1 interaction. Thus, our results argue that the CKI-2 degradation is mediated by RNF-1 in an ubiquitin-dependent manner, where SUMO may be involved in this process through its ability to associate with CKI-2.

Appendix

Appendix I

Supplemental data for chapter II: Cell cycle regulators control centrosome elimination during oogenesis in *C. elegans*

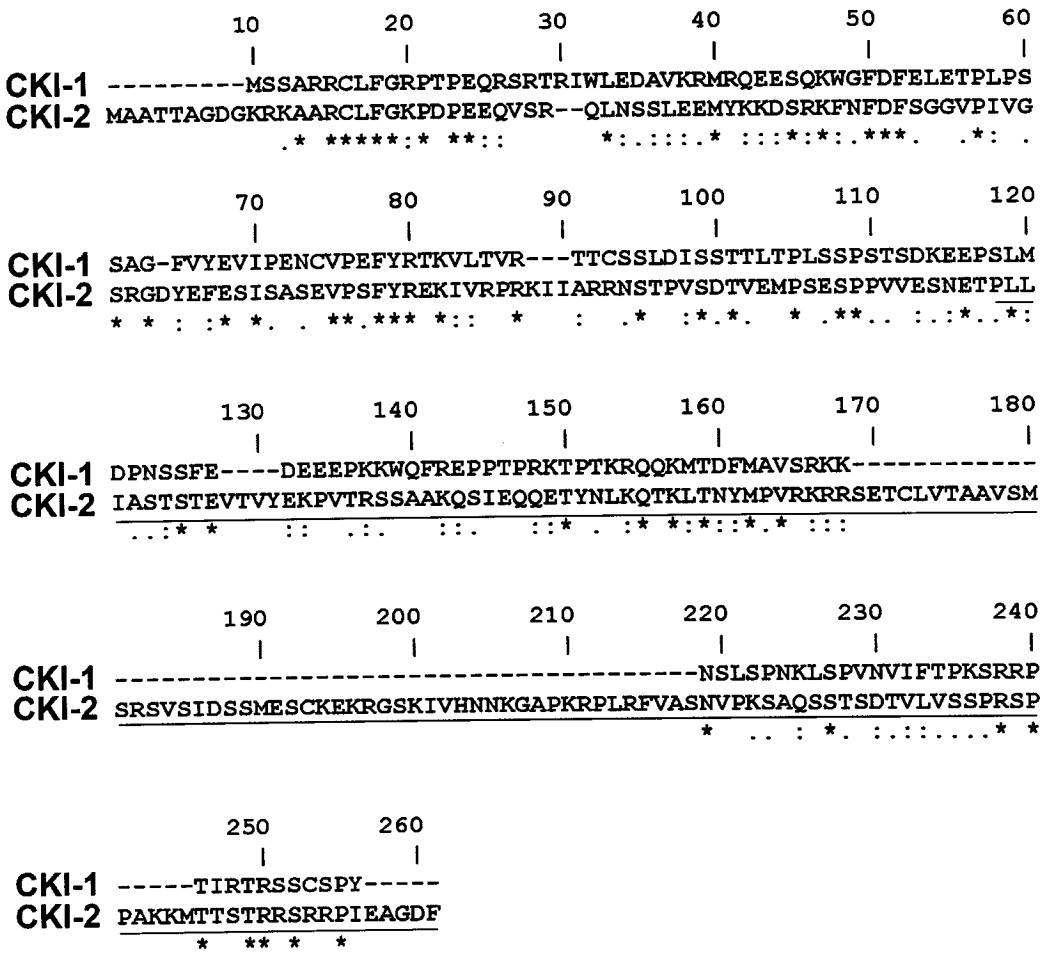
1.1. Legends to Supplemental Figures

Figure S1.1. **Protein sequence alignment of CKI-2 with CKI-1 shows that the C-termini are divergent.** The protein sequence of CKI-2 was aligned to that of CKI-1 using Clustal W (Thompson et al., 1994). Asterisks (*) mark identical residues between the two CKIs. Strongly (:), or weakly (.) similar residues are shown. The underline marks the C-terminus of CKI-2 (CKI-2C) used for *cki-2* co-suppression. The global sequence identity (%) of the two CKIs was 20.31 (53/261). At the N-terminus the identity (%) was 29.91 (35/117) while 12.5 % (18/144) was shown at the C-terminus. The nucleotide sequence alignment of *cki-2* with *cki-1* using MAFFT (v5.667) (Katoh et al., 2002; data not shown) revealed that the identity (%) in the 5′ region was 38.15% (132/346) while it was 29.4% (127/432) in the 3′ region. This level of identity is far below the threshold for cross-reactivity of RNAi or co-suppression.

Figure S1.2. **Centrosomal material persists on the meiotic spindle in *cki-2cs* one-cell embryos and is associated with abnormal morphology of the meiotic spindle.** (A,B) (A) Wild-type, or (B) *cki-2cs* one-cell embryos stained with anti-SPD-2 and DAPI during the first meiotic division at fertilization. (C) The embryo shown in (B) is counterstained with anti-alpha-tubulin antibody (open arrowhead, red) (Matthews et al., 1998). The rectangular boxed region is magnified to provide greater detail. The asterisks (*) and arrows represent the paternal centrosome (green) and DNA (blue), respectively. The closed arrowhead represents SPD-2. S, sperm DNA.

Figure S1.3. **Both anti-SAS-6 and anti-SAS-4 recognize centrioles, and co-localize with γ-tubulin in the early embryo.** (A) a wild-type embryonic cell (the P1 blastomere in a two-cell embryo) labelled with GFP-γ-tubulin (green), Cy3-conjugated anti-SAS-6 (red), and Cy5-conjugated anti-SAS-4 (blue). The rectangular boxed region is magnified to provide greater detail.

Figure S1.1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CKI-1 ---------MSSARRCLFGRPTPEQRSRTRIWLEDAVKRMRQEESQKWGFDFELETPLPS
CKI-2 MAATTAGDGKRKAARCLFGKPDPEEQVSR--QLNSSLEEMYKKDSRKFNFDFSGGVPIVG
70 80 90 100 110 120
CKI-1 SAG-FVYEVIPENCVPEFYRTKVLTVR---TTCSSLDISSTTLTPLSSPSTSDKEEPSLM
CKI-2 SRGDYEFESISASEVPSFYREKIVRPRKIIARRNSTPVSDTVEMPSESPPVVESNETPLL
130 140 150 160 170 180
CKI-1 DPNSSFE----DEEEPKKWQFREPPTPRKTPTKRQQKMTDFMAVSRKK----------
CKI-2 IASTSTEVTVYEKPVTRSSAAKQSIEQQETYNLKQTKLTNYMPVRKRRSETCLVTAAVSM
190 200 210 220 230 240
CKI-1 ----------NSLSPNKLSPVNVIFTPKSRRP
CKI-2 SRSVSIDSSMESCKEKRGSKIVHNNKGAPKRPLRFVASNVPKSAQSSTSDTVLVSSPRSP
250 260
CKI-1 -----TIRTRSSCSPY-----
CKI-2 PAKKMTTSTRRSRRPIEAGDF

Figure S1.2

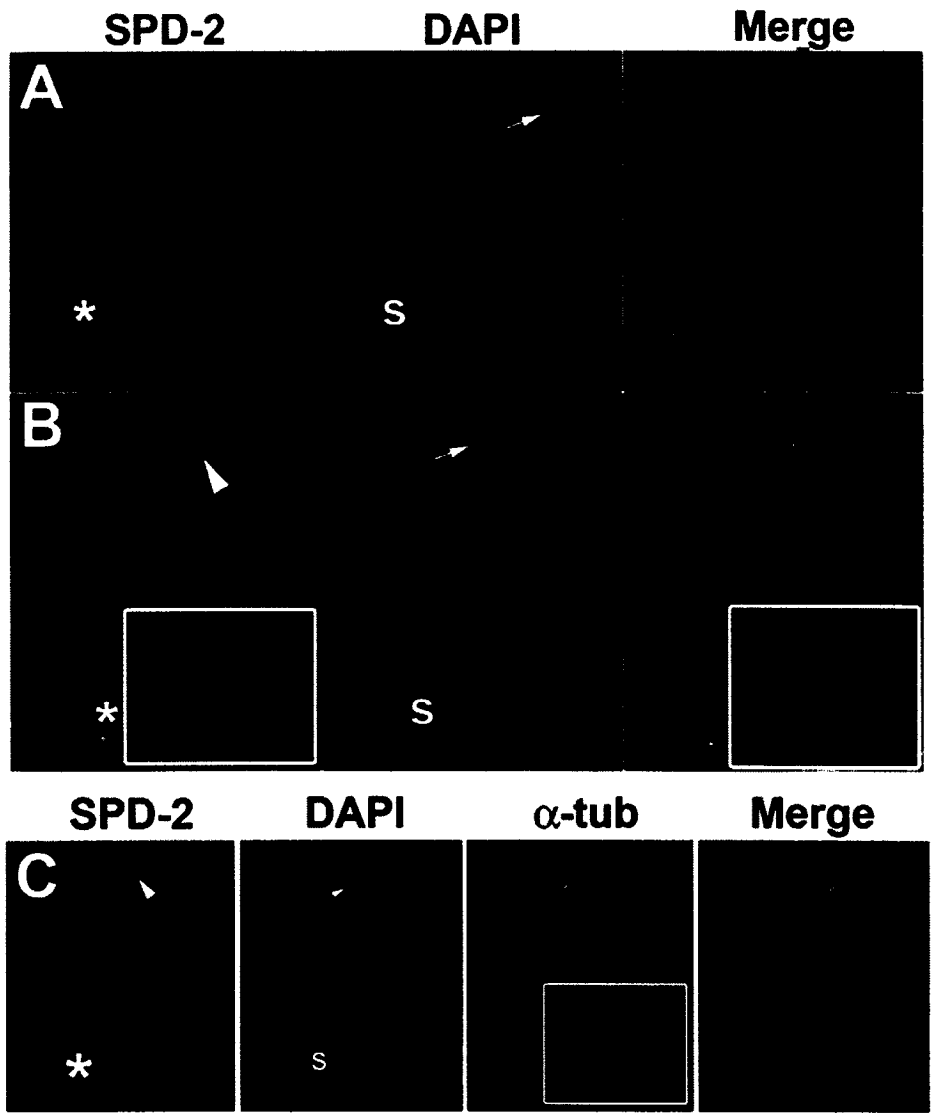
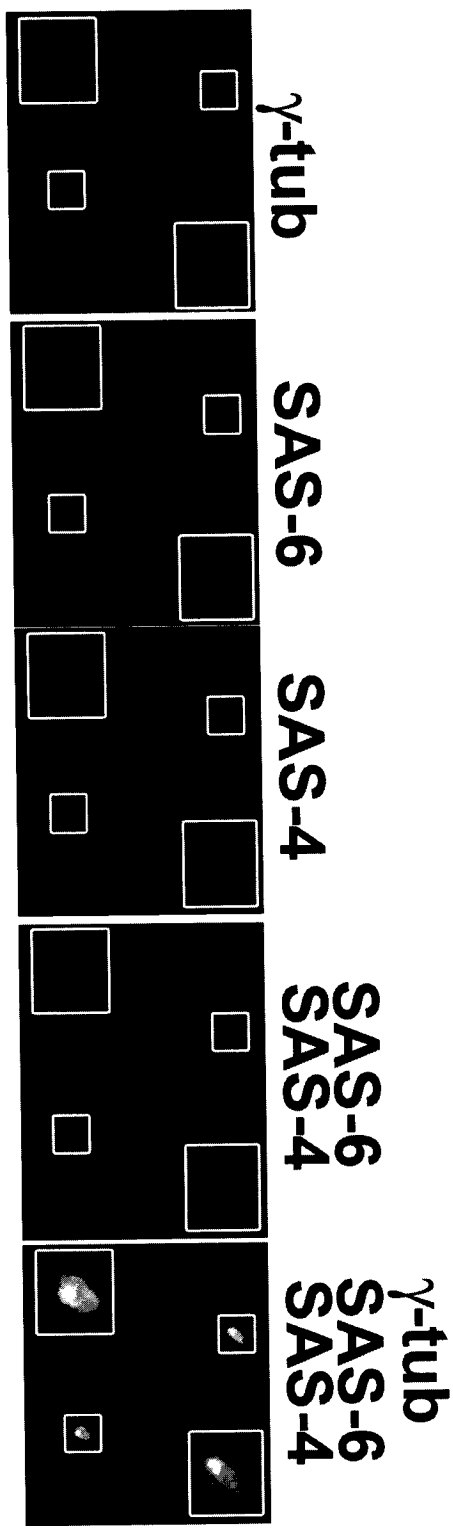


Figure S1.3



1.3. Supplemental References

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Caenorhabditis elegans protein required for microtubule organization and function is a component of meiotic and mitotic spindle poles. J. Cell Biol. **141**, 1159-1168.

Thompson JD, Higgins DG, and Gibson TJ. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**, 4673-4680.

Appendix II

Supplemental data for chapter III: CKI-2 regulates embryonic cell divisions and is modulated by SUMO-mediated nucleolar localization and subsequent degradation

2.1. Legends for Supplemental Figures

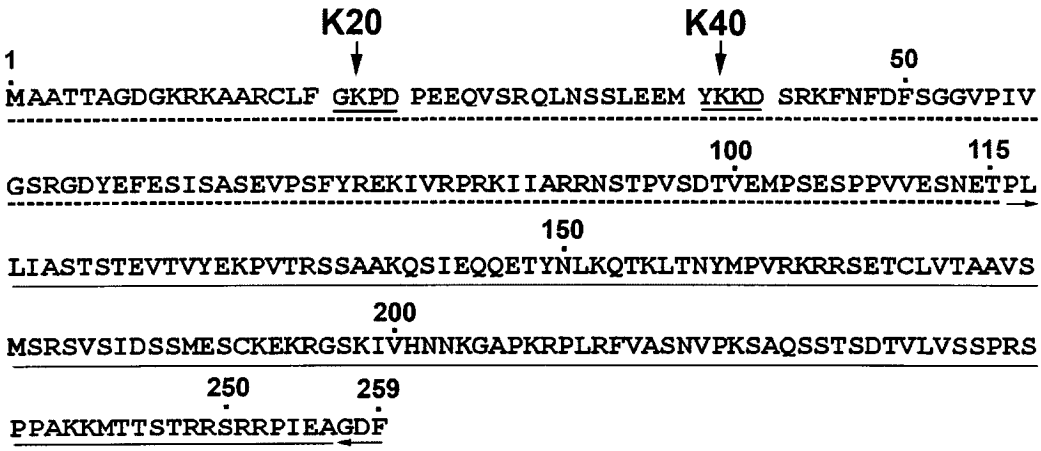
Figure S2.1. **The primary structure of CKI-2 and the CKI-2 variants (CKI-2N and CKI-2C), and summary of the yeast two-hybrid screen.** (A) Amino acid sequence of CKI-2 and the CKI-2 variants (CKI-2N and CKI-2C). The dotted line marks the region of CKI-2 (1-115) used as the N-terminal variant (CKI-2N) while the line indicated by arrow shows the region of CKI-2 (116-259) used as the C-terminal variant (CKI-2C). Asterisks (*) indicate the lysine residues (K) of the consensus SUMOylation target sites (bold underlined). (B) Summary of the yeast two-hybrid screen. Yeast two-hybrid screens were performed with three different CKI-2 variants (full length CKI-2 (amino acids 1-259) and the N-terminus of CKI-2 (CKI-2N, amino acids 1-115)), and the C-terminus of CKI-2 (CKI-2C, amino acids 116-259)) as baits. Two *lacZ* positives obtained with full length CKI-2 (1-259) corresponded to the *C. elegans* orthologue of PCNA (PCN-1), *W03D2.4*. Among three *lacZ* positives from the N-terminus of CKI-2 (1-115), one of the interactors interacted with full length CKI-2 and corresponded to *K12C11.2* (SMO-1), the *C. elegans* orthologue of SUMO-1 (small ubiquitin-related modifier-1). The remaining two interactors did not interact with the full length CKI-2. The C-terminus recovered two lacZ positives, one of which corresponded to PCNA (PCN-1).

Figure S2.2. **The anti-CKI-2 antibody is specific for CKI-2.** (A) Western analysis with anti-CKI-2 (α-CKI-2, left panel) or anti-GFP (α-GFP, right panel, top) of embryos obtained from heat shock induced (+) or non-induced (-) animals that carry a *hs*::CKI-2::GFP transgene. CKI-2C::GFP expressing embryos were examined in an

identical manner (right panel, bottom). (B) Embryos carrying [*hs*::CKI-2::GFP] transgenic array was induced by heat shock followed by labelling with DAPI (blue) and pre-immune serum (P.I). (C) Embryos carrying [*hs*::CKI-2C::GFP] transgenic array was induced by heat shock followed by labelling with DAPI (blue) and anti-CKI-2 antibody (α-CKI-2).

Figure S2.3. ***cki-2* co-suppressed (*cki-2cs*) embryos arrest with expanded endodermal and pharyngeal fields.** (A,B) GFP/DIC overlay image captured at ~250 minute post-fertilization in wild-type (A) or *cki-2cs* embryos (B) visualized with *elt-2*::GFP, which is expressed in wild-type intestinal cells beginning at the 16E stage (~250 minute after fertilization) and maintained throughout development thereafter. (C,D) Immunofluorescence/DIC overlay image showing embryonic pharyngeal cell nuclei in wild-type (C) or *cki-2cs* (D) embryos detected by anti-PHA-4 antibody (red). Embryos are all ~250 minute post-fertilization. Scale bar, 10μm.

A



B

Type of Bait	CKI-2(1-259)	CKI-2(1-115)	CKI-2(116-259)
Total screen #	$6x10^6$	$8.8x10^5$	$3.5x10^6$
LacZ positives	2	3	3
Interactor	PCN-1	SMO-1	PCN-1

Figure S2.2

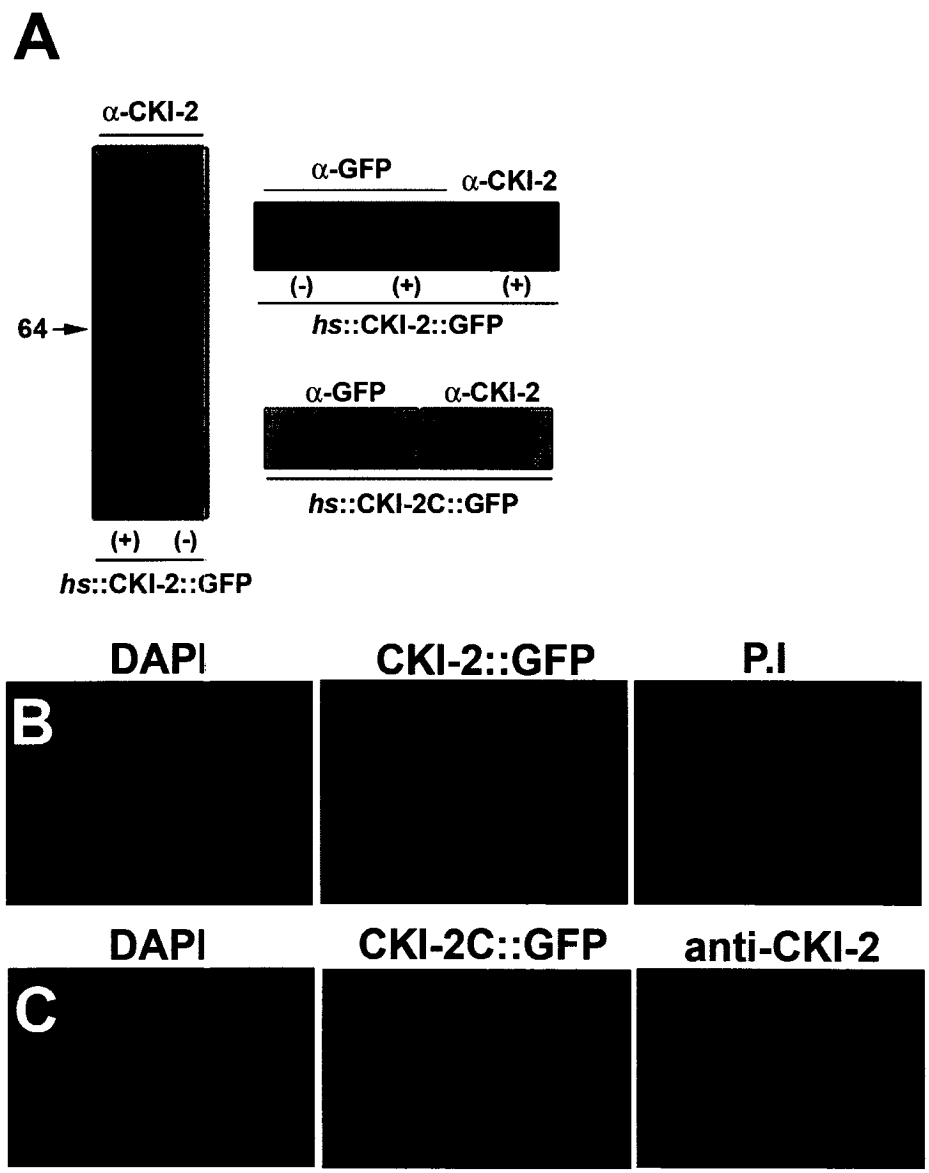
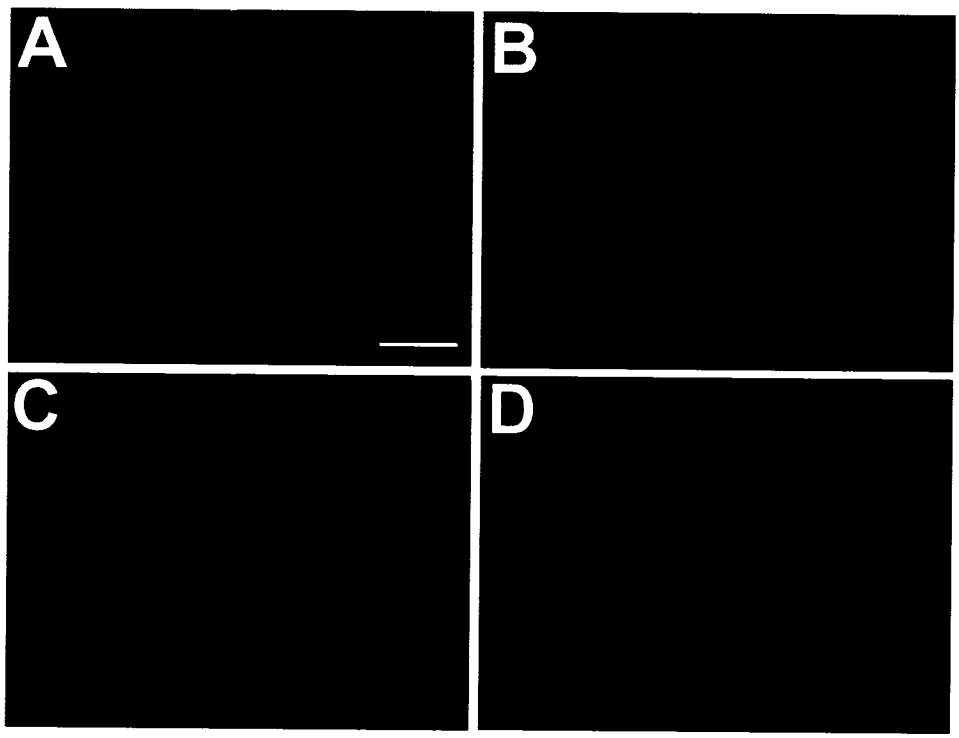

A
α-CKI-2
64
(+) (-)
hs::CKI-2::GFP
α-GFP
α-CKI-2
(-) (+) (+)
hs::CKI-2::GFP
α-GFP
α-CKI-2
hs::CKI-2C::GFP
DAPI
CKI-2::GFP
P.I
B
DAPI
CKI-2C::GFP
anti-CKI-2
C

Figure S2.3



Appendix III

Supplemental data for chapter IV: RNF-1, a *Caenorhabditis elegans* RING finger protein, modulates CKI-2 through ubiquitin-dependent proteolytic pathway

3.1. Legends to Supplemental Figures

Figure S3.1. **The primary structure of CKI-2 and the CKI-2 variants (CKI-2N and CKI-2C).** Amino acid sequence of CKI-2 and the CKI-2 variants (CKI-2N and CKI-2C). The dotted line marks the region of CKI-2 (1-115) used as the N-terminal variant (CKI-2N) while the line indicated by arrow shows the region of CKI-2 (116-259) used as the C-terminal variant (CKI-2C).

Figure S3.2. **SMO-1 does not antagonize the interaction between CKI-2C and RNF-1.** An *in vivo* competition assay using a galactose-inducible system in yeast. *Gal1*, *Gal1* promoter. Ade and Gal, Adenine and Galactose, respectively. CKI-2C, C-terminus of CKI-2. (+) or (-) indicates possession (+) or deficiency (-) of the component on the media.

Figure S3.1

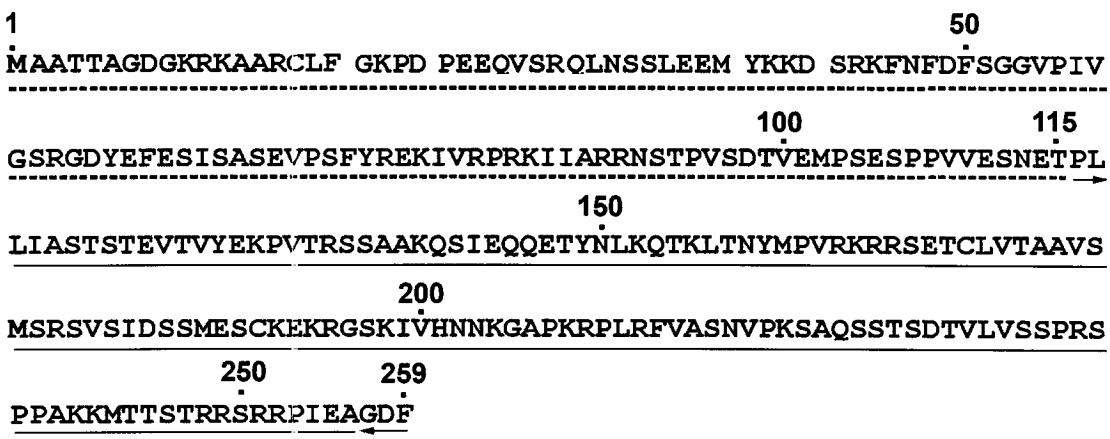
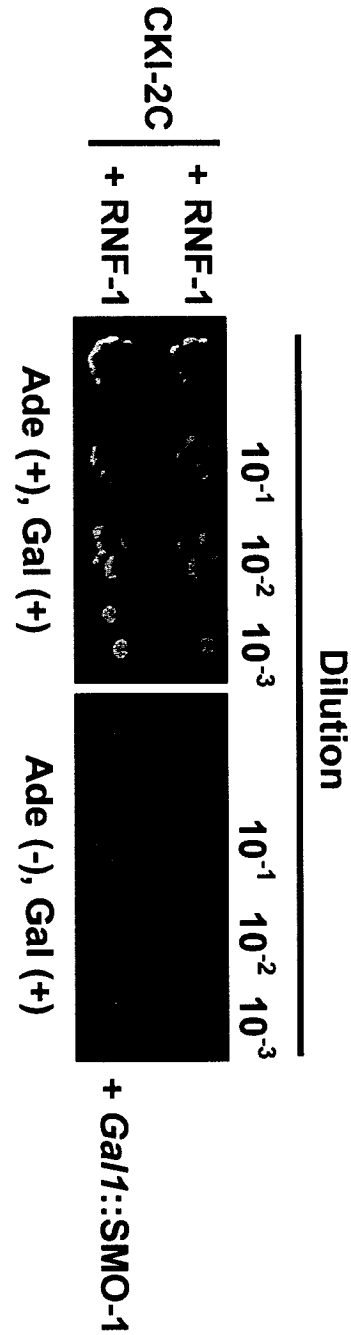

1
50
MAATTAGDGKRKAARCLF GKPD PEEQVSRQLNSSLEEM YKKD SRKFNFDFSGGVPIV
100
115
GSRGDYEFESISASEVPSFYREKIVRPRKIIARRNSTPVSDTVEMPSESPPVVESNETPL
150
LIASTSTEVTVYEKPVTRSSAAKQSIEQQETYNLKQTKLTNYMPVRKRRSETCLVTAAVS
200
MSRSVSIDSSMESCKEKRGSKIVHNNKGAPKRPLRFVASNVPKSAQSSTSDTVLVSSPRS
250
259
PPAKKMTTSTRRSRRPIEAGDF

Figure S3.2


Dilution
10^{-1} 10^{-2} 10^{-3}
10^{-1} 10^{-2} 10^{-3}
CKI-2C
+ RNF-1
+ RNF-1
Ade (+), Gal (+)
Ade (-), Gal (+)
+ *Gal1*::SMO-1

Cell cycle regulators control centrosome elimination during oogenesis in *Caenorhabditis elegans*

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In many animals, the bipolar spindle of the first zygotic division is established after the contribution of centrioles by the sperm at fertilization. To avoid the formation of a multipolar spindle in the zygote centrosomes are eliminated during oogenesis in most organisms, although the mechanism of this selective elimination is poorly understood. We show that *cki-2*, a *Caenorhabditis elegans* cyclin-dependent kinase (Cdk) inhibitor, is required for their appropriate elimination during oogenesis. In the absence of *cki-2*, embryos have supernumerary centrosomes and form multipolar spindles that result in severe aneuploidy after anaphase of the first division. Moreover, we demonstrate that this defect can be suppressed by reducing cyclin E or Cdk2 levels. This implies that the proper regulation of a cyclin E–Cdk complex by *cki-2* is required for the elimination of the centrosome that occurs before or during oogenesis to ensure the assembly of a bipolar spindle in the *C. elegans* zygote.

Introduction

Experiments performed by Boveri (1900) over a century ago revealed the essential requirement for accurate centrosome inheritance and its role in regulating genome integrity in the developing embryo. In many metazoans, the establishment of the bipolar spindle during the first zygotic cell division is dependent on the paternal contribution of a microtubule organizing center. After fertilization, this organelle will recruit pericentriolar material present within the oocyte cytoplasm to assemble the two functional centrosomes that will define the first mitotic spindle. In addition to this essential role of the centrosome in organizing the spindle, in *Caenorhabditis elegans*, this structure is also required to specify the anterior/posterior axis after sperm entry in a microtubule-dependent and -independent manner (O'Connell et al., 2000; Wallenfang and Seydoux, 2000; Cowan and Hyman, 2004a). Therefore, the appropriate regulation of centrosome number is pivotal because aberrations in these controls result in asymmetrical chromosome segregation and/or severe polarity defects.

Although centrosomes are associated with most nuclei in *C. elegans*, including those in the germ line, they are absent in oocytes, whereas they are clearly detectable and required for fertility in the sperm (Kemp et al., 2004). The loss of the centrosome from the oocyte is common to many species, but the mechanism responsible for this elimination is currently unknown. During our characterization of a *C. elegans* Cdk inhibitor (CKI; *cki-2*) we noticed that compromise of *cki-2* function caused embryos to arrest at the one-cell stage with a multipolar spindle. We show that this defect is due to a role of *cki-2* in centrosome elimination, and our data provide pioneering evidence on how centrosomes are appropriately eliminated from the developing oocyte.

Results and discussion

Recently, large-scale screens using RNAi-based strategies have provided a framework for understanding many maternally controlled embryonic processes (Sonnichsen et al., 2005). However, not all genes respond equally to RNAi. Our initial use of RNAi analysis to understand the role of a *C. elegans* CKI called *cki-2* was not informative because of the variable penetrance and frequency of the RNAi-related phenotypes. Furthermore, no loss-of-function *cki-2* alleles are currently available. We therefore turned to an alternative reverse genetic approach called cosuppression, which is an RNAi-related posttranscriptional gene-silencing mechanism that is conserved among many phyla (Ketting and Plasterk, 2000). In wild-type animals, *cki-2* mRNA is normally present in the hermaphrodite germ line but is excluded from the distal mitotic zone (Fig. 1 A). To test whether *cki-2* could be compromised through the cosuppression pathway, we expressed the 3′ portion of the *cki-2* gene (Dernburg et al., 2000), which could not encode a functional protein and shared a very low degree of sequence conservation with *cki-1*, a second *C. elegans* CKI (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200512160/DC1). The cosuppression transgenic array included a GFP marker facilitating our

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Abbreviations used in this paper: CKI, Cdk inhibitor; dsRNA, double-stranded RNA.

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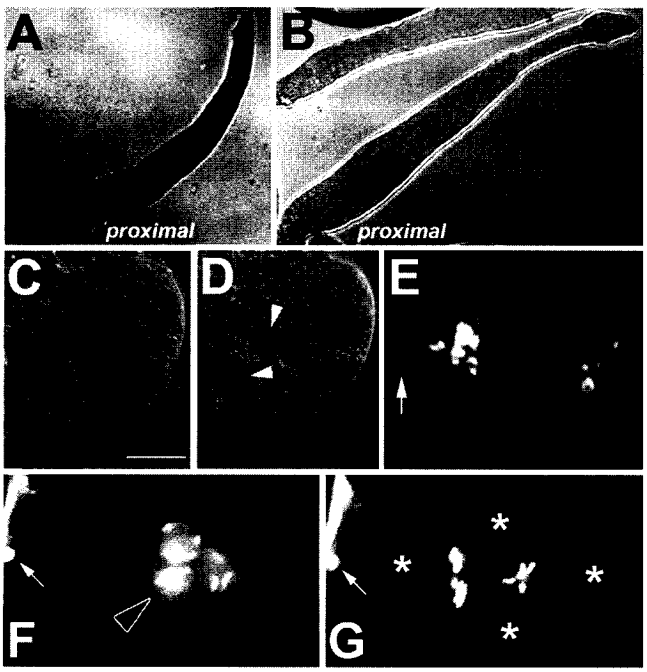
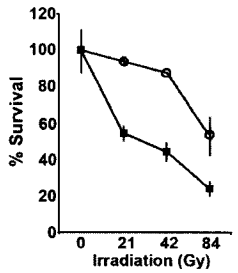
http://www.jcb.org/cgi/doi/10.1083/jcb.200512160



H



Radiation (Gy)	% Survival (GFP-)	% Survival (GFP+)
0	99.3±0.3 (n=766)	69.5±11.3 (n=1409)
21	92.5±1.2 (n=1349)	37.8±4.2 (n=1084)
42	87.1±0.02 (n=1334)	30.8±5.0 (n=527)
84	52.2±10.8 (n=1515)	16.7±4.0 (n=685)

Figure 1. ***cki-2cs* causes multiple phenotypes typical of a negative cell cycle regulator.** (A and B) In situ RNA hybridization using an antisense *cki-2* probe on wild-type (A) or *cki-2cs* (B) gonads extruded from adult hermaphrodites. (C and D) Sequential differential interference contrast images of a *cki-2cs* one-cell embryo showing normal pronuclear meeting (C) and nuclear divisions without appropriate cytokinesis giving rise to supernumerary nuclei (D, arrowheads) with variable DNA content based on staining with DAPI (E). (F and G) A sequential GFP fluorescence image of *cki-2cs* one-cell–arrested embryo that expresses [H2B::GFP; β-tubulin::GFP]. The open arrowhead indicates an extra maternal pronucleus, asterisks mark centrosomes, and the arrows indicate polar bodies. (H) Irradiation sensitivity of *cki-2cs* (GFP+; closed square) or wild-type sibling (GFP−) animals (open circle). The values are presented as the percentage of embryos that hatched from a total population of embryos laid from irradiated or not parents that were examined at each point. At point zero in each experiment, the survival percentage was normalized to 100%. The error bars represent the standard deviation of two independent experiments ($P < 0.05$; 95% confidence). Bar, 10 μm.

detection of animals that possessed the transgene. We obtained several transgenic lines in different genetic backgrounds, all of which indicated that reduction of *cki-2* consistently resulted in reproducible embryonic lethality wherein ~60% of the GFP transgene-bearing embryos (GFP+) failed to complete embryogenesis (Table I). The abundance of *cki-2* mRNA was reduced substantially throughout the gonad in these GFP+ animals (Fig. 1 B), whereas the observed embryonic lethality could be reversed by genetically disrupting this silencing mechanism using mutants in the downstream components of the cosuppression pathway (*mut-7* and *rde-2*), indicating that the observed lethality was specifically due to the reduction of *cki-2* through cosuppression (Table I). We therefore refer to these GFP+ animals as *cki-2* cosuppressed (*cki-2cs*). Although ~40% of the *cki-2cs* embryos survive embryogenesis and continue larval development without visible abnormalities, we found that these animals are irradiation sensitive (Fig. 1 H). This indicates that despite their wild-type appearance, the DNA damage response in *cki-2cs* animals is nonetheless compromised. Therefore, reduction of *cki-2* function results in cell cycle–related abnormalities that reflect the various thresholds of *cki-2* activity required to appropriately execute these cellular processes. Among the embryonically arrested embryos, we noticed that 7% of the embryos ($n = 558$) arrested at the one-cell stage with multiple micronuclei (9.1%; $n = 66$), consistent with abnormal chromosome segregation and/or cytokinesis (Fig. 1, C–E). Examination of the affected zygotes by differential interference contrast indicated that early events (contractions of the anterior membrane or ruffling and pseudocleavage) before the pronuclear meeting were not significantly different from wild type (unpublished data). Shortly after nuclear envelope breakdown, however, the two pronuclei reformed and several de novo micronuclei became apparent. Cleavage furrows appeared occasionally but would regress, and ~50% ($n = 18$) of the micronuclei-containing embryos did not form a cleavage furrow. The remaining 50% were defective in cleavage plane orientation, although both classes did undergo multiple rounds of karyokinesis (Fig. 1, C–E). To better understand the basis of the "one-cell" arrest phenotype, we imaged *cki-2cs* embryos that harbored GFP-histone and GFP–β-tubulin transgenes. In some embryos, we observed a second maternal pronucleus (4.5%; $n = 66$), a meiotic defect that arises because of abnormal polar body exclusion (Fig. 1 F). We also noted that chromosomes failed to align correctly after nuclear envelope breakdown, whereas the spindle microtubules appeared to be organized around multiple foci, typical of extra microtubule organizing centers or centrosome-like structures (Fig. 1 G and Video 1).

To confirm that this unique multipolar spindle phenotype was due to the reduction of *cki-2* and not due to cosuppression-related phenomena or nonspecific effects on *cki-1*, we used an RNAi-sensitive strain (Simmer et al., 2002) to reduce either *cki-1* or *-2* levels to reproduce the *cki-2cs*–associated multipolar spindle phenotype. We did detect one-cell embryos with supernumerary centrosomes after *cki-2(RNAi)* in *rrf-3* (Table II and see Fig. 3, E and F), although the penetrance of the defect was considerably lower than that observed in *cki-2cs* animals. On the other hand, despite causing a high frequency of embryonic arrest in the *rrf-3* background, *cki-1(RNAi)* never caused a one-cell arrest or a multipolar spindle phenotype (Table II). Therefore, we conclude that the supernumerary centrosomes and the resulting multipolar spindle defect observed in *cki-2cs* embryos were not due to effects on *cki-1* function or due to cosuppression per se but, rather, to a loss or reduction of *cki-2* function.

To address whether *cki-2* affected the centrosome cycle during spermatogenesis or, alternatively, during oogenesis, we examined centrosome numbers in early pronuclear stage embryos using

Table I. ***cki-2* cosuppression causes embryonic lethality**

Genotype	Embryonic lethality	
	GFP+	GFP−
	%	%
N2	NA	0.29 (*n* = 1384)
N2; cki-2 (RNAi)	NA	5.5 (*n* = 710)
N2; [fem-1::GFP] (0/4)	0 (*n* = 244)[a]	ND
N2; [fem-1::cki-2C] (3/3)		
line #1	26.9 (*n* = 466)[a]	0.7 (*n* = 280)
line #2	23.3 (*n* = 103)	ND
line #3	8.1 (*n* = 186)	ND
rrf-3; [fem-1::cki-2C] (2/2)		
line #1	55.3 (*n* = 159)[a]	27.6 (*n* = 116)
line #2	42.2 (*n* = 436)	25.1 (*n* = 231)
TH27 (*pie-1::*γ-tub::GFP); *[fem-1::cki-2C]* (5/5)		
line #1	29.1 (*n* = 1257)[a]	1.7 (*n* = 232)
line #2	21.5 (*n* = 395)	ND
line #3	19.2 (*n* = 198)	ND
rde-2; [fem-1::cki-2C] (0/2)		
line #1	5.7 (*n* = 357)	7.5 (*n* = 374)
line #2	11.6 (*n* = 404)	17.7 (*n* = 561)
mut-7; [fem-1::cki-2C] (0/3)		
line #1	17.9 (*n* = 313)	20.2 (*n* = 325)
line #2	11.4 (*n* = 245)	12.1 (*n* = 440)
line #3	12.7 (*n* = 181)	9.4 (*n* = 276)

A *C. elegans* strain that harbors an extrachromosomal array containing the *[fem-1::cki-2C]* cosuppression transgene segregates animals that possess the array (GFP+) or not (GFP−), as indicated by the presence of the dominant *elt-2::GFP* cotransformation marker. Embryonic lethality was presented as the percentage of unhatched embryos from total progeny obtained from GFP+ or GFP− young adult animals. The frequency of the embryonic lethality phenotype in the various trangenic lines obtained is shown in parentheses. The embryonic lethality from GFP− animals was determined from only one transgenic line of each tested genotype.
[a]The transmission frequency (%) of the transgenic array in these strains was scored as the number of GFP+ progeny from the total number of progeny, and the transmission rate of the *cki-2cs* strain used throughout the study was ~50%.

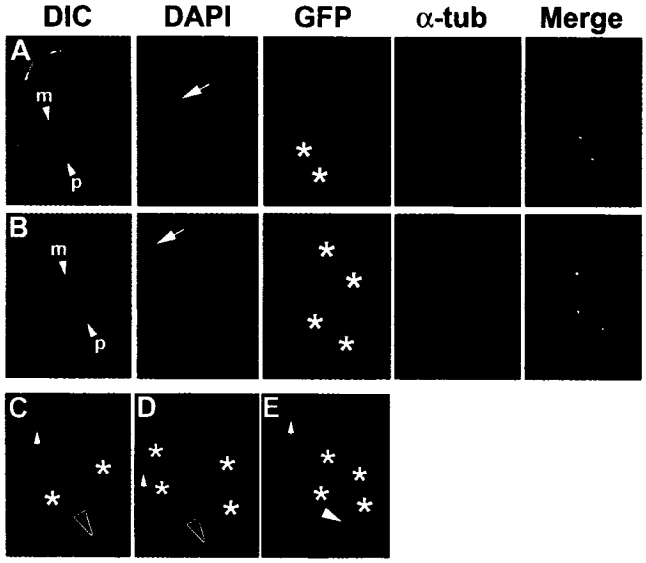
an antibody against SPD-2, a coiled-coil protein that associates with the centrosome (Kemp et al., 2004). We noticed that unlike wild-type embryos, strong SPD-2 expression was visible at distinct foci in both the paternal and maternal pronuclei (pronuclear meeting stage; Fig. 2, A and B). To ascertain whether the presence of the extra centrosomes was indeed due to their contribution from the maternal pronucleus, as opposed to defects associated with failed cytokinesis (Skop et al., 2004), we imaged embryos from meiosis to pronuclear meeting using GFP–γ-tubulin, revealing that GFP–γ-tubulin was associated with the maternal pronucleus in prepronuclear migration stage embryos obtained from *cki-2cs* animals (6.7%; $n = 60$; Fig. 3, B and C), whereas we never observed GFP–γ-tubulin associated with the maternal pronucleus in wild-type embryos ($n = 80$; Fig. 3 A).

Collectively, these results indicate that the supernumerary centrosomes were already associated with the maternal pronucleus at the time of fertilization in *cki-2cs* embryos, possibly because they were not appropriately eliminated in the maternal

Table II. **Supernumerary centrosomes are present in the one-cell embryo of *cki-2cs* animals**

Genotype	Embryonic lethality	Supernumerary centrosome[a]
	%	%
rrf-3	23.0 ± 1.2 (*n* = 374)	0 (*n* = 76)
rrf-3; cki-1 (RNAi)	94.7 (*n* = 570)	0 (*n* = 40)
rrf-3; cki-2 (RNAi)	27.5 ± 3.7 (*n* = 734)	4.5 (*n* = 111)
N2; [fem-1::cki-2C][b]	26.9 (*n* = 466)	13.5 (*n* = 133)
TH27; *[fem-1::cki-2C]*[b]	29.1 (*n* = 1257)	6.7 (*n* = 60)

One-cell embryos obtained from the *cki-2* cosuppression transgene-bearing animals (GFP+) were examined to score the frequency (%) of the supernumerary centrosomes. For RNAi of *cki-1* or *-2*, each dsRNA was injected into *rrf-3* hermaphrodites as described (see Materials and methods), and the frequency (%) of both the embryonic lethality and the supernumerary centrosomes was scored. The embryonic lethality was presented as the percentage of unhatched embryos from total progeny obtained from the RNAi-treated mothers.
[a]Embryos were stained with anti–SPD-2 or γ-tubulin::GFP, and the results are presented as the percentage of the total number of one-cell stage embryos examined. All one-cell embryos examined were at or before the first cell division.
[b]The frequency of the supernumerary centrosome defect was determined in the most penetrant cosuppressed lines (line #1 of *N2; [fem-1::cki-2C]* and TH27; *[fem-1::cki-2C]*) for comparison.



F

Genotype	One-cell arrest (%)	Supernumerary centrosome (%)
cki-2cs	1.59±0.45 (n=1860)	14.07±1.85 (n=133)
cki-2cs;cye-1 (RNAi)	0.82±0.22 (n=1720)	5.05±3.99 (n=87)
cki-2cs	n.d	9.48±4.21 (n=102)
cki-2cs; K03E5.3 (RNAi)	n.d	4.58±3.83 (n=55)

Figure 2. **Supernumerary centrosomes observed in *cki-2cs* embryos are contributed by the maternal pronucleus in a cyclin E/Cdk2-dependent manner.** (A and B) Late pronuclear stage wild-type (A) or *cki-2cs* (B) one-cell embryo stained with DAPI (blue), anti–SPD-2 (green), and anti–α-tubulin (red). The small arrowheads indicate the pronuclei at different stages. Arrows indicate polar bodies, and asterisks indicate centrosomes. p and m, paternal and maternal pronuclei, respectively. (C and D) PAR-2::GFP (red) in the posterior cortex (open arrowheads) of a wild-type (C) or a *cki-2cs* (D) one-cell embryo. (E) Anti–P-granule staining (red spots; closed arrowhead) of a *cki-2cs* one-cell embryo. The arrows indicate polar bodies (anterior), and the asterisks mark centrosomes. (F) Frequency (%) of *cki-2cs*–associated one-cell arrest and the persistence of maternal centrosome after *cye-1(RNAi)* or *K03E5.3(RNAi)*. Standard deviation of at least three independent experiments is shown, and asterisks represent significant differences compared with *cki-2cs* controls ($P < 0.05$; 95% confidence). The one-cell arrest phenotype was presented as the percentage of unhatched one-cell embryos from the total number of progeny (embryos and larvae). The embryos from injected or uninjected (control) animals were labeled with DAPI and anti–SPD-2 antibody 24 h after dsRNA microinjection, and the resulting one-cell embryos were examined for supernumerary centrosomes. The results are presented as the percentage of the total number of embryos examined at the one-cell stage. All one-cell embryos examined were at or before the first cell division. The variation observed in the penetrance of the centrosome defect is due to the progressive silencing of the cosuppression transgene over time.

germ line as a result of a reduction in *cki-2* function. However, because we could not show definitive live images of an embryonic cell division beginning in the prepronuclear stage to the first mitotic division, we cannot formally rule out the possibility that the supernumerary centrosomes may arise from a cytokinesis failure after the first mitotic division.

Therefore, to test whether centrosome elimination is defective in *cki-2cs* oocytes, we stained the gonads of affected (GFP+) and unaffected (GFP−) animals with an anti–SAS-4 antibody to determine whether centrioles were abnormally present in the oocytes of *cki-2cs* animals. SAS-4 is associated with all centrioles in *C. elegans* and is required for their duplication

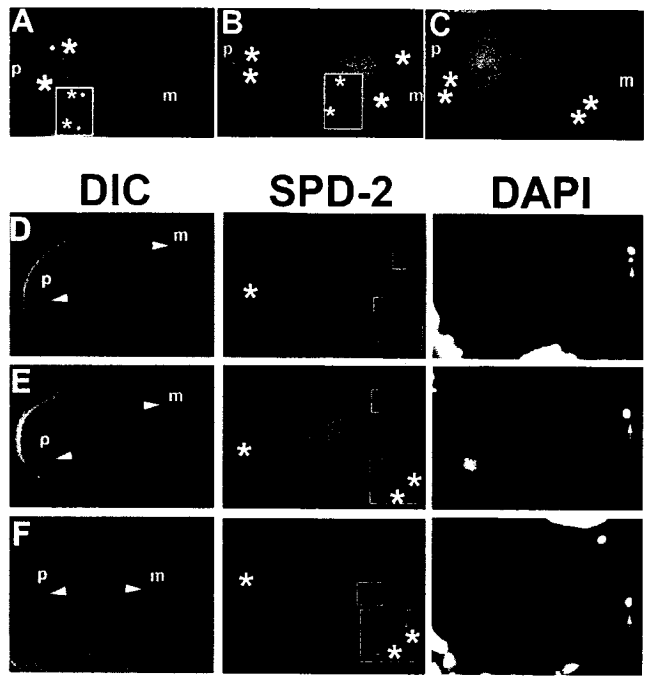


Figure 3. ***cki-2(RNAi)* causes defects in the elimination of the maternal centrosome.** (A–C) Early wild-type one-cell embryo (A; prepronuclear migration stage) or *cki-2cs* embryos that express GFP–γ-tubulin to visualize centrosomes (B and C). (D–F) Early one-cell embryos (prepronuclear migration stage) from *rrf-3* (D) or *rrf-3; cki-2(RNAi)* (E and F) adult hermaphrodites stained with anti–SPD-2 antibody. The arrows indicate polar bodies stained with DAPI (anterior). Asterisks mark centrosomes (maternal [m] and paternal [p]). The white rectangular box in A shows the paternal centrosome that could not be observed in the same focal plane. The rectangular boxed regions in B and D–F were magnified to show greater detail.

(Leidel and Gonczy, 2003). In wild-type animals, SAS-4 is associated with all germ cell nuclei, although SAS-4 staining foci were noticeably absent from oocytes (Fig. 4 A). The absence of the SAS-4/centriole staining in oocytes is consistent with previous observations that the centrosomes are eliminated from the germ cell nuclei at or around the stage of oocyte commitment (Albertson and Thomson, 1993).

Anti–SAS-4 staining of the oocytes from the *cki-2cs* hermaphrodite animals revealed that SAS-4 staining structures were present next to the oocyte nuclei at a frequency consistent with the penetrance of the extra centrosome defect caused by the *cki-2cs* transgene (8.9%; $n = 79$), whereas no obvious SAS-4 foci were ever observed in oocytes in wild-type animals (Fig. 4 B and not depicted). Although this is the strongest evidence that *cki-2* is required for appropriate centriole elimination during oogenesis, we wanted to further confirm that the anti–SAS-4 staining recognized bona fide centrioles and not simply SAS-4 aggregates in the oocyte. We therefore stained the oocytes of wild-type and *cki-2cs* animals using anti–SAS-4 and anti–SAS-6, both of which recognize the centriole (Dammermann et al., 2004; Leidel and Gonczy, 2005). Both antibodies recognized the centrioles of embryos, where they colocalize with γ-tubulin (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200512160/DC1). After double staining, we compared the number of overlapping signals between wild-type and *cki-2cs* germ lines (Fig. 4, C–E). Consistent with our previous observation (Fig. 4 B), we noted that

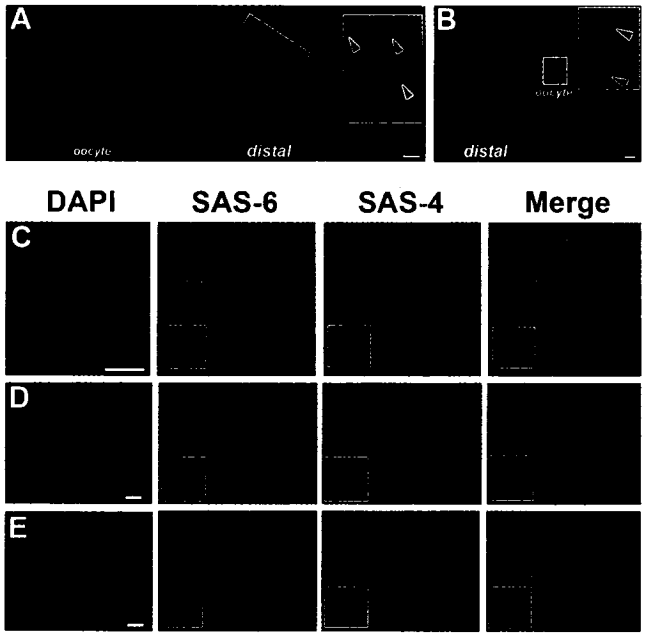


Figure 4. **Centrioles are not appropriately eliminated during oogenesis in *cki-2cs* animals.** (A and B) Extruded gonads from wild-type (A) or *cki-2cs* (B) adult hermaphrodites stained with DAPI (red) and anti–SAS-4 (green). The bracket in A delineates the region that corresponds to oocyte commitment, where ~50% of the germ cell nuclei stain positively for SAS-4. The region within the rectangular box is shown in detail, and the open arrowheads indicate SAS-4 foci (centrioles) in this inset and throughout. The inset in B shows a magnified oocyte (from the white frame) with two SAS-4 staining foci. (C–E) A wild-type meiotic germ cell (C), a wild-type oocyte (D), or an oocyte from a *cki-2cs* adult hermaphrodite (E). All were stained with DAPI (blue), Cy3-conjugated anti–SAS-6 (green), or Cy5-conjugated anti–SAS-4 (red). The region within the rectangular box is shown at higher magnification. Bars: (A and B) 10 μm; (C–E) 2.5 μm

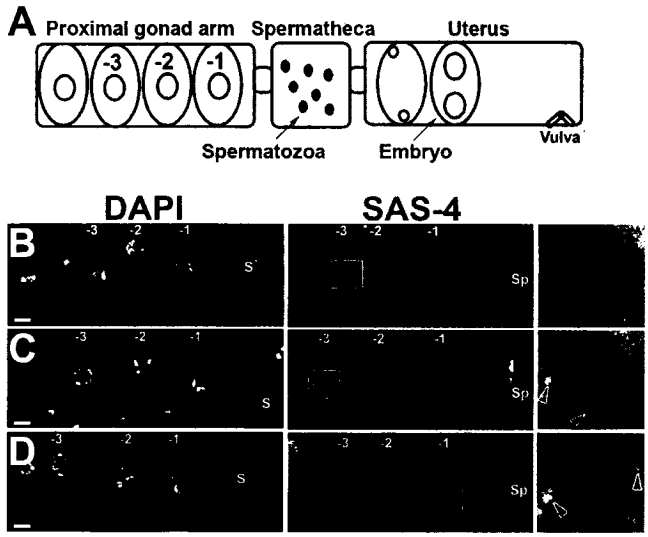


Figure 5. **Centrioles persist into the later stages of oogenesis in *cki-2cs* animals.** (A) Diagram of late-stage oogenesis in the proximal gonad arm. The number indicates the position of the oocyte undergoing meiotic maturation. Oocytes in diakinesis of meiotic prophase I before maturation (−3, −2); the oocyte adjacent to the spermatheca is designated as −1. (B–D) A proximal gonad arm from a wild-type animal (B) or *cki-2cs* animals (C and D) stained with anti–SAS-4 antibody. S, Spermatozoa and/or Spermatids; Sp, Spermatheca. Open arrowheads indicate SAS-4 foci detected in the oocyte nuclei (C and D). The white rectangular boxed region was magnified to provide greater detail. Bars, 10 μm.

significantly more SAS-6 staining oocytes showed overlapping positive signals with anti–SAS-4 in the *cki-2cs* animals (14/55 SAS-6–positive oocytes) compared with wild-type (1/29 SAS-6–positive oocytes; this single overlapping SAS-4 signal may be due to juxtaposition of the signals during the deconvolution process; Fig. 4, D and E). Therefore, our staining with two independent centriole-specific antibodies suggests that the observed foci are indeed centrioles, which are not appropriately eliminated in the *cki-2cs* oocytes.

In *C. elegans*, oogenesis occurs in an assembly line–like fashion (Fig. 5 A; Schedl, 1997). We observed that the SAS-4 staining structures persisted into the late stages of oogenesis in *cki-2cs* hermaphrodites (Fig. 5, B–D). These data are consistent with *cki-2* playing a critical role in the timely elimination of the maternal centrioles during oogenesis, and when its activity is reduced below a critical threshold, the centrioles persist and eventually will give rise to the supernumerary centrosomes. Although our results strongly argue that *cki-2* is involved in the elimination of maternal centrioles, ultrastructural studies would provide more definitive evidence of centriolar perdurance. Intriguingly, although the maternally contributed centrosomes are the likely cause of the abnormal division observed in the one-cell–arrested *cki-2cs* embryos, we have been unable to show that these supernumerary centrosomes can nucleate microtubules and/or duplicate beyond the first division. We also noticed that the polarity of the affected embryos seems consistently normal based on GFP–PAR-2 (100%; n = 17; Fig. 2, C and D) or P-granule staining (Fig. 2 E; Cowan and Hyman, 2004b). Our observation that anterior/posterior polarity does not seem to be affected in *cki-2cs* zygotes suggests that although the maternally contributed centrosomes appear competent to organize a mitotic spindle, they are seemingly not equivalent to the paternal centrosome in providing the polarity cue in the zygote. The basis of this difference between the centrosome pairs is currently unknown, as no difference in centrosomal morphology or molecular composition has been identified between the centrosomes of paternal and maternal origin.

Our observations, although obtained with fixed embryos, suggest that a functional difference may distinguish the maternal and the paternal centrosome in establishing the anterior/posterior polarity at fertilization. However, we have been unsuccessful in imaging the maternally contributed centrosomes into and beyond the first division while simultaneously monitoring the establishment of the PAR-2 domain. Therefore, we cannot formally rule out the possibility that the polarity is established early by the sperm and that the extra centrosomes we observe in the multinucleate embryos are paternal in origin that have duplicated and appear later due to cytokinesis defects (Fig. 2, A–E).

Because meiotic defects were also observed in *cki-2cs* embryos, we determined whether the abnormal presence of centrosomal components on the meiotic spindle might disrupt the normal mechanism of the acentriolar meiotic division. We found that the morphology of the meiotic spindle in early *cki-2cs* zygotes is disorganized (Fig. S2 C, available at http://www.jcb.org/cgi/content/full/jcb.200512160/DC1), whereas SPD-2 was detectable as a diffuse haze surrounding the spindle (Fig. S2, A and B).

We also found that ZYG-1, a protein that is also required for centrosomal duplication (O'Connell et al., 2001), was similarly present on the meiotic spindle in *cki- 2cs* zygotes (unpublished data), suggesting that the atypical presence of these ectopic centrosomal materials may be responsible for the meiotic spindle abnormalities and the consequent meiotic defects observed in *cki-2cs* embryos.

The loss of *cki-2* could result in misregulated levels of Cdk activity within the oocyte, causing a centrosomal anlage to persist and eventually form the tetrapolar spindle that results in a one-cell arrest. To test this scenario, we compromised G1/S Cdk activity by performing *cye-1(RNAi)*, which is the only E-type cyclin in *C. elegans* (Fay and Han, 2000). Loss of cyclin E has no effect on the first cell division in *C. elegans* (Fay and Han, 2000). However, after *cye-1(RNAi)* in *cki-2cs* animals, the characteristic one-cell arrest phenotype was suppressed substantially, which was also reflected in the nearly twofold reduction in the frequency of the multipolar spindle defect (Fig. 2 F). A similar degree of suppression was also observed after *K03E5.3(RNAi)*, where *K03E5.3* is the predicted *C. elegans* Cdk2 homologue (Liu and Kipreos, 2000; Fig. 2 F). Control animals injected with double-stranded (dsRNA) corresponding to cyclin D showed no such effect (unpublished data).

That this effect of cyclin E occurs independently of Cdk activity (Matsumoto and Maller, 2004) seems unlikely based on the current accepted mechanism of CKI function and our observation that *K03E5.3(RNAi)* suppressed the frequency of the persistence of the maternal centrosomes to levels comparable to *cye-1(RNAi)*. Our data are thus consistent with the loss of *cki-2* resulting in misregulated cyclin E/Cdk2 activity in the germ line that consequently allows centrioles to perdure into the developing oocyte.

That both ZYG-1 and SPD-2 persist during oogenesis and are present on the meiotic spindle in *cki-2cs* embryos suggests that their levels may be regulated by cyclin E/Cdk activity, in a manner similar to Mps1 (Fisk and Winey, 2001). The loss of *cki-2* therefore reveals a previously undescribed function of cyclin E–Cdk complexes in centrosome stabilization in the *C. elegans* germ line. Through the timely regulation of this activity, the maternal centrosomes are eliminated as the germ cell acquires its oocyte fate.

This novel function of Cdks and CKIs in centrosome inheritance would probably not have been uncovered through conventional gene targeting in mouse models. Unlike most animals, the sperm does not contribute the centrioles in the mouse; instead, they arise de novo in the fertilized zygote (Schatten, 1994). Why, then, do most metazoans selectively eliminate the centrosomes within the maternal germline? The answer may come from species that can develop parthenogenetically, where the oocyte is thought to harbor a centriolar anlage (Delattre and Gonczy, 2004). This would be selected against in species that undergo a biparental mode of development based on sperm-specific centriolar contribution. The elimination of the maternal centrosomes, either through CKI-mediated or related mechanisms, would block the ability of the oocyte to develop parthenogenetically and strongly favor the union of sperm and egg to trigger the onset of cell division in the zygote. Because the mode of centrosome inheritance in *C. elegans* shares considerable parallels with that of many animals, identification of the Cdk targets in this model may provide invaluable insight pertinent to the mode of centrosome inheritance shared by most metazoans, including humans.

Materials and methods

Nematode strains

The following *C. elegans* strains were used: N2 Bristol was used as the wild type throughout. MR258 (N2; *rrEx258* [*fem-1*::*cki-2C*; *elt-2*::GFP]), MR306 (N2; *rrEx306* [*fem-1*::GFP; *elt-2*::GFP]), MR294 (*rde-2*; *rrEx294* [*fem-1*::*cki-2C*; *elt-2*::GFP]), MR303 (*mut-7*; *rrEx303* [*fem-1*::*cki-2C*; *elt-2*::GFP]), NL917 (*mut-7* [*pk204*]), WM29 (*rde-2* [*ne221*]), MR446 (*unc-119*; *ruIs32* [*unc-119*(+); *pie-1*::GFP::H2B]; *ojIs1* [*unc-119*(+); *pie-1*::GFP::TBB-2]; *rrEx258* [*fem-1*::*cki-2C*; *elt-2*::GFP]), XA3501 (*unc-119*; *ruIs32* [*unc119*(+); *pie-1*::GFP::H2B]; *ojIs1* [*unc-119*(+); *pie-1*::GFP::TBB-2]), TH27 (*unc-119*; *ddIs6* [*unc-119*(+); *pie-1*::GFP:: TBG-1]), MR628 (*itIS153* [*rol-6*(+); *pie-1*::PAR-2::GFP]; *rrEx258* [*fem-1*::*cki-2C*; *elt-2*::GFP]), MR824 (*unc119*; *ddIs6* [*unc-119*(+); *pie-1*::GFP::TBG-1]; *rrEx824* [*fem-1*::*cki-2C*; *elt-2*::GFP]), NL2099 (*rrf-3(pk1426)*), and KK866 (*itIS153* [*rol-6*(+); *pie-1*:: PAR-2::GFP]). All *C. elegans* strains were cultured using standard techniques and maintained at 20°C unless stated otherwise (Brenner, 1974).

Constructs

For *cki-2* cosuppression, 3 kb of genomic sequence upstream of the *fem-1* translational start site was PCR amplified from N2 genomic DNA followed by SphI–PstI digestion and insertion into pPD49.26 to yield pMR220. The *cki-2C* fragment (amino acids 116–259; lacking a translational start site; Fig. S1) was prepared by PCR and then inserted into pMR220 at the BamHI–XmaI sites to create pMR221. The *fem-1* promoter fragment was inserted into pPD95.77 at SphI–PstI sites to yield pMR266. For RNAi of *cki-2*, a *cki-2* template for dsRNA synthesis was generated by subcloning the *cki-2* cDNA into the PstI–KpnI sites of pBluescript II to generate pMR215. *cye-1* dsRNA was prepared as described previously (Fay and Han, 2000). *cki-1* dsRNA was prepared as described previously (Hong et al., 1998). *K03E5.3* dsRNA template was amplified from a clone of the bacterial feeding RNAi library (I-1D09) using PCR and inserted into the SacI–SacII sites of pBluescript II to generate pMR330.

cki-2 cosuppression and RNAi

pMR220 and pMR221 were coinjected (50 μg/ml) with 100 μg/ml *elt-2*::GFP as a coinjection marker into N2 hermaphrodites as described previously (Mello et al., 1991). F1 progeny expressing *elt-2*::GFP were singled, and their progeny (F2) were scored for transmission of the extrachromosomal array. Embryonic lethality was scored from each transgenic line. dsRNA was obtained by in vitro transcription reactions, annealing, and injection as described previously (Fire et al., 1998). Injected animals were transferred to new plates every 24 h, and the F1 progeny was examined for visible abnormalities that affected development or cell division.

Antibodies and immunological methods

The following primary antibodies were used: anti–α-tubulin (Sigma-Aldrich), polyclonal anti–rabbit SPD-2 (a gift from K. O'Connell, National Institutes of Health, Bethesda, MD), rabbit polyclonal anti–SAS-4 (a gift from P. Gonczy, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland), Cy3-conjugated anti–SAS-6 and Cy5-conjugated anti–SAS-4 (a gift from K. Oegema, University of California, San Diego, La Jolla, CA), and rabbit polyclonal anti–P-granule (a gift from S. Strome, Indiana University, Bloomington, IN). Secondary antibodies were anti–rabbit or anti–mouse Texas red or FITC-conjugated secondary antibodies or anti–rabbit Alexa Fluor 594 secondary antibody (all obtained from Invitrogen). DAPI (Sigma-Aldrich) was used to counterstain slides to reveal DNA. Embryos or hermaphrodite gonads were fixed and stained as described elsewhere (Couteau et al., 2004). Indirect immunofluorescence microscopy was performed using a 60× oil-immersion objective lens in a compound microscope (DMR; Leica) equipped with a digital camera (C4742-95; Hamamatsu), imaging an ~0.5-μm-thick optical section. Image analysis, computational deconvolution, and pseudocoloring were performed using Openlab 4.0.2 software (Improvision). All images using Cy3-conjugated anti–SAS-4 and Cy5–conjugated anti–SAS-6 were acquired (using a 60× oil-immersion objective lens) and deconvolved using an image restoration system (DeltaVision; Applied Precision). Data were collected as a series of 35 optical sections in increments of 0.25 μm under standard parameters using the SoftWoRx 3.0

program (Applied Precision). Images were processed using Photoshop 8.0 (Adobe). All microscopic works were performed at 20°C.

In situ hybridization

Digoxigenin-labeled antisense and sense probes were generated using T7 and T3 kits with digoxigenin-11-UTP (Roche). In situ hybridization was performed on the gonads dissected from wild-type or *cki-2cs* (GFP+) adult hermaphrodites as described previously (Feng et al., 1999).

Online supplemental material

Fig. S1 shows protein sequence alignment of CKI-2 with -1. Fig. S2 depicts centrosomal material persisting on the meiotic spindle in *cki-2cs* one-cell embryos. Fig. S3 shows an embryonic cell labeled with GFP–γ-tubulin, anti–SAS-6, and anti–SAS-4. Video 1 shows a *cki-2cs* one-cell embryo labeled with GFP histones and GFP–β-tubulin. Video 2 shows a wild-type one-cell embryo (pronuclear migration stage) labeled with GFP–γ-tubulin. Video 3 shows a *cki-2cs* one-cell embryo (pronuclear migration stage) labeled with GFP–γ-tubulin. Video 4 shows a *cki-2cs* one-cell embryo (prepronuclear migration stage) labeled with GFP–γ-tubulin. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200512160/DC1.

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