THE MOLECULAR AND GENETIC ANALYSIS OF C. ELEGANS CULLINS CUL-2

AND CUL-4

by

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(Under the Direction of Edward T. Kipreos)

ABSTRACT

Members of the cullin family function as integral components of cullin/RING finger ubiquitin ligase complexes. Here we demonstrate that two C. elegans cullins, CUL-2 and CUL-4, are key cell cycle regulators. We discovered that CUL-2 promotes the G1-to-S phase transition by negatively regulating CKI-1, a member of the CIP/KIP family of cyclin-dependent kinase inhibitors. CUL-2 is also required for mitotic chromosome condensation. In cul-2 mutant embryos, mitotic chromosomes fail to condense, leading to multiple nuclei, DNA bridges, and unequal DNA segregation. To explore the composition of CUL-2/RING finger complexes, potential C. elegans CUL-2 interactors were uncovered. By BLAST search and RNAi, all C. elegans orthologs of human CUL2/VCB components but VHL were suggested to function with CUL-2. In addition, over ten proteins that associate with FLAG-tagged CUL-2 were identified by affinity purification and mass spectrometry. The identification of four BC-box proteins as CUL-2 interactors suggests that CUL-2 forms multiple CUL-2/RING finger complexes, using Elongin BC to link BC-box proteins to the core complex. Interestingly, three transcriptional regulators were identified as CUL-2 interactors, suggesting a role for CUL-2 in transcription control. Another C. elegans cullin family member, CUL-4, is essential for maintaining genome stability by restraining DNA replication licensing in S phase. Inactivation of CUL-4 leads to an S phase arrest and massive DNA re-replication. To explore the mechanism of CUL-4 function, we investigated the regulation of the replication licensing factor CDT-1. We demonstrated that the C. elegans CDT-1 ortholog is required for DNA replication. Further, C. elegans CDT-1 is present in G1 phase nuclei but disappears as cells enter S phase. The absence of CDT-1 in S phase cells prevents DNA from undergoing re-replication. In cells lacking CUL-4, CDT-1 levels fail to decrease as cells enter S phase but instead remain constant in the re-replicating cells. Removal of one genomic copy of the cdt-1 gene suppresses the cul-4(RNAi) rereplication phenotype, suggesting that the aberrant presence of CDT-1 protein in S phase cells promotes re-replication. We propose that CUL-4 functions to prevent aberrant reinitiation of DNA replication, at least in part, by facilitating the degradation of CDT-1.

INDEX WORDS: *Caenorhabditis. elegans*, cell cycle, cullin, CUL-2, CUL-4, ubiquitin-mediated proteolysis, Cullin/RING finger complex, SRC, BC-box motif, affinity purification, mass spectrometry.

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DEDICATION

This dissertation is dedicated to my parents. Without their encouragement, support, and discipline, it would be far more impossible for me, a child full of rebellion and laziness, to change myself and to make achievements. My deep gratitude goes to my father specially. Without his open mind, deep love, and sacrifice to his children, I would not be able to realize any of my dreams.

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CHAPTER I

GENERAL INTRODUCTION

EUKARYOTIC CELL CYCLE PROGRESSION

The cell is the basic unit of life. Cell division is the process by which one cell divides into two daughter cells after duplicating genetic information (DNA) and organelles. The cell cycle is a progression of ordered events and can be divided into four periods or phases: G1, S, G2, and M. While non-dividing cells exit the cell cycle from the G1 phase and enter a quiescent G0 state, most proliferating cells proceed through the four phases successively.

G1

G1 is the first "gap" interval between mitosis (M) and DNA replication phase (S). G1 is a growth phase where the cell grows to a critical size and prepares itself for entry into the S phase. In addition, centrosomes start to duplicate in G1. In sexual diploid organisms, G1 cells have two copies of each chromosome (2n), one maternal and one paternal.

S

DNA duplication occurs in the S phase, where the chromosomes duplicate and the ploidy increases from 2n to 4n. The replication machinery is accurately controlled, which

allows the cell replicates its DNA once, and only once, during each cell cycle. Unless DNA replication is complete, the cell will not progress further into the next cell cycle stage, G2. The centrosome duplication generally completes in the end of S [1].

G2

The next cell cycle interval is the G2 phase. When DNA damage occurs, the cell's checkpoint machinery detects it and arrests cell division in G2 phase until the damaged DNA is repaired [2], [3].

Μ

Dramatic cellular changes take place in M phase, although it is the shortest period among the four cell cycle phases. The duplicated chromosomes are condensed and the cell segregates the chromosomes equally to two daughter cells. The events of mitosis can be divided into five stages: prophase, prometaphase, metaphase, anaphase, and telophase.

Prophase

Prophase is the beginning of mitosis. The replicated chromosomes undergo condensation; the nucleolus begins to disappear; the duplicated centrosomes also separate and move apart to set up the mitotic spindle.

Prometaphase

There are three things to distinguish prometaphase from prophase: The mitotic spindle is set up, with the two centrosomes on the opposite poles and microtubules

nucleated from the two centrosomes; the chromosomes are fully condensed; the nuclear envelope breaks down.

Metaphase

In metaphase, sister chromatids are aligned on the mid-plate of the cell by the mitotic spindle with microtubules attached to the kinetochores.

Anaphase

Sister chromatids separate from each other and move to opposite spindle poles during Anaphase A. During Anaphase B, the two centrosomes anchoring the mitotic spindle separate further from each other. If microtubules are not attached to kinetochores properly, a spindle checkpoint detects the defect and cells arrest prior to anaphase [4].

Telophase

Cell division is completed in telophase. The nuclear envelope reforms around each set of chromosomes. Cytokinesis splits the mitotic cell into two genetically identical daughter cells (each 2n) and the cell exits mitosis.

CONTROL OF THE EUKARYOTIC CELL CYCLE

The cell division cycle has to be well controlled for organisms to reproduce and develop normally. With multiple regulatory pathways working hand-in-hand, the clock of a normal cell cycle is finely-tuned. The activity of a class of serine/threonine kinases, the cyclin-dependent kinases (CDKs), oscillates in different cell cycle phases to control

multiple cell cycle events [5]. The critical cell cycle regulators are synthesized in a cell cycle specific manner under transcriptional control [6]. The ubiquitin-mediated proteolysis pathway targets the various cell cycle regulators for degradation, hence influencing CDK activity and promoting cell cycle progression irreversibly from one phase to the next [7].

CDKs

CDK was first discovered in yeast. Both Cdc28 in budding yeast and Cdc2 in fission yeast were uncovered by isolating and analyzing cell division cycle (cdc) arrest mutants [8], [9]. Each of these two Cdk mutants arrest at both the G1/S phase and the G2/M phase transitions [8], [9]. Although only a single CDK is clearly required for the regulation of the cell cycle in fission yeast (Cdc2) and budding yeast (Cdc28), there are other cellular CDKs that have roles in transcriptional regulation, such as Pho85, Kin28, Srb10, and Ctk1 in budding yeast [5]. Vertebrates have multiple cell cycle related CDKs, e.g., CDK1, CDK2, CDK3, CDK4, and CDK6. Other vertebrate CDKs, such as CDK7, CDK8, and CDK5, are responsible for non-cell cycle events [5].

The study of CDK crystal structure provides an understanding of how CDKs function and how their activity is regulated. CDK family members share 35-65% identity to the prototypes Cdc2 and Cdc28. Human CDK2, whose major structural features are conserved in all CDKs, was extensively studied crystallographically [10]. Like other protein kinases, CDK2 contains two domains: a smaller N-terminal lobe and a larger helical C-terminal lobe [10]. The N-terminal lobe is composed of a beta sheet and a large PSTAIRE helix [10]. The ATP is positioned into the hydrophobic pocket within the cleft

between the lobes [10]. The activity of unmodified CDK is restrained by two mechanisms: first, rising from the C-terminal lobe, a flexible loop (the T-loop) blocks protein substrate binding at the entrance of the active site; second, key residues needed for ATP phosphate binding are not correctly positioned [10].

The regulation of CDK activity

The cell employs at least four mechanisms to control the CDK activity. The primary mechanism for regulating CDK activity is the binding of an activating cyclin regulatory subunit [5]. Complete activation of most CDKs also requires phosphorylation at a conserved threonine by CDK-activating kinases (CAKs) [5], [11]. The activity of the cyclin/CDK complex can be inhibited by several classes of CDK-inhibitors (CKIs) [12]. The cyclin/CDK complex can also be inactivated by inhibitory phosphorylation [5], [13].

The association of positive cyclins

Cyclins are a diverse protein family with size ranging from 35 to 90kDa. In budding yeast, there are G1 cyclins Cln1-3, S phase cyclins Clb5-6, and M phase cyclins Clb1-4 [14]. Each of these cyclins associates with a single Cdk, Cdc28, in different phases of the cell cycle. The cyclins in fission yeast that bind Cdc2, are Gic1, Gic2, and Puc1 for G1 and S phase, and Cdc13 for M phase [15]. In vertebrates, G1 cyclins include three D-type cyclins, which associate with CDK4 and CDK6 [16]. Cyclin E functions in both late G1 and S phase, and cyclin A functions in late S phase, both coupling with CDK2 [16]. A and B class cyclins function in M phase with CDK1 [16]. The studies of the crystal structural of two cyclins, cyclin A and cyclin H, revealed the structural similarities among cyclins. The cyclin box, a sequence of 100 amino-acid residues with sequence homology among the different cyclins, is necessary for CDK binding and activation [17], [18], [19]. The core of all cyclins contains a pair of 5-helix domains, with the first 5-helix bundle corresponding to the conserved cyclin box; this central fold is flanked at each end by short regions with various position and secondary structure in different cyclins [17], [18], [19].

The CDK2-cyclin A crystal structure reveals the basis of how the binding of cyclin increases kinase activity. Upon the binding of cyclin A, several major changes occur in the conformation of the CDK2 active site [17], [18]. The most obvious change occurs in the T-loop, which is no longer an obstacle to the protein substrate-binding site but lies almost flat at the entrance of the cleft; the binding of cyclin A to CDK2 also exposes the activating threonine, Thr160 on the T-loop, to the outer solvent thus allowing its phosphorylation by CAK; major changes also occur in the ATP-binding site: The small helix (L12) is melted to allow the PSTAIRE helix of the small lobe to move inward, allowing the correct positioning of the ATP phosphates for the phosphotransfer reaction [17], [18].

CKIs, negative regulatory subunits for CDKs

CDK activity is inhibited by binding to negative inhibitory subunits, CKIs [20]. There are three CKIs identified in budding yeast: Far1 which binds Cdc28-Cln complexes [21]; Sic1 which associates with Cdc28-Clb5-6 complexes [22]; and Pho81 which inactivates Pho85-Pho80 complexes under low phosphate conditions [23]. In *S. pombe*, the CKI Rum1 inhibits mitotic Cdc2-Cdc13 complexes [24]. In vertebrates, there are two classes of CKIs: the INK4 (for inhibitor of CDK4) protein family and the CIP/KIP protein family (for CDK interacting protein/Kinase inhibitory protein) [20]. The INK4 family contains four proteins, $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$, with ankyrin repeats as the common structural domain [20]. The INK4 proteins compete with D-type cyclins to bind CDK4, thus inhibiting the activity of all three CDK4/Cyclin D complexes [20]. Three proteins constitute the CIP/KIP family: $p21^{Cip1}$, $P27^{Kip1}$, and $p57^{Kip2}$ [20]. $p21^{Cip1}$ was the first CKI identified in vertebrates and is involved in p53-dependent DNA damage induced G1 arrest [25], [26]. $p21^{Cip1}$ associates with the CDK2-cyclin E complex, while $p27^{Kip1}$ binds both CDK2-cyclin E/A and CDK4-cyclin D complexes [20]. $p57^{Kip2}$ mainly binds to the CDK2-cyclin E complex and was cloned by searching for homologs of p21 and p27 [27].

Crystal structure studies of CKIs have mainly focused on the mammalian CIP/KIP family. Analysis of deletion mutants indicates that the N-terminal fold of p21 and p27 is primarily responsible for CDK inhibition [28]. The N-terminal domain contains two subsections: a short motif required for cyclin binding and a more complex segment required for interacting with the CDK subunit [28]. The crystal structure of the CDK2-Cyclin At-p27 complex reveals three approaches that p27 employs to disrupt the active site of CDKs [28]. The p27 stretches across the top of the CDK-cyclin complex in an extended conformation [28]. Without influencing the cyclin A structure, p27 alters the structure of CDK [28]. First, a beta turn in p27 interacts with the beta sheet at the top of the kinase, producing a flattening of the beta sheet [28]. Second, a short beta strand in

p27 displaces the first beta strand in the CDK2 sheet, further disrupting the conformation of the upper lobe and the ATP-binding sites [28]. Finally, a small helix in the most C-terminal segment of the p27 peptide binds deeply within the cleft of the CDK active site to block ATP binding [28].

Regulation of CDK activity through phosphorylation

1. Positive phosphorylation

The assembly of a CDK with its positively-activating cyclin only yields partial activity. Full CDK activity can only be achieved upon phosphorylation at the activating CDK threonine residue in the T-loop (Thr172 in CDK4/6, Thr160 in CDK2, and Thr161 in CDK1) [29], [30], [31], [32], [33]. In addition, this modification also promotes tight association of CDK-cyclin complexes [5]. The conserved threonine phosphorylation further flattens the T loop, allowing the active cleft to be more accessible to ATP [34].

The activating phosphorylation of CDKs is carried out by CAK. In *S. cerevisiae*, Cak1/Civ1 phosphorylates Cdc28 throughout the cell cycle [35], [36]. In addition, it also phosphorylates Kin28, a CDK with a role in transcription [37]. There are two CAKs in *S.pombe*, Mcs6 and Csk1 [38], [39]. While Csk1 activates the CDK Mcs6 by phosphorylation, both Mcs6 and Csk1 can phosphorylate Cdc2 to activate it [38], [39], [40]. In vertebrates, the Cdk7-cyclin H complex has CAK activity *in vitro* and functions as a CAK *in vivo*, although there appear to be other unidentified CAK(s) [41].

2. Negative phosphorylation

Phosphorylation can also inhibit CDK activity. Inhibitory phosphorylation occurs on the N-termini of all CDKs (Tyr15 and Thr14 on CDK2 and CDK1) [33]. Phosphorylation on these residues causes inhibition of CDK activity despite the presence of cyclins and activating phosphorylation. The inhibitory phosphorylation of CDK1 contributes to mitotic entry timing in many organisms, and is carried out by Wee1 and Myt1 kinases [42], [43], [33]. The phosphates on Tyr15 and Tyr14 are removed by phosphatases of the Cdc25 family, which act to trigger mitosis entry [44], [45].

Transcriptional regulation of the cell cycle

Although CDK is a master player for the cell cycle, gene transcription controls the abundance of many key cell cycle regulators, thereby contributing to cell cycle regulation [6]. Gene transcription is the process of RNA synthesis, using specific gene sequences as template. Gene transcription is involved in cell cycle control mostly through influencing CDK activity [5]. In turn, the varied CDK activity can stimulate or inhibit transcription, forming a positive or negative feedback loop.

Transcription alters the activity of CDK by interfering with the availability of the positive cyclins or inhibitory CKIs [5]. In *S. cerevisiae*, except for Cln3, which remains at relatively constant levels, the levels of most cyclins rise and fall during the cell cycle [6], [46]. The transcriptional activation of most cyclins is under the control of the transcription complexes SBF and MBF that are regulated by Cln3/Cdc28 [6], [47], [46]. In response to transcriptional regulation, Cln1-2 peaks in late mitosis and in G1 phase; Clb5-6 peaks in G1 and early S phases; and the other four B-type cyclins, Clb1-4, peak in

G2 and early mitosis [16], [48]. In vertebrates, the E2F family of transcription factors promotes the cell cycle, especially the G1-to-S phase transition [49]. E2F activity increases the level of a number of cell cycle regulators: cyclin E, cyclin A, and even E2F itself [5].

Similar to cyclins, the abundance of CKIs is also subject to transcriptional regulation. In yeast, the level of CKI Sic1, responding to transcriptional regulation, peaks at late mitosis and early G1; the increased Sic1 binds Cdc28/Cln5-6 complexes and keeps them inactive [22]. Mammalian CKI p21^{Cip} is mainly regulated at the transcriptional level and is induced by p53, a transcriptional regulator mediating cell cycle arrest after DNA damage [50]. Transcriptional regulation is also important for the level of another CKI, p15^{Ink4}, which associates with and inhibits CDK4/cyclin D complexes [51].

Protein degradation in the cell cycle

Cell cycle regulators are not only synthesized at a particular period, but are often selectively destroyed through regulated protein degradation [52], [53]. The degradation of key cell cycle regulators occurs almost exclusively through ubiquitin-mediated proteolysis, which is introduced in the next section [53].

UBIQUITIN-MEDIATED PROTEOLYSIS

For the past ten years, great achievements have been made to demonstrate the important roles of ubiquitin-mediated proteolysis in controlling aspects of the cell cycle. Ubiquitin-mediated degradation allows specific and efficient removal of key regulators from subcellular compartments to regulate multiple cellular processes, including the cell

cycle, signal transduction, and transcription [54]. To undergo ubiquitin-mediated degradation, a substrate has to be covalently linked to a poly-ubiquitin chain which allows the recognition and subsequent degradation of the substrate by the 26S proteasome [55], [56], [54]. Ubiquitin-protein ligation requires the sequential action of three enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) [55], [56], [54].

Ubiquitin

Ubiquitin is a highly conserved protein with 76 amino acid residues [55]. The modification of a protein by covalent linkage to ubiquitin has at least three consequences. In most cases, multiple ubiquitins covalently link to proteins through a K48-G76 isopeptide bond and mark the proteins for degradation by the 26S proteasome [57]. In other cases, poly-ubiquitin modification occurs through K63-G76 which does not target proteins for degradation but rather for subcellular compartment sorting [58], [54]. Finally, single ubiquitin can be added to certain proteins, such as histones, to act as a modifier [59].

Besides ubiquitin, there are multiple ubiquitin-like (UBL) proteins, which can also be covalently attached to substrates. However, these ubiquitin-like proteins use different enzyme systems and they do not mark proteins for proteolysis [60]. Ubiquitinlike proteins include Nedd8/Rub1p, UCRP, SUMO-1/Smt3p, and Apg12p, etc. [60]. Modification by a UBL often directs proteins for correct subcellular localization [60]. In most organisms, a single E1 enzyme (Uba1) initiates the activation of ubiquitin for an array of downstream conjugating enzymes [61], [62]. The ubiquitin-E1 conjugation is carried out by a two-step ATP-dependent reaction. First, an intermediate ubiquitin-adenylate is formed with the release of PPi; subsequently, while AMP is being released, ubiquitin forms an intermolecular thioester linkage that involves a Cys residue of E1 and the C-terminal Gly residue of ubiquitin [55]. The activated ubiquitin is then transferred to an E2 [63].

E2s

The E2 receives ubiquitin from E1 and forms a thioester linkage with ubiquitin through a Cys residue [55]. The E2 can either transfer ubiquitin to an E3 which then transfers ubiquitin to the substrates, or the E2 can directly transfer ubiquitin to substrates with the help of an E3 [55]. In a few cases, E2s can directly interact with substrates without the presence of an E3 [64]. In contrast to E1, there are multiple E2s, which generally interact with multiple downstream E3s. This forms a hierarchical cascade: A single E1 transfers ubiquitin to several E2s, and each E2 conjugates ubiquitin to substrates ubiquitines with the help of multiple E3s [55], [54], [65]. All E2s share an active-site, ubiquitin-binding Cys residue, and are distinguished by the presence of a UBC domain required for associating with distinct E3s [65].

Eleven E2s have been identified in yeast [55]. Many more E2s have been described in higher organisms. Among them, yeast Ubc3/Cdc34 and Ubc11 function in the cell cycle, working with SCF and APC E3 complexes, respectively [65], [54]. Human

cell cycle E2s include: E2-C/UBCH10 and UBCH4 for APC E3 complexes; UBCH3, UBCH4, and UBCH5C for SCF E3 complexes; and UBCH5A and UBCH5B for CUL2/VCB E3 complexes [65], [54], [66]. In addition to cell cycle roles, E2s are involved in multiple cellular processes, ranging from cell growth, cell viability, protein transportation, to DNA repair [65].

E3s

The specificity of ubiquitin-mediated proteolysis is determined by E3 ubiquitin ligases that bind substrates and act as conduits to bring the E2 close to the substrate, thereby allowing the E2 to transfer ubiquitin to the substrate [55]. There are at least two major classes of E3s: those with a HECT-domain and those with a RING finger motif. Several other minor groups of E3s also exist. For instance, the E4 or the U box-containing E3s, which serve as a scaffold to aid in transfer of ubiquitin from the E2 to a previously conjugated ubiquitin moiety to effect the elongation of polyubiquitin chains [67].

Among all E3s, only those containing the HECT-domain have been shown to form a thioester bond with ubiquitin and then transfer the ubiquitin to substrates which they bind [55], [54], [65]. The prototype of E3s with HECT-domains is E6-AP (E6 associating protein). E6-AP targets p53 for degradation in the presence of the HPV oncoprotein E6 [68]. All HECT-domain containing E3s harbor a C-terminal domain with sequence homologous to the E6-AP C-terminus. The conserved Cys residue that receives ubiquitin from the E2 is located in this region. The N-terminus of HECT-domain E3s is unique for different E3s and is believed to bind substrates. Members of another major class of E3s contain a RING finger motif [54], [65]. RING (the Really Interesting New Gene) finger motif is a cross-brace structural region which displays a series of His and Cys residues with a characteristic spacing that allows for the coordination of two zinc ions [69], [70]. The RING finger motif-containing E3s can be further classified into two groups, single-subunit RING E3s and multisubunit RING E3s [54], [65]. Single-subunit RING E3s contain both a RING finger domain and the substrate-binding/recognition site in the same molecule [65]. These E3s can bind E2s to catalyze ubiquitination on the proteins with which they associate, or on themselves [65].

Most RING finger motif-containing E3s are composed of multiple proteins. Examples of these multisubunit RING E3s are SCF complexes, CUL2/VCB complexes, and APC complexes. Among them, SCF complexes and APC complexes have been shown to regulate the cell cycle. Cullin/RING finger complexes are referred as a class of E3 ubiquitin ligases containing both a cullin and the RING H2 finger protein, Rbx1, as common subunits. Both SCF and the CUL2/VCB complexes are Cullin/RING finger complexes [71], [66]. SCF Cullin/RING finger complexes have been more thoroughly investigated. Subsequently, multiple SCF Cullin/RING finger complexes have been discovered; their cellular roles have been described; crystal structures have been analyzed; and mechanisms of action have been proposed. The following is an overview of the subunits, functions, and structural features of Cullin/RING finger complexes, mostly based on SCF complexes.

Composition of Cullin/RING finger complexes

SCF Cullin/RING finger complexes contain SKP1, a cullin, CUL1 or Cdc53, the RING finger protein RBX1, and an F-box protein [71], [66], [72]. Throughout this dissertation, the F-box protein in an SCF complex is superscripted, such as SCF^{Cdc4}, where Cdc4 is the F-box protein. Another class of Cullin/RING finger complex, CUL2/VCB complex, is composed of a cullin CUL2, a SKP1-like protein Elongin C, a ubiquitin-like protein Elongin B, RBX1, and the von Hippel-Lindau tumor suppressor protein (VHL), which is a BC-box protein [73], [74], [66]. In SCF complexes, the F-box protein functions as a substrate recognition component (SRC), directly recognizing and binding substrate [75]; whereas in CUL2/VCB complex, VHL is the SRC [76].

1. Cullins

Members of the cullin family are conserved among species. There are five major classes of cullins in *C. elegans* and in humans, CUL-1 through CUL-5 [77]. Yeast Cdc53 and metazoan CUL1 are the best-characterized members of the family. They are the only ones to interact with SKP1 [78]. The name cullin comes from the verb "to cull", which means to examine carefully so as to select or reject. Based on the compositions of the above two prototypes of Cullin/RING finger complexes, it seems reasonable to suspect that all cullins will associate with a RING-H2 finger protein, a SKP1/SKP1 -like protein, and an SRC capable of interacting with the SKP1/SKP1-like protein to form multiple Cullin/RING finger E3 complexes that cull or sort numerous substrates for ubiquitin-mediated proteolysis.

2. Ring H2 finger proteins

RING finger domains can be classified into two groups: RING HC and RING H2, depending on whether a Cys or a His occupies the fifth coordination site for zinc ion binding. The RING H2 protein, an essential component of Cullin/RING complexes, was discovered by several groups and was given various names, HRT1/ROC1/RBX1 [79], [80], [81], [82], [73]. RBX1 is a small [83] protein containing a N-terminal domain and a C-terminal domain, where the RING H2 domain lies in the C-terminus of the protein. In spite of its small size, RBX1 is at the heart of SCF complexes, interacting with cullin, Cdc53 or CUL1, and E2 UBCH3/Cdc34 [71], [66], [84].

RBX1 is critical for protein degradation as it allows the E2 to bind the E3 complex thereby promoting the transfer of ubiquitin to substrates. In an *in vitro* reconstituted system, purified ROC1/RBX1 was able to activate UBCH5 to synthesize polyubiquitin chains [85]. The RBX1 protein has been found to bind all six human cullins [73], [80]. In addition, all cullins, through their binding with RBX1, constituted active ubiquitin ligases [85]. This suggests the *in vivo* existence of a large number of cullin-RING finger ubiquitin ligases. Besides RBX1, there is another RING H2 protein family, ROC2/RBX2 [72]. Like RBX1, RBX2 can bind all cullins. However, the potential Cullin/RING complexes in which RBX2 exists are not well characterized.

3. SKP1 and SKP1-like proteins

SKP1 or a SKP1-like protein, which links the SRC to the cullin, is the core component of known Cullin/RING finger complexes. [66], [84]. SKP1 interacts only with CUL1 (or yeast Cdc53) but not with other cullins [86]. Both yeast and vertebrate SKP1

can directly bind multiple F-box proteins [87], [86], [83]. Interestingly, although there is only a single SKP1 in yeast and humans, over 21 SKP1-related genes exist in *C. elegans* [88], [89]. Most of them are able to interact with *C. elegans* CUL-1 but not with other cullins [88], [89]. Currently, Elongin C is the only SKP1-like protein that has been identified, and it functions in the CUL2/RING finger complexes [90], [91].

4. SRCs

The specificity of ubiquitination is determined by the SRC in each Cullin/RING finger E3 ubiquitin ligase [71], [66]. Different SRCs recognize and bind a repertoire of unique substrates. SRCs in SCF complexes harbor a F-box motif. The F-box is a 45-50 amino acid long degenerate sequence which is conserved among species and functions as a site of protein-protein interaction [75]. The number of F-box proteins is much greater than that of any other component of SCF complex. This diversity corresponds to its role as SRCs to target a range of different substrates. For instance, there are 11 F-box proteins in *S. cerevisiae*, 22 in *Drosophila*, and at least 38 in humans [75]. Astonishingly, there are 326 *C. elegans* proteins containing a F-box [75].

Another class of Cullin/RING finger complexes, CUL2/VCB like complexes, use BC-box proteins as SRCs [92], [93], [94], [95]. Like the F-box, the BC-box motif is a 12amino-acid long degenerate sequence that is also conserved among species. The BC-box motif was found in several classes of proteins, such as Elongin A, VHL, and SOCS-box proteins. [95], [76], [96]. The BC-box containing SRCs bind Elongin C instead of SKP1 [97], [95], [66]. Besides the four major components described above, Cullin/RING finger complexes also contain several other proteins. These include Nedd8/Rub1, Sgt1, and/or Elongin B. Nedd8/Rub1, a UBL protein, covalently modifies all cullins and exists in all Cullin/RING finger complexes [98]. The Nedd8 modification is required for CUL1 function in mammalian cells [99]. The assembly of an active E3 ubiquitin ligase by VHL promotes Nedd8 modification of CUL2 [100]. Sgt1 is a modifier of SKP1 [101]. Finally, Elongin B is a UBL protein that associates with Elongin C in CUL2/RING finger complexes [102].

Functions of Cullin/RING finger complexes

Cullin/RING finger complexes have been demonstrated to regulate both cell cycle and non-cell cycle events. In *S. cerevisiae*, Cdc53 forms at least three distinct SCF complexes, SCF^{Cdc4}, SCF^{Gr1}, and SCF^{Met30}, using Cdc4, Grr1, and Met30 as SRC molecules, respectively [83], [66]. Through these complexes, Cdc53 regulates the G1-to-S phase transition by targeting both positive and negative cell cycle regulators, G1 cyclins (Cln1p, Cln2p) and CKIs (Far1p, Sic1p), for degradation [103], [104], [105], [106], [107]. In *S. pombe*, cullin Pcu1 associates with Pop1/Pop2 heterodimer as SRC to form a SCF complex, targeting CKI Rum1 and replication licensing factor Cdc18 for degradation [66]. Human CUL1 forms multiple SCF complexes with four SRC molecule, β TrCP, β TrCP2, SKP2, and hCdc4/Fbw7/hAgo, to target the degradation of the signaling protein β -Catenin, the transcriptional repressor IkB α , the cell surface receptor CD4, the CKIs p27^{Kip1} and P21^{Cip1}, and positive G1 regulators cyclin E, D, and E2F [72] [108] [86], [109]. *C. elegans* CUL-1 functions as a negative cell cycle regulator that is required for exit from the cell cycle [77]. Inactivation of either *cul-1* or a F-box protein *lin23* causes hyperplasia in all tissues [77] [110].

The CUL2/VCB-like Cullin/RING finger complexes contain the core complex, CUL2 or CUL5, Elongin C, Elongin B, Rbx1, and different SRCs, which are currently found to be BC-box proteins [92], [93], [94], [95]. The human CUL2/VCB complex possesses ubiquitin-ligase activity, targeting hypoxia-inducible transcription factors (HIF1 α and 2 α) for degradation [102], [111], [73], [112]. CUL5/RING finger complexes are probably involved in the regulation of signal transduction [95], [93].

The crystal structure of Cullin/RING finger complexes

The crystal structure of human SCF^{SKP2} has been determined [113], [84]. From the study of the SCF^{SKP2} complex, we can gain insights into possible structural features of other Cullin/Ring finger complexes. The crystal structural study revealed that the SCF^{SKP2} complex is organized by CUL1, which functions as a scaffold protein to interact with SKP1/SKP2 and RBX1 [84]. CUL1 can be divided into an N-terminal domain and a C-terminal domain [71]. The N-terminal 415 amino acids of CUL1 forms an extended, stalk-like α -helical domain, comprising three repeats (cullin repeats) of a novel five-helix bundle [84]. The first N-terminal cullin repeat of CUL1 forms the binding site for SKP1 [84]. This binding site is conserved among CUL1 orthologs, for instance, human CUL1 and *C. elegans* CUL-1. However, other cullins show conservation in this same region with their orthologs, suggesting that SKP1-like proteins bind cullins in this region [114]. The C-terminal domain of CUL1 is composed of a four-helix bundle (4HB), an α/β

domain, and two copies of a "winged-helix" motif (WH-A and WH-B) [84]. The Nedd8 modification of CUL1 occurs in the WH-B motif. RBX1 binds CUL1 by inserting its β -strand deeply into four-strand β sheets within the CUL1 α/β domain, explaining the tight association of CUL1/RBX1 [84]. The CTD of CUL1, highly conserved among different cullins, forms the V-shaped groove to bind the RING fingers; the WH-A domain and part of the 4HB and α/β domain comprise the cullin homology (CH) region, which exists in all cullins [115].

The structural study of SCF^{SKP2} complex also shed light on how the SCF complex promotes the ubiquitination of substrates. The overall structure of the SCF complex lacks flexibility, with each cullin repeat packing against one another at a rigid angle to form a stalk of over 100 A° [84]. Replacement of cullin repeats with a flexible linker inactivates the SCF^{SKP2} complex E3 activity [84]. This may indicate that an extended rigid structure of the cullin is essential for positioning the substrate binding and catalytic domains of the SCF complex.

26S Proteasome

Ubiquitinated substrates are recognized and degraded by the 26S proteasome [116]. The 26S proteasome is a 2.5-mDa molecular machine built from over 32 different subunits [116]. Two major structural subcomplexes, the 20S core particle (CP) and the 19S regulatory particle (RP), constitute the 26S proteasome [55], [54], [65]. The 20S CP contains the protease subunits and the 19S RP regulates the function of the former. Both the structure and subunits of 26S proteasome are highly conserved among species,

suggesting the importance and conservation of the ubiquitin-mediated proteolysis pathway [55], [65].

20S core particle

The 20S proteasome is a barrel-shaped core complex with 28 subunits, some of which have proteolytic activity [117]. Inside the 20S CP hollow cylindrical structure, ubiquitinated proteins are degraded into peptides of 3-23 amino acids long [55]. The cylindrical structure of the 20S CP is made up of four heptameric rings: two outer identical α -rings and two identical inner β -rings [117], [55], [65]. Each of the above rings is made of seven distinct homologous subunits. The protease active sites in certain β -subunits face inward into the proteolytic chamber of the 20S CP [118], [55], [65]. In addition, the N-termini of the α -subunits of the outer rings obstruct access to the proteolytic chamber, suggesting that the proteasome channel is gated [119], [118].

19S regulatory particle

The 19S regulatory particle caps one end or both ends of the 20S CP to form the 26S proteasome [120]. The 19S RP recognizes ubiquitinated proteins, unfolds them, and translocates them into the interior of the 20S CP, where they are degraded [55], [65]. The 19S RP consists of two eight-subunit subcomplexes, the lid and the base [121]. The base, which contains all six proteasomal ATPases and two non-ATPase subunits, attaches to the α -ring of the 20S CP and functions to open the central channel and translocate substrates into the 20S CP [65]. The lid of the 19S RP is a 400-kD complex, made up of eight of the remaining non-ATPase subunits [121]. These subunits, the role of which is

not clear, can be released from the proteasome or rebound under certain conditions [65]. Interestingly, the 19S RP was also found to interact with chaperones and components of the ubiquitination machinery [65].

THE RATIONALE AND PURPOSE OF THIS RESEARCH

Well controlled cell cycle progression is critical for the viability of both single cell and multicellular organisms. The more that ubiquitin ligase functions are uncovered, the more we realize the importance of ubiquitin-mediated proteolysis in cell cycle regulation and the more we fully understand how the cell cycle is precisely controlled. Cullins, as the central subunit of Cullin/RING finger complexes, play critical roles in cell cycle regulatory pathways. Analysis of the yeast Cdc53 E3 complex linked cell cycle regulatory pathways with cullin and ubiquitin-mediated proteolysis [105]. The extensive study of SCF cullin/RING finger complexes highlighted additional cell cycle regulatory roles for cullins [71], [122]. However, except for yeast Cdc53 and vertebrate CUL-1, whose functions have been well-studied, the cell cycle regulatory functions of other cullin complexes remained largely unknown prior to the work presented in this dissertation.

My dissertation project focused on exploring the functions of two members of the cullin family, CUL-2 and CUL-4, with special focus on their roles in cell cycle regulation. Both CUL-1 and Cdc53 form multiple Cullin/RING finger complexes, SCF complexes, to target different substrates for ubiquitin-mediated degradation [71]. We propose that CUL-2 and CUL-4 function to regulate the cell cycle through multiple Cullin/RING finger complexes. Therefore, another goal of my project was to identify

CUL-2/RING finger complex components in order to gain insight into the mechanism of CUL-2 functions.

Through this research, I expected to uncover new functions of *C. elegans* CUL-2 and CUL-4. The following questions were asked prior to the research: do CUL-2 and CUL-4 also regulate aspects of the cell cycle? If so, in which particular cell cycle stage do they function? And which cell cycle events do they control? Does *C. elegans* CUL-2 also form multiple Cullin/RING finger complexes as its human counterpart does? What CUL-2/RING finger complexes will CUL-2 form? The related work to address the above questions is described in Chapter II (CUL-2 cellular functions), Chapter III (possible CUL-2/RING finger complexes), and Chapter IV (CUL-4 functions) of this dissertation, respectively.

My project used the nematode *C. elegans* as a model organism for the following reasons. First, members of the cullin family and aspects of cell cycle regulatory mechanisms are conserved in *C. elegans* [77]. Second, *C. elegans* is a multicellular organism, whose genes can be studied in a developmental context. Third, *C. elegans* has unique physiological advantages as a model organism: small size, simple and short life span, and rapid development [123]. Fourth, molecular and genetic approaches for studying this organism have been well developed over the past few decades. Most importantly, the ability to perform robust RNAi (RNA-mediated interference [124]) greatly facilitates the study of genes' functions. Fifth, with the completed genome sequenced, *C. elegans* can be applied to high-throughput genomic and proteomics analyses [125]. Finally, with its invariant cell lineage, small number of defined somatic cells (595 in the newly hatched larvae) [126], and transparent appearance, *C. elegans* is

particularly well-suited for cell cycle study, allowing us to study defined cell divisions in live animals by DIC microscopy and *in vivo* GFP-labeling.

CHAPTER II

THE CHARACTERIZATION OF C. ELEGANS CUL-2 FUNCTIONS

BACKGROUND

Eukaryotic cell cycle transitions are regulated in large part by the activity of cyclin-dependent kinases (CDKs) [127]. The transition from one cell cycle state to another is made irreversible by the ubiquitin-mediated degradation of cyclins and cyclin-dependent kinase inhibitors (CKIs). Much is currently known about how ubiquitin-mediated degradation promotes the cell cycle transition through the study of two classes of E3 complexes, the anaphase-promoting complex (APC) or cyclosome, which is required for the metaphase-to-anaphase transition and for exit from mitosis; and Cullin/Ring finger complexes, which are required for both the G1-to-S and G2-to-M phase transitions [128], [129], [130], [22]. In this chapter, I report that *C. elegans* CUL-2 functions as a positive cell cycle regulator. In the absence of *cul-2*, germ cells undergo G1 phase arrest, which is correlated with a post-transcriptional accumulation of CKI-1, a member of the CIP/KIP family of CKIs. Surprisingly, *cul-2* is also required for mitotic chromosome condensation. The following summarizes the general regulation of the G1-to-S phase transition and mitotic chromosome condensation

The G1-to-S phase transition

In order to maintain constant nuclear/cytoplasmic ratios, eukaryotic cells couple growth to DNA synthesis by preventing S phase entry until cells have reached a critical size [131]. Like many other cell cycle events, the G1-to-S phase transition is regulated by CDK activity. CDK activity that is essential for the G1-to-S phase transition is upregulated through the synthesis of the G1 and S phase cyclins and the degradation of the negative regulators, the CKIs. The activation of CDK/Cyclin complexes triggers S phase entry with the initiation of DNA replication. However, the detailed regulation of the G1to-S phase transition differs between species. In yeast, the degradation of CKIs is essential for S phase entry, while the initiation of S phase in vertebrates requires the release of an active transcription factor E2F from its inhibitory subunit retinoblastoma (Rb) protein.

The G1-to-S phase transition in yeast

Great insights into the G1-to-S phase transition were made by studying the budding yeast *S. cerevisiae*. Both environmental conditions and CDK activity contribute to the G1-to-S phase transition [132], [5]. When there is nutrient deprivation or pheromone present, yeast cells arrest before START (START is the time point when the cell commits itself for division). Cells that pass START are resistant to environmental changes and are able to proceed further. Under favorable environmental conditions, the single cell cycle CDK, Cdc28 in budding yeast and Cdc2 in fission yeast, assembles into active holoenzyme complexes with G1 and S phase cyclins to direct the G1-to-S phase transition [16]. In budding yeast, S phase entry requires the activity of the S phase
promoting complex Cdc28/Clb5, which is inactive in G1 due to association of the inhibitory subunit Sic1 [22]. While the other regulatory pathways work together to promote the G1-to-S phase transition, the degradation of CKIs, Sic1 in budding yeast, is the most important step for S phase entry [133], [134], [135].

1. The synthesis and degradation of G1 and S phase cyclins

The activation of Cdc28/Cdc2 requires the association with cyclins. There are three G1 cyclins: Cln1, Cln2, and Cln3. The level of most cyclins is temporally controlled during the cell cycle by regulated transcription [6]. In late G1, Cdc28/Cln3 complex increases the transcription of the G1 cyclins, Cln1 and Cln2, by activating the Swi6/Swi4 transcription factor complex (SBF) [5]. Cdc28/Cln1-2 complexes can also stimulate the transcription of Cln1 and Cln2, forming a positive feedback loop. By activating another transcriptional complex, Swi6/Mbp1 (MBF), the Cdc28/Cln3 complex up-regulates the transcription of the S phase cyclins Clb5 and Clb6, which complex with Cdc28 and inhibit the transcription of the G1 cyclins [5], [16].

While G1 and S phase cyclins are regulated at the transcriptional level, they are also targeted for proteolysis. The Cdc28/Cln1-2 complexes in *S. cerevisiae* and Cdc2/Gic2 in *S. pombe* can self-phosphorylate the cyclins that they bind, which triggers the destruction of Cln1, Cln2, and Gic2 by ubiquitin-mediated proteolysis [108]. Cdc34 was the first protein to be implicated in the control of cell cycle progression by proteolysis, and it was later characterized as an E2. The E3 ubiquitin ligase SCF^{Grr1} complex, formed by the yeast cullin Cdc53, Skp1, Rbx1, and one of the F-box proteins Grr1, targets Cln1 and Cln2 for degradation, with the help of the E2 Cdc34 [136], [137], [66].

2. CKI degradation in late G1

In late mitosis, Sic1 accumulates since its transcription is up-regulated upon the activation of the transcription factor Swi5 [138]. During the G1 phase, Sic1 binds Cdc28/Clb5-6 complexes to keep them inactive. The degradation of Sic1 in budding yeast is necessary and sufficient for the G1-to-S phase transition [133], [134], [135]. A cdc34 mutant is arrested at G1 phase because of an inability to eliminate Sic1 [22]. Upon deletion of the Sic1 gene, this cdc34 temperature sensitive mutant can progress into S phase successfully. Also, overexpression of a non-degradable Sic1 can block the yeast at the G1 phase [22]. Similar to that of G1 cyclins, the phosphorylated Sic1 is targeted for ubiquitin-mediated proteolysis by Cdc34-SCF^{Cdc4} complex, using Cdc4 as the F-box protein [107] [72]. Sic1 destruction then triggers the activation of Cdc28/Clb5 complex that is required for S phase entry [7]. Another G1 CKI, Far1, is also targeted for ubiquitination and degradation by the SCF^{Cdc4} complex. Far1 binds Cdc28/Cln1-2 complexes and keeps them inactive upon nutrient deprivation or in the presence of mating pheromone [139]. A mutant form of Far1 that is non-degradable can prevent efficient S phase re-entry after α -factor arrest [108].

Unlike in budding yeast, where the degradation of Sic1 is required for the onset of the S phase, in fission yeast, the destruction of CKIs is more important for the proper custodial control of the cell cycle. In *S. pombe*, the CKI Rum1 inhibits the mitotic Cdc2/Cdc13 complex in G1 phase to prevent premature mitotic entry [5]. Rum1 is expressed periodically and is degraded during S phase in a phosphorylation-dependent manner [140], [141]. Similar to Sic1, a non-degradable Rum1 arrests cells at the G1

phase but with polyploidization due to inappropriate reinitiation of DNA replication [140], [142].

The G1-to-S phase transition in vertebrates

Mammalian cells become committed to enter S phase upon the stimulation of extracellular mitogenic signals. Unlike yeast, there are multiple cell cycle CDKs, CDK4, CDK6, and CDK2, the activity of which is required for the successful G1-to-S phase transition [16]. The association of cyclins activates the G1- and S-phase CDK complexes, and the degradation of cell cycle regulators is also important for proper timing of progression into S phase. However, the exact regulation for the G1-to-S phase transition largely remains unknown in vertebrates.

1. Rb phosphorylation and transcription activation by E2F

Rb phosphorylation is a critical requirement for the G1-to-S phase entry in vertebrates. Unphosphorylated Rb inhibits transcription by two means. First, it directly binds the transactivation domain of E2F, thereby preventing E2F from activating transcription. Second, Rb can also bind to the promoter region in a complex with E2F and actively represses transcription [49]. E2F is a transcription factor family, composed of at least five DNA binding proteins, E2F1-5, and two heterodimeric proteins DP1 and DP2 [143].

The phosphorylation of Rb is carried out by G1 CDK complexes. There are three mammalian D-type cyclins, cyclin D1, D2, and D3, which assemble with CDK4 or CDK6 into holoenzymes in G1, and one cyclin E, which partners with CDK2 in late G1

[16]. In the presence of growth factor stimulation, D-types cyclins are synthesized and only moderately oscillate during the cell cycle with peak levels achieved near the G1-S transition [49]. Unlike the D-type cyclins, cyclin E is expressed periodically and induces maximal cyclin E-CDK2 activity at the G1-to-S phase transition [49]. Once Rb is phosphorylated, it then releases E2F. The free form of E2F can activate the transcription of many genes required for DNA replication and genes of key cell cycle regulators needed for S phase entry. The elevated transcription prepares the cells for S phase entry and the initiation of DNA replication.

2. Proteolysis of the G1 and S phase regulators

Besides the transcriptional activation of key cell cycle genes, the proteolysis of G1 and S phase regulators is also important for the G1-to-S transition in vertebrates. Upon treatment with proteasome inhibitors, several mammalian cell types arrest before S phase entry. In *Xenopus laevis*, immunodepletion of Cdc34 inhibits the initiation of DNA replication [144]. Many regulators of the G1-and S-phases, such as P27^{Kip1}, P21^{Cip1}, E2F, Cdc6, Cyclin E, and Cyclin D, are targeted for ubiquitin-mediated proteolysis although the biological significance of these regulators' degradation has not been clearly defined [108].

The degradation of the following regulators, Geminin, P21^{Cip1}, P57^{Kip2}, P19^{Ink4d}, and P27^{Kip1}, may be required for the onset of DNA replication. Geminin is a protein first identified in *Xenopus laevis*. Geminin is degraded in late mitosis and, when overexpressed, prevents loading of MCM proteins onto the pre-replication complex [145]. The degradation of Geminin is believed to contribute to onset of DNA replication

due to releasing replication licensing factor CDT1 in vertebrates [146]. Except for P19^{Ink4d}, which binds only CDK4-cyclin D, all three CKIs, P27^{Kip1}, P21^{Cip1}, and P57^{Kip2}, are able to inhibit CDK4/6-cyclin D, CDK2-cyclin E, and CDK2-cyclin A complexes [108]. Similar to the case in yeast, the association of CKIs with CDK/Cyclin complex inhibits CDK activity and the removal of the CKIs by proteolysis is needed for full activity of CDK/Cyclin complexes. The degradation of P27^{Kip1} may be important for the G1-to-S phase transition since overexpression of P27^{Kip1} arrests mammalian cells in G1 [72] [108]. In both human and mice, it is suggested that P27^{Kip1} is ubiquitinated by SCF^{Skp2} E3 ubiquitin ligase with Cdc34 as E2 [72] [108] [86]. However, Skp2^{-/-} mice are viable, and their cells don't undergo G1 arrest despite elevated levels of cyclin E and P27^{Kip1} in certain cells. Therefore, it is debatable if the degradation of P27^{Kip1} for proteolysis. Currently, it is unknown who targets P57^{Kip2} and P19^{INK4d} for degradation [108].

Mitotic chromosome condensation

Mitotic chromosome condensation is the process of condesing interphase chromatin into an orderly compact structure. Starting in S phase, as DNA completes its replication and sister chromatids pair up, chromosomes already begin to fold in an axialloop structure [147]. Further folding of the loops mainly takes place in early prophase and is completed in prometaphase.

The significance of condensation

It is very important that mitotic chromosomes condense properly for two reasons. First, the length of uncondensed chromatin is very long. For example, human DNA, over 3000MB, is about 4 meters long. If not compacted, it would be impossible to fit chromatin into a tiny nucleus with a diameter less than 10 µm. In addition, it would be impossible to separate the sister chromatids in anaphase since the length of the dividing cell is much shorter than the uncondensed chromatin. Second, if the chromosomes were not condensed, the clean segregation of chromosomes during mitosis would be virtually impossible due to the tremendous tangles arising from DNA replication and the simple diffusion of interphase chromosomes. Once the DNA damage occurred by chromosome breakage, cell division would either arrest or continue with an abnormal amount of DNA, producing genome instability and inducing diseases in organisms.

The regulation of condensation

To ensure proper mitotic chromosome condensation, the process has to be wellregulated. It is generally believed that condensation is initiated at specific chromosomal foci, so-called *cis*-acting sites, which served as landmarks to direct condensation [148], [147]. Heterochromatic regions have been proposed to be such *cis* elements [149]. Also heterochromatin-independent regions and other novel sites could also exist [148]. Currently, only three chromosomal components, histones, topoisomerase II, and the condensin complex containing structural maintenance of chromosomes (SMC) proteins, have been implicated in condensation.

1. Histones and histone modifications

The function of histories in DNA condensation is controversial. There are four types of core histones (H2A, H2B, H3, and H4), which together with DNA form the nucleosome core particle, the basic chromosome subunit. The first indication of histones' participation in condensation is from the observation that the 11-nm long chromatin is converted into 30-nm fiber upon the addition of histone H1. Furthermore, histone H1 on condensed chromosomes is hyperphosphorylated [150]. However, when histone H1 was depleted in *Tetrahymena*, mitotic condensation proceeded unperturbed [151]. Later, the focus was shifted from histone H1 to histone H3 since histone H3 was found to be phosphorylated prior to metaphase. Fostriecin-induced condensation is blocked by a kinase inhibitor staurosporine, which blocks H3 but not H2A phosphorylation [150]. The phosphorylation of H3 is carried out by INCENP-aurora B kinases in many species [152]. Although phosphorylation of H3 is generally believed to be required for mitotic chromosome condensation, there is evidence against this model [153], [154], [155]. Another two histones, H2A and H2B, are also suggested to play a role in chromosome condensation because that they are modified by ubiquitin in anaphase but lose their ubiquitin modification at metaphase [156]. Nevertheless, as in the case of H1, further evidence for the involvement of H2A and H2B in chromosome condensation is needed for a causal relationship.

2. Topoisomerase II

The activity of topoisomerase II is involved in chromosome condensation even though there is evidence suggesting that chromosomes can undergo condensation in the absence of topoisomerase II [160]. Topoisomerase II is able to create a transient doublestrand break in a DNA molecule that allows the passage of one DNA strand through another. This activity can be detected by the resolution of catenated DNA circles in vitro. It is generally believed that topoisomerase II facilitates the formation of proper folds needed for condensation by removing catenation with its enzymatic domain. Several observations support this model. First, mutants defective in topoisomerase II fail to condense their chromosomes in prophase [157]. Second, condensation is blocked by a class of inhibitors that interact specifically with the enzymatic active site of topoisomerase II [158]. Third, inactivation of topoisomerase II prior to condensation leads to partially compacted chromosomes that are apparently tangled with each other [156]. Besides the contribution to condensation based on its enzymatic activity, topoisomerase II has also been proposed to facilitate condensation by forming the chromosome scaffold, an insoluble complex in the chromosomal core region [159]. This latter proposal has not been experimentally confirmed.

3. The SMC proteins

The discovery of SMC proteins is an important milestone in the study of chromosome structure and chromosome condensation. The necessity of SMC proteins in chromosome condensation is quite clear [160]. SMCs are also essential for sister-chromatid cohesion, recombination, DNA repair, and epigenetic silencing of gene expression [160].

3.1. The structure and classification of SMC proteins

The basic structure of SMC proteins, which is shared from bacteria to humans, consists of the following distinct domains: the Walker A motif (GxxGxGKS/T) at the N-terminus, Walker B motif ($\Phi\Phi\Phi\Phi$ D, where Φ represents any hydrophobic residue) and a ATP-binding cassette (ABC)-like motif at the C-terminus, and two coil-coiled domains separated by one central flexible hinge. When the N-terminal and C-terminal domains come together in a heterodimer, SMCs share not only sequence but also structural similarities to ABC proteins. For instance, the crystal structure of a modified *Thermotoga maritima* SMC shows structural homology to ABC proteins and to the Rad50 DNA-repair protein [161]. In addition to their ATPase activity, SMCs are also capable of binding to DNA, with the DNA-binding motif located at the C-terminal.

There is one SMC in prokaryotes and at least six in eukaryotes. Most of the bacterial and *archaeal* genomes contain a single *smc* gene, the protein product of which forms a homodimer. Although some Gram-negative bacteria, such as *E. coli*, lack SMC proteins, a gene product MukB plays an analogous role to that of SMCs [162]. In eukaryotes, at least six SMCs are found in individual organisms. Since each of them has a specific partner to form an SMC heterodimer, SMCs in eukaryotes are classified into three distinct groups: SMC1-SMC3, SMC2-SMC4, and SMC5-SMC6 [161]. These heterodimers further associate with different non-SMC subunits to assemble fully functional SMC holocomplexes. Among them, the SMC2-SMC4 holocomplex is involved in chromosome condensation, dosage compensation, and gene silencing [163], [161]. The SMC1-SMC3 holocomplex functions in sister-chromatid cohesion and DNA

recombination [163], [161]. The function of SMC5-SMC6 complex is not clear but with some indication in DNA repair and checkpoint responses [161], [164].

3.2. The condensin complex and its molecular actions

The first SMC proteins exhibiting a role in chromosome condensation were found in *S. pombe*. The *cut3* (SMC4) and *cut14* (SMC2) mutants displayed a "cut" phenotype in which chromosome segregation was incomplete with subsequent chromosome loss [165]. Similar phenotypes were later observed in other organisms including *smc2* and *smc4* mutants of *S. cerevisiae* [149]. The first condensation holocomplex was idenitified in *Xenopus* eggs. This 13S condensin contains XCAP-E (SMC2-type), XCAP-C (SMC4type), and three non-SMC proteins: XCAP-D2, XCAP-H, and XCAP-G [166]. A condensin complex has also been detected in *S. pombe* lysates, with a subunit composition similar to that of the *Xenopus* 13S condensin complex [167]. In addition, there is another *C. elegans* SMC4, DPY27, which is involved in dosage compensation with MIX-1, DPY-26, and DPY-28 [168].

The exact mechanism through which the condensin complex facilitates chromosome condensation remains to be determined. However, the mechanism is different from that used by topoisomerase II since the uncondensed state of chromosomes in the absence of condensin is clearly distinct from that observed in the absence of topoisomerase II [166]. In *S. cerevisiae*, condensin concentrates in the rDNA region during mitosis and interphase, implying that condensin may have a specialized function in organizing this highly repetitive locus that has properties of heterochromatin [169]. Condensin subunits are also found enriched in the nucleolus in human cells [164]. The

antiparallel arrangement of SMC dimers with their flexible hinge allows them to form Vshaped cross-linkages, functioning as an intramolecular DNA cross-linker in the case of condensin and as an intermolecular DNA cross-linker in the case of the related cohesin complex [170]. The different conformation of SMCs in condensin and cohesin may help them to recognize either a single DNA molecule or two DNA molecules. In the presence of topoisomerase II *in vitro*, the condensin complex converts nicked circular DNA into positive knots by using the energy of ATP hydrolysis [171], [172]. Although DNA supercoiling and knots, induced by condensin, may be important for chromosome condensation, other activities are also required. The *Drosophilia SMC4* mutant exhibited a chromosome condensation defect with normal shortening of the chromosome axis, indicating that the shortening of the chromosome axis is independent of condensin [173].

3.3. The regulation of the condensin

The activity of condensin is directly influenced by its non-SMC subunits. The full activity can be achieved only when the core complex (SMC2-SMC4) successfully assembles with its regulatory subcomplex (non-SMC proteins, such as XCAP-D2, XCAP-G, and XCAP-H in *Xenopus*). The SMC core complex can bind to naked DNA and shows ATPase activity. However, both DNA binding and ATPase activity are weaker than in the complete condensin complex. In addition, the core complex alone is not able to reconfigure DNA *in vitro* [171], [172]. Antibodies against XCAP-H prevent association of the regulatory complex with the core complex, indicating that the interaction between the two is mediated via XCAP-H [174]. The association of subcomplex with the core complex not only increases the latter's DNA binding and

ATPase activity, it also is required for the binding of condensin to DNA packed into nucleosomes or into chromatin [174], [164].

The activity of condensin is also influenced by the phosphorylation of SMCs and histone H3. *Xenopus* condensin must be phosphorylated by Cdc2 kinase in mitosis to become active for condensation [175]. In *S. Pombe*, condensin is excluded from the nucleus if it is not phosphorylated by Cdc2 [167]. In humans and flies, phosphorylation of histone H3 on serine 10 is important for the recruitment of condensin onto mitotic chromosomes since the condensin and phosphorylated histone H3 co-localize in the immunostaining experiments. AKAP95, an anchoring protein which interacts with protein kinase A, can also recruit condensin [176].

4. Other factors related to condensation

Besides histones, topoisomerase II, and condensin, there are a number of other factors involving in chromosome condensation. Most importantly, three lines of evidence indicate DNA replication in condensation. First, the length of a mitotic chromatin loop, between 30 and 90 kb, is comparable to the length of replicons [177], [178]. Second, through examination of DNA cell fusion studies by electron microscopy, it was discovered that S-phase progression is required for complete chromosome condensation [179]. Fusion of cells in M and G2 phases produces condensed DNA, whereas DNA remains extended with an M-G1 fusion. Fusion of S and M phase cells yields DNA that is partially condensed and partially extended. Third, the most direct evidence is from recent phenotypic analysis of DNA replication mutants in *Drosophila*. Defects in chromosome condensation were observed in *Orc2*, *Orc3*, *Orc5*, *Cdt1*, or *Mcm* mutants. All of these

genes participate in DNA replication. In *S. cerevisiae*, cohesin, a distinct but structurally similar protein complex to condensin, is also required for full chromosome condensation [180]. This suggests cross talk between DNA condensation and sister-chromatid cohesion.

RESULTS

cul-2 developmental expression.

The developmental expression of *cul-2* mRNA was determined by *in situ* hybridization. In adults, the major source of *cul-2* mRNA expression is the germline (Fig 2.1a,b). *cul-2* mRNA is provided to embryos as maternal product, and during embryogenesis the levels of *cul-2* mRNA decrease (Fig 2.1f,g). During larval development, *cul-2* mRNA is found in proliferating tissues, with the highest level in the intestine, which endoreplicates at each larval molt [181], as well as in a few postmitotic neurons in the nerve ring (Fig 2.1c-e).

Recombinant CUL-2 protein was purified and used as an antigen to produce polyclonal anti-CUL-2 serum. Anti-CUL-2 antibodies were affinity-purified to reduce non-specific background staining. We used anti-CUL-2 immunofluorescence staining to determine the CUL-2 protein expression pattern. In adults, CUL-2 is observed in the germline, with higher levels in meiotic cells (data not shown). The highest CUL-2 levels are found in oocytes, where CUL-2 is primarily nuclear with some cytoplasmic staining (Fig 2.1j,k). The CUL-2 staining is not seen in oocytes from *cul-2/cul-2* homozygous hermaphrodites (Fig 2.11,m). Disruption of *cul-2* expression by double stranded RNA-mediated interference (RNAi) [124] severely reduced the levels of both nuclear and

cytoplasmic anti-CUL-2 staining with a residual 2% of the protein level compare to wild type (Fig 2.1m,n). Similar to *cul-2* mRNA, CUL-2 protein is provided to embryos as maternal product and the level decreases during embryogenesis (Fig 2.1h,i). During larval development, CUL-2 is found in proliferating tissues: P cells during the L1 stage (data not shown); seam cells when they divide at every molt (Fig 2.1p,q); vulval and somatic gonad cells in late L3 and L4 stages (Fig 2.1o); and in intestinal cells throughout larval development (Fig 2.1o).

Isolation of a *cul-2* deletion mutant

To gain insight into *C. elegans* gene function, we screened for a *cul-2* deletion mutant to allow analysis of the loss-of-function phenotype. A single *cul-2* allele, *ek1*, was recovered after screening a 370,000 genome deletion library. The *ek1* mutation is a 1.2 kb deletion that removes exons 2, 3, and 4, as well as the 5' splice site of exon 5 (Fig 2.2a). The mutant allele is completely recessive. From heterozygous parents, *cul-2* homozygous progeny have normal embryonic and postembryonic somatic cell divisions (Fig 2.2b; data not shown), although germ cells and embryos are affected (see below).

G1 arrest of *cul-2* germ cells

Beginning in the L3 stage, *cul-2(ek1)* homozygote mutants have fewer and larger germ cells than wild-type (Fig 2.3a,b). At 61 hrs post-hatch, adult *cul-2* homozygotes have on average 270 germ cells while *cul-2* heterozygotes have 1040 germ cells. *cul-2* germ cell nuclei are two fold larger than wild-type at the L4 stage (54 ± 16 vs. $27\pm6.1 \mu m^3$, n=30, respectively; Fig 2.3a,b).

To determine whether the *cul-2* phenotype of fewer, larger germ cells was due to a G1 arrest or endoreplication, we measured the genomic DNA content of germ cells in *cul-2(ek1)* homozygotes and wild-type. Wild-type germ cells have a bimodal distribution with the majority of cells having either 2n or 4n DNA content corresponding to G1 and G2/M phases, respectively (Fig 2.3c). In contrast, *cul-2* mutants have a single peak at 2n, indicating that *cul-2* germ cells undergo a G1 phase arrest, with cells either severely delayed or blocked in entry into S phase.

C. elegans cki-1/2

The finding that *cul-2* mutant germ cells undergo a G1 arrest, coupled with the insight that the Cullin Cdc53p functions to degrade CKIs, raised the possibility that CUL-2 also functions to degrade CKIs. To address this possibility, we identified two CIP/KIP homologs in the *C. elegans* genome, *cki-1* and *cki-2* (Fig 2.4a-c).

Inactivation of cki-2 by RNAi produced a poorly penetrant embryonic arrest phenotype. Approximately ten percent of cki-2 RNAi embryos arrested development with fewer cells than wild-type at hatch, 242 ± 51 cells (n=13) vs. 558 cells for wild-type at hatch. No larval mutant phenotypes were observed in the cki-2 RNAi animals that hatched. cki-1 RNAi produced a low penetrance of embryos that arrested with hyperplasia (>750 cells/embryo) and a high penetrance of larval hyperplasia of the somatic gonad, vulva, and hypodermis (Fig 2.4d-f; data not shown), indicating that cki-1functions as a negative cell cycle regulator. While our project was underway, Hong et al reported similar cki-1 RNAi results [182].

Post-transcriptional accumulation of CKI-1 in *cul-2* mutants

To determine if the level of CKI-1 was increased in *cul-2* germ cells, we generated anti-CKI-1 antibodies and checked CKI-1 level with immunofluorescence staining. We used embryos homozygous for *mnDf100*, a deletion that removes *cki-1*, to demonstrate that the anti-CKI-1 antibody staining was specific, with anti-CKI-1 staining significantly reduced in *mnDf100* embryos compared to the same age of wild-type (Fig 2.5a-d). In the distal mitotic germ cells of wild-type, CKI-1 level is low. As germ cells enter meiosis, the level of CKI-1 increases and attains the highest intensity in oocyte nucleus (Fig 2.5e). In *cul-2(ek1)* gonad arms the level of CKI-1 is higher, particularly in the distal region (Fig 2.5f). The amount of anti-CKI-1 signal per distal nuclei in *cul-2(ek1)* is 13-fold higher than in wild-type (13 ± 10 , n=50, vs. 1.0 ± 0.9 arbitrary units, n=36).

To distinguish whether the accumulated CKI-1 protein in *cul-2* mutants is due to a higher level of mRNA synthesis or a defect in protein turnover, we checked the *cki-1* mRNA levels in both wild type and *cul-2* mutants. In contrast to the increased CKI-1 protein level in *cul-2* mutants, the *cki-1* mRNA *in situ* signal in *cul-2* gonads was lower than that in wild-type $(1.0\pm0.5, n=8, vs. 2.1\pm0.7 a.u., n=10, respectively)$ (Fig 2.5i-k). The above results suggest that the increased CKI-1 protein levels in *cul-2* gonads are the result of post-transcriptional accumulation. We also produced affinity-purified antibodies to the CKI-2 protein. We did not observe an increase of CKI-2 protein in *cul-2* gonads (data not shown).

Suppression of the *cul-2* G1 arrest by *cki-1* RNAi

If the increased level of CKI-1 was contributing to the G1 arrest, then reducing the level of CKI-1 should suppress the phenotype. To test this, we created the strain *cul-2(ek1)/unc-64(e246)*; *mnDf100 unc-4(e120)/mnc1*, wherein one chromosomal copy of *cki-1/-2* was deleted by the deficiency *mnDf100*. In this strain, germ cell number increased 30% relative to *cul-2(ek1)* (353 ± 25 vs. 278 ± 59 cells, n=11, respectively). This increased germ cell number correlated with a 3.3-fold higher egg production (57 ± 18 vs. 17 ± 13 eggs, n=22), indicating a partial suppression of the *cul-2* germ cell arrest. Introducing T05A6, a cosmid that contains *cki-1* and *cki-2*, into this strain abolished the suppression (15 ± 20 eggs, n=22). To distinguish the role of *cki-1* and *cki-2* in the suppression of *cul-2*, we injected dsRNA for either gene into L4 stage *cul-2* hermaphrodites and then determined the number of eggs produced as adults. *cki-1* RNAi was capable of partially suppressing the *cul-2* arrest (58 ± 21 eggs, n=10). In contrast, *cki-2* RNAi did not suppress the germ cell arrest (15 ± 26 eggs, n=11). Injection of either *cki-1* or *cki-2* dsRNA into wild-type animals did not increase egg number (data not shown).

cul-1 and cul-2 are not functionally redundant

cul-1 is the closest paralog of *cul-2* and both are believed to share a common ancestor with the yeast *CDC53* gene [77]. In contrast to the *cul-2* G1 arrest phenotype, *cul-1* mutants have hyperplasia of blast cell lineages[77]. We observed no increase in the level of CKI-1 in *cul-1* mutants (data not shown). To explore possible redundancy of function, we created a double heterozygous strain with *cul-1(e1756)* and *cul-2(ek1)*. The germ cells of *cul-1/-2* double homozygotes arrest in G1 with a phenotype and CKI-1 level

identical to that of *cul-2(ek1)* (data not shown). The larval somatic blast cells of *cul-1/-2* double homozygotes have hyperplasia, although less severe than in *cul-1* mutants alone (L4 *cul-1/-2* have 53 ± 8 vulval cells, n=11, vs. 82 ± 16 , n=20, for *cul-1*[77]). These observations indicate that there is no significant functional redundancy between the two genes and that the *cul-2* G1 arrest phenotype is epistatic to the *cul-1* hyperplasia phenotype.

Embryonic *cul-2* phenotype

Because of a lack of oocytes and sperm, cul-2(ek1) homozygotes lay only 17 fertilized eggs on average, compared to almost 300 eggs in wild-type. All cul-2 embryos arrest development at an extremely early stage with only 24 ± 4.6 cells, n=20 (Fig 2.6f). Wild type hermaphrodites injected with cul-2 RNAi produce 100% arrested embryos (Fig 2.2b). cul-2 homozygotes from heterozygous parents have normal development while cul-2 RNAi produces 100% arrested embryos in the immediate progeny (Fig 2.2b). This suggests that cul-2 maternal product is sufficient to allow normal embryonic development. The embryonic arrest does not appear to be due to CKI-1 accumulation as the level of CKI-1 in cul-2 embryos is the same as in wild-type embryos (data not shown). CKI-1 protein is maternally provided and does not appear to inhibit the rapid cell cycle divisions of the early embryo (data not shown). It is known that substrates of SCF E3 complexes are targeted for ubiquitination by phosphorylation[128], and it is therefore possible that the maternal CKI-1 is not marked (by phosphorylation) for degradation in the early embryos which lack G1 phase but have only S/M phases. During mitotic embryonic cycles, multiple nuclei of radically different sizes are present in *cul-2* cells (Fig 2.6a,b,h). These multinuclei do not arise from a failure in cytokinesis as is found in other cell cycle mutants, but rather they form immediately after cytokinesis has completed. We observe between one to five nuclei per cell (Fig 2.6a,b). The extra nuclei that do form have nuclear pores, as revealed by immunostaining with the anti-nuclear pore antibody MAb414 (Fig 2.6h), suggesting that nuclear membrane formation around chromosomes may occur normally in *cul-2* mutants. Mitosis is dramatically lengthened in *cul-2* mutants. Much of the increase in mitotic length is due to an increase in the length of prometaphase. The percentage of *cul-2* cells in prometaphase increased 1.9-fold relative to wild-type, from 35% to 65% (n=100).

During *cul-2* embryonic mitotic cycles, DNA bridges are frequently formed between dividing cells (Fig 2.6i,j). Subsequently, following mitosis, chromosomal DNA is not segregated evenly into two daughter cells (Fig 2.6f,g). In addition, multiple centrosomes are present, possibly as a result of failed mitosis (Fig 2.6c,e). We have observed up to 16 centrosomes/cell. The presence of multiple centrosomes creates multivalent mitotic spindles (Fig 2.6c,d). It should be noted that even in cells with two centrosomes, mitosis delay and unequal DNA segregation still occurs (Fig 2.6f,g). (For a more detailed description about mitotic delay and the extensive cytoskeletal movement in *cul-2* mutant embryos, please check Weiwei Zhong's dissertation or our paper published in *Nature Cell Biology*, 1: 486-92, 1999.)

Defect in mitotic chromosome condensation

During prometaphase, sister chromatids are aligned on the metaphase plate by the mitotic spindle. To distinguish if defects in the mitotic spindle were present, we compared the structure of the wild-type mitotic spindle to that of *cul-2(ek1)* embryos by costaining with anti-tubulin antibody and DAPI. At a gross morphological level, the mitotic spindle appears normal in *cul-2* mutants compared to wild type (Fig 2.7a,c). However, the chromosomal DNA in *cul-2* mutants is strikingly non-condensed relative to wild type mitotic chromosomes (Fig 2.7b,d). Further, during anaphase, *cul-2* chromosomes do not segregate cohesively to mitotic poles, and sheared strands of chromosomes and chromosomal fragments apparently contributes to the observed multiple nuclei, some of which have very little DNA (Fig. 2.6g,h). Chromosome condensation defects are also observed in the germline of old *cul-2* adults (4-5 days post-larvae) (data not shown).

To examine the initial state of mitotic DNA condensation, chromosomes were observed at the beginning of the first prometaphase, immediately after fusion of pronuclei in the zygote. Wild-type zygotes had condensed chromosomes while *cul-2(ek1)* zygotes had uncondensed chromosomes, including those that had apparently normal meiosis and entered mitosis with the normal 4n content of genomic DNA (Fig 2.7e,g; data not shown). At the first mitosis for *cul-2* RNAi embryos, chromosomes entered a prolonged state of partial condensation, presumably due to the effect of residual CUL-2 protein remaining after RNAi (Fig 2.7f). Antibody staining revealed that there was on average 2% of CUL-2 protein remaining after RNAi, visible upon longer exposure as faint

nuclear staining $(2.0 \pm 2.3 \text{ a.u.}, n=10, \text{ vs.} \text{ wild type}, 100 \pm 57, n=10)$, whereas *cul-2* mutants had no nuclear staining. In later mitosis, *cul-2* RNAi embryos had chromosomes that were as non-condensed as those in *cul-2(ek1)* embryos (data not shown).

The *cul-2* embryonic phenotype is superficially similar to that of meiosis defective mutants, such as *mei-1*, which also have multiple nuclei [183]. To address whether a failure of meiosis can lead to an inability to condense chromosomes in mitosis, we analyzed chromosome condensation in progeny from homozygous *mei-1(b284)*. We observed that multiple nuclei occurred in *mei-1(b284)* when there was an excess number of chromosomes (generally \geq 30); however, the chromosomes underwent normal condensation (Fig 2.7h). Therefore, a failure of meiosis does not lead to defects in chromosome condensation.

DISCUSSION

We have demonstrated that *C. elegans cul-2* is required in germ cells for the G1-to-S phase transition. The *cul-2* G1 arrest is correlated with increased levels of CKI-1. *cul-2* is also required for mitotic chromosome condensation in early embryos, which lack a G1 phase [184]. *cul-2* is expressed specifically in dividing larval cells. It is currently not known if somatic larval mutant phenotypes are masked by *cul-2* maternal product. Germ cells, which divide substantially more than other tissues, only undergo G1 arrest in later larval stages, suggesting that *cul-2* maternal product is only diluted to non-functional levels after extensive proliferation.

CUL-2 is required for the G1-to-S phase transition by negatively regulating CKI-1 in *C. elegans*

cul-2 has fewer and bigger germ cells with only 2n DNA content, compared to the 2n-4n DNA distribution in wild type. This indicates that *cul-2* germ cells are defective or severely delayed for S phase entry and the initiation of DNA replication. *C. elegans* has two members of the CIP/KIP family of cyclin-dependent kinase inhibitors, *cki-1* and *cki-2*. Overexpression of CKI-1 inhibits cell division [182]. RNAi of *cki-1* but not *cki-2* results in hyperplasia of multiple larval lineages, indicating that only *cki-1* functions as a general negative cell cycle regulator in *C. elegans* (Fig 2.4 d-f) [182]. In *cul-2* germ cells, there is a 13-fold post-transcriptional increase in the level of CKI-1 that is correlated with the G1 arrest phenotype. CKI-2 does not have increased protein levels in *cul-2* mutants. These data suggest that the *cul-2* G1 arrest is due to elevated CKI-1 level. In support of this, the *cul-2* G1 arrest can be suppressed by reducing CKI-1 protein level with RNAi of *cki-1* or the removal of one copy of the *cki-1* gene with a deficiency.

CUL-2 is a member of the cullin gene family. The yeast cullin Cdc53 functions in a SCF^{Cdc4} complex to degrade the CKIs Sic1 and Far1 to promote the G1-to-S phase transition [16] [107]. Parsimony analysis suggests that Cdc53 gave rise to the higher eukaryotic cullins *cul-1* and *cul-2* [77]. *C. elegans* CUL-1 functions as a negative cell cycle regulator. In the absence of CUL-1 function, blast cells undergo excessive cell divisions due to an inability to exit the cell cycle [77]. CUL-1 is therefore likely to act through the degradation of positive cell cycle regulators, such as G1 phase cyclins, but is unlikely to degrade negative cell cycle regulators whose accumulation would inhibit cell cycle progression. Therefore, CUL-2 may function in an E3 complex to directly target CKI-1 for degradation, although an indirect role in the negative regulation of CKI-1 has not been ruled out. Further experiments are needed to confirm if CUL-2 directly targets CKI-1 for degradation and, if so, which SRC (substrate recognition component) in the CUL-2/RING finger complex recognizes CKI-1.

CUL-2 is required for mitotic chromosome condensation in C. elegans

cul-2 mutant embryos arrest at an early stage with multiple nuclei, DNA bridges, and unequal DNA distribution (Fig 2.6a-j). There is no G1 phase in the early embryo cell cycle, which may explain why there is no G1 arrest observed in the embryos. In addition, CKI-1 is expressed only later in embryogenesis (by transcriptional reporter construct) and so is unlikely to be required in the early embryonic divisions [185]. The *cul-2* mitotic embryonic phenotypes, DNA bridges (Fig 2.6i-j), unequal DNA distribution (Fig 2.6f-g), incomplete chromosome segregation (Fig 2.7d), and broken DNA fragments (Fig 2.6i and Fig 2.7d), are extremely similar to yeast condensation mutants, such as *smc2* and *smc4* in *S. cerevisiae* and *cut3* and *cut14* in *S. pombe*. The observation of spread-out chromosomes in *cul-2* mutants (Fig 2.7g) compared to tightly compacted wild-type chromosomes (Fig 2.7h) argues that mitotic chromosomes are indeed not condensed in *cul-2* mutants. The partial chromosome condensation observed in *cul-2* RNAi mitotic embryos (Fig 2.7f) correlates with the 2% residual CUL-2 protein, further indicating that the degree of chromosome condensation depends on sufficient levels of CUL-2 protein.

The observation that mitotic chromosome condensation requires CUL-2, a ubiquitin-ligase component, suggests that ubiquitin-mediated proteolysis is required for this process *in vivo*. Currently it is not known how CUL-2 facilitates chromosome

condensation and which protein is the substrate of the CUL-2 E3 ligase for mitotic chromosome condensation. Are histones CUL-2 targets? The phosphorylation of histone H3 on mitosis specific sites is an early event that is required for proper condensation [186], [187]. In *cul-2* embryos this phosphorylation is still observed, indicating that *cul-2* is not required for this process (data not shown). In addition, the level and distribution of AIR2 (aurora B ortholog in *C. elegans*), a kinase required to phosphorylate histone H3, is normal in *cul-2* (Liu and Kipreos, personal communication). This further demonstrates that histone H3 phosphorylation is not impaired in *cul-2* mutants. However, it is still possible that CUL-2 may regulate condensation through pathways involving other histone modifications, such as H2A and H2B ubiquitination [156].

Currently, we have not obtained any evidence to suggest if CUL-2 influences the activity of topoisomerase II, SMC subunits, or non-SMC subunits although they are all potential CUL-2 targets. In *cul-2* mutant embryos, DNA bridges, unequal DNA segregation, and DNA fragments were frequently observed. Those phenotypes are similar to those seen in topoisomerase II and *smc* mutants [163], [157], [164]. The activity of SMCs is regulated by both phosphorylation of CDC2/cyclin A kinase and the association of non-SMC subunits [161]. Nevertheless, it is unlikely that the phosphorylation of SMC is disrupted in *cul-2* because *cul-2* embryos can progress into mitosis successfully, indicating that the activity of mitosis promoting factor, Cdc2/cyclinA, is not impaired. Disruption of one of the SMC subunits or any non-SMC subunits can impair mitotic chromosome condensation [161]. Therefore, it is still possible that CUL-2 regulates mitotic chromosome condensation through regulating activity or stability of condensin.

Our data suggest a link between the ubiquitin-mediated proteolysis pathway and mitotic chromosome condensation. It would be very interesting to do further investigations to gain insights into the following questions. How does CUL-2 regulate the mitotic chromosome condensation, through proteolysis or by other unknown mechanisms? If CUL-2 does function as an E3 in this process, then who is the SRC(s) and which protein is targeted for degradation? Finally, is this function of CUL-2 conserved in other species?

MATERIALS AND METHODS

C. elegans observation and genetics

Egg number was determined by transferring hermaphrodites to new plates once a day and counting the eggs on the recently vacated plates by removing them with a pick, to avoid over-counting the eggs. Cell number in embryos was determined by fixation of embryos in methanol and acetone (10 min each), staining DNA with propidium iodide, and observing nuclei in serial sections from a confocal microscope. To observe *cul-1*, *cul-2* double homozygotes, we created a strain with *cul-1* and *cul-2* in *cis* balanced by a deficiency located between the genes, *cul-1(e1756) cul-2(ek1)/eDf2*.

RNA interference

RNA was synthesized from linearized plasmids containing the full-length clones of *cul-2*, *cki-1*, and *cki-2* with either the T3 or T7 MegaScript Kit (Ambion) to produce sense and antisense RNA transcripts, respectively. Equal amounts of sense and antisense RNA were annealed to create dsRNA by incubation at 95°C for 5 min and then at 70°C

for 20 min. dsRNA was injected into the distal gonad arms of young wild-type adults at a concentration of 0.5 to 1 mg/ml. For suppression of the cul-2(ek1) phenotype by injection of cki dsRNA, L4 hermaphrodites were injected in their intestines with dsRNA at 0.5 to 1.0 mg/ml.

Isolation of *cul-2* deletion mutant

The deletion screen was based on an outline presented by Carl Johnson (NemaPharm, Inc.) at the 1997 International C. elegans Meeting. Synchronized L4 animals [77] were mutagenized with 50 mM ethylmethanesulfonate for four hrs. Eggs from the mutagenized animals were collected by treatment with hypochlorite. The F1 progeny were seeded at 20 animals per well in a 96 well plate in NGM media supplemented with OP50 bacteria. Ninety six 96-well plates were created to produce a library of 370,000 mutagenized genomes. Half of the F2 progeny were frozen and half were used to make DNA [188]. Two rounds of nested PCR were performed on pooled DNA from each 96-well plate with primer pairs separated by 4.5 kb of genomic sequence. Wells containing deletion bands were identified by PCR of row and column DNA from the 96-well plate. Animals were recovered from the frozen well and cloned. Single worm PCR was performed on animals after they had produced progeny to identify animals harboring the *cul-2* mutation. The mutant allele, ek1, was outcrossed six times and balanced with unc-64(e264). The site of deletion was determined by sequencing PCR products.

Isolation of cki cDNAs

The *cki-1* and *cki-2* genes were identified by homology searches of the *C. elegans* genome database [189]. The 3' sequences of *cki-1* and *cki-2* were obtained by sequencing cDNA clones obtained from the *C. elegans* EST Project, yk490e9 and yk374f4, respectively. The 5' ends of both genes were cloned by PCR from a cDNA library with internal primers and a 5' primer corresponding to the trans-spliced leader SL1 [190]. The presence of SL1 indicates that both clones are full-length. The GenBank accession numbers for the *cki-1* and *cki-2* cDNA sequences are AF179358 and AF179359, respectively. Phylogenetic analysis was performed by aligning CKI proteins with the clustalW sequence alignment program [191]. The most parsimonious tree was obtained with the exhaustive search method using the PAUP parsimony program [192].

Antibody production, immunofluorescence, and *in situ* hybridization

Antisera to full-length CKI-1, CUL-2, and the N-terminal 135 amino acids of CKI-2 were produced in rabbits using bacterially derived His-tag fusion proteins as antigens (pET32a+ vector, Novagen). Fusion proteins were isolated under denaturing conditions using Ni-NTA agarose (Qiagen) according to manufacturer instructions. Sera were precleared of anti-His-tag antibodies by passage over Affigel-10 (BioRad) linked to Histag, and then affinity purified against the antigen linked to Affigel-15, as described [193].

Embryos were obtained by hypochlorite treatment and frozen on poly-lysine coated slides. Gonad arms were dissected from adult hermaphrodites by placing animals in M9 solution on a poly-lysine-coated slide and cutting the animals behind the pharynx so that the extruded gonads attached to the slide. Slides were processed using the "freeze-crack"

method, followed by methanol and acetone fixation in -20°C. We used 45 min methanol and 30 min acetone for anti-CKI-1 and anti-CKI-2 fixation; 20 min methanol and 5 min acetone fixation, then 2 min rehydration in 100%, 90%, 70%, 50%, 30%, and 10% ethanol/PBS at room temperature for monoclonal anti-tubulin N356 (Amersham) and polyclonal anti-AIR1, a centrosome marker [194]; 20 min methanol and 10 min acetone followed by air drying for monoclonal anti-nuclear pore MAb414 (BAbCo) staining. All the slides were blocked with 3% BSA at room temperature for 45 min. Slides were incubated with primary antibody then secondary antibodies at 4° over night or at room temperature for 2 hr as described [195].

The following primary antibodies were also used: monoclonal anti-histone MAB052 (Chemicon International) and polyclonal anti-phospho-histone H3, a mitotic marker [186] (Upstate Biotechnology). The secondary antibodies used were goat anti-mouse conjugated to rhodamine (Cappel) or FITC (Boehringer Mannheim) and goat anti-rabbit rhodamine or FITC (Boehringer Mannheim). Secondary antibodies were precleared with acetone-fixed *C. elegans* powder as described [195].

To determine the percentage of cells in the various mitotic stages, wild-type and *cul-2(ek1)* embryos were co-stained with anti-phosphohistone H3 antibody [186], anti-AIR-1 [194], and DAPI. Only cells with two centrosomes were analyzed.

Antisense and sense digoxigenin-labelled RNA probes were created from fulllength *cul-2* and *cki-1* cDNAs using digoxigenin-11-UTP (Boehringer Mannheim) according to the manufacturer's protocol. Whole animals and dissected gonad arms were frozen immediately beneath a coverslip and processed for *in situ* hybridization as described by Seydoux and Fire [196], except that the hybridization temperature and washes were at 55°C.

Microscopy and signal quantitation

Germ cell DNA levels were quantitated in young adult hermaphrodites that were fixed using the "freeze-crack" method described above. Fixed specimens were incubated with 40 μ g/ml RNAase A in PBS for 1 hr at 37°C, stained with 50 μ g/ml propidium iodide (Sigma) for 30 min at room temperature, and then rinsed with PBS. Specimens were mounted in 1 mg/ml p-phenylenediamine in 90% glycerol and observed with an MRC600 Biorad confocal microscope. Germ cells within five cell diameters from the DTC were serially scanned. The same confocal settings were used for all specimens of a given experiment and set so that no image saturated. A 100x objective was used with a scan depth of 0.5 µm. Quantitation was accomplished with Comos software version 7.0 (Biorad). The signal for a nucleus in a given slice was determined by multiplying the average intensity for the nuclei (minus background intensity) by the area. The total signal for a nucleus was obtained by adding the signal from serial sections. DNA content was normalized to haploid genome equivalents by using the DNA content from somatic cells, other than hypodermis or intestine, as internal 2n standards; mitotic germ cells were used as internal 4n standards. Quantitation of anti-CKI-1 protein levels in gonad arms was accomplished by dissection of *cul-2* and wild-type gonad arms on the same slide and processing for immunofluorescence with polyclonal anti-CKI-1 and monoclonal antinuclear pore antibodies. The anti-nuclear pore antibody was used as control for permeabilization. Quantitation was as above.

Microscope images were captured on TechPan film (Kodak) and digitally scanned with a Nikon LS-2000 scanner. The images were processed with Adobe Photoshop version 4.0. For matched images, e.g., CKI-1 level in *cul-2* and wild-type, the same shutter time (in seconds) was used and the images were processed identically.

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Figure 2.1: cul-2 developmental expression. a, b, In situ hybridization of adult wildtype hermaphrodites with control *cul-2* sense RNA (**a**), or *cul-2* antisense RNA (**b**). **c**, **d**, In situ hybridization of L2 stage larva with cul-2 sense RNA (c), or cul-2 antisense RNA (d). e, DAPI stain of L2 larvae in (d). Bracket denotes the longitudinal extent of the gonad. f, g, In situ hybridization of zygote (f) and 2-fold stage embryo (g) with cul-2 antisense RNA. h, i, Zygote with two pronuclei (h) and 1 1/2-fold stage embryo (i) stained with anti-CUL-2 antibody. j, k, Wild-type oocytes co-stained with anti-CUL-2 antibody (j) and DAPI (k). I, Oocytes from cul-2/cul-2 homozygous hermaphrodites stained with anti-CUL-2 antibody (I). m, n, Oocytes from wild-type hermaphrodites that were injected with cul-2 dsRNA are co-stained with anti-CUL-2 antibody (**n**) and DAPI (m). Proximal oocytes are to the left. o, L4 stage hermaphrodite stained with anti-CUL-2 antibody. V, U, and I denote vulva, uterus, and intestinal nuclei, respectively. p, q, Lateral hypodermis of early L3 stage larva co-stained with anti-CUL-2 antibody (**p**) and DAPI (q). Seam cells (marked) have high levels of CUL-2 at the molt. Anterior is to the left. Scale bars, 10 µm.



Figure 2.2: Inactivation of cul-2 by RNAi or gene deletion. a, cul-2(ek1) deletion mutant. Line drawing of the cul-2 genomic region on chromosome III with exons represented as boxes; introns and flanking regions as horizontal lines. Shaded areas represent coding regions. The cDNA sequence of *cul-2* is from Kipreos et al. (1996) [78], and the genomic sequence is from cosmid ZK520 (GenBank acc. no. Z92822). The region deleted in *cul-2(ek1)* is represented as the missing region bounded by dashed lines and includes exons 2, 3, and 4, as well as the first four base pairs of exon 5. The deletion is inclusive of base pairs 12,166 to 13,393 of cosmid ZK520. Locations of primers used for the deletion screen are presented below the genomic line drawing. b, Skematic drawing to represent *cul-2* phenotypes. *cul-2* double stranded RNA injection (dsRNAi) produces 100% arrested embryos 16 hrs post-injection (left panel). cul-2 homozygotes from a heterozygous parent have normal embryogenesis and normal somatic cell divisions, probably due to maternal cul-2 product (right panel). Only a germ cell phenotype was observed in these homozygotes. The eggs laid by these animals were arrested at the 24-cell stage, indistinguishable from embryos produced by *cul-2* dsRNAi.



a.

b.



Figure 2.3: G1 arrest of *cul-2* **germ cells. a**, **b**, Differential Interference Contrast (DIC) micrographs of the distal gonad arms of wild-type (**a**) and *cul-2(ek1)* (**b**) L4 stage hermaphrodites. The distal tip cell (DTC) is denoted by an arrow. Scale bars, 10 μ m. **c**, Histogram of DNA content of germ cells from young adult *cul-2(ek1)* and wild-type hermaphrodites.









Figure 2.4: cki-1 and cki-2 cloning and RNAi phenotype. a, Location of cki-1 and cki-2 in a nine kb region of cosmid T05A6. Exons are presented as boxes and coding regions are shaded. *cki-1* corresponds to gene T05A6.1 and *cki-2* corresponds to T05A6.2. **b**, The predicted protein sequences from the cki-1 and cki-2 cDNAs. cki-1 and cki-2 share 21 to 27% sequence identity with mammalian $p21^{CIP1}$ and $p27^{KIP1}$ and share 26% identity between themselves. c, Phylogram of CKI proteins from C. elegans (CKI-1 and CKI-2), H. sapiens (p21^{CIP1}, p27^{KIP1}, and p57^{KIP2}), and D. melanogaster (Dacapo) depicting the most parsimonious tree. d, e, Uterine hyperplasia induced by cki-1 RNAi. DIC micrograph of the vulva and uterus of an L4 stage progeny from an uninjected wild-type hermaphrodite (d) and a wild-type hermaphrodite injected with cki-1 dsRNA (e). f, Extra distal tip cells producing extra gonad arms in *cki-1* RNAi progeny. DIC micrograph of anterior gonads of an L4-stage progeny of a wild-type hermaphrodite injected with cki-1 dsRNA. Distal tip cells (DTCs), which direct the migration of the developing gonad arms, are denoted by arrows. This exceptional individual had four gonad arms (three anterior shown) rather than the two gonad arms that are observed in wild-type. Scale bars, 10 µm.


Figure 2.5: Level of CKI-1 protein in *cul-2* mutants. a-d, Specificity of anti-CKI-1 affinity-purified antibody demonstrated by the absence of specific staining in embryos homozygous for *mnDf100*, a deletion that removes *cki-1*. 12 hr old wild-type (\mathbf{a} , \mathbf{b}) and homozygous *mnDf100* (\mathbf{c} , \mathbf{d}) embryos co-stained with affinity-purified anti-CKI-1 antibody (\mathbf{a} , \mathbf{c}) and anti-nuclear pore antibody as a control (\mathbf{b} , \mathbf{d}). We obtained the same staining pattern with anti-CKI-1 antibodies from two different rabbits. \mathbf{e} - \mathbf{h} , Dissected gonad arms from wild-type (\mathbf{e} , \mathbf{g}) and *cul-2(ek1)* (\mathbf{f} , \mathbf{h}) adult hermaphrodites were co-stained with affinity-purified anti-CKI-1 antibody (\mathbf{e} , \mathbf{f}) and DAPI (\mathbf{g} , \mathbf{h}). For wild-type (\mathbf{e}), the mitotic zone at the distal end is bracketed. \mathbf{i} , \mathbf{j} , *in situ* hybridization with digoxigenin-labelled *cki-1* antisense probe of wild-type (\mathbf{i}) and *cul-2* (\mathbf{j}) adult distal gonads. \mathbf{k} , Control *in situ* hybridization with *cki-1* sense probe of wild-type adult distal gonad. Scale bars, 10 µm.



Figure 2.6: *cul-2* mutant embryonic phenotype **a**, DIC micrograph of *cul-2(ek1)* 2-cell stage embryo. Black and white arrows indicate cytoplasmic extensions of leftward and rightward cells, respectively. Small arrowheads indicate multiple nuclei. **b**, DIC micrograph of embryo from wild-type parent injected with *cul-2* dsRNA. **c-e**, Extra centrosomes, resulting from failed mitoses, are present in 59% (n=44) of *cul-2* cells. *cul-2(ek1)* embryo observed with DIC (**c**), and co-stained with anti-tubulin antibody (**d**) and anti-AIR-1 antibody (**e**), which localizes to centrosomes [159]. The two leftward cells each had four centrosomes, three of which are visible for each cell in this focal plane. **f-h**, Embryonic arrest phenotype of *cul-2(ek1)*. *cul-2(ek1)* embryo 12 hours after being laid was fixed, observed with DIC (**f**), and co-stained with 1 μ g/ml DAPI to detect DNA (**g**) and anti-nuclear pore antibody (**h**). Note the unequal DNA distribution among nuclei. **i-j**, DNA bridge in *cul-2(ek1)* mutant. Fixed *cul-2(ek1)* embryo stained with DAPI (**i**) and observed by DIC (**j**). DNA bridge in (**i**) is marked by large arrowhead. Scale bars, 10 µm.



Figure 2.7: Chromosome condensation is impaired in *cul-2* mutant embryos. a-d, Anaphase in *cul-2* and wild-type two-cell stage embryo. Wild-type (**a**, **b**) and *cul-2(ek1*) (\mathbf{c}, \mathbf{d}) two-cell stage embryos were fixed and co-stained with anti-tubulin antibody (\mathbf{a}, \mathbf{c}) and DAPI (**b**, **d**). In wild-type (**a**, **b**), the leftward cell, AB, is in anaphase while the rightward cell, P₁, is in prophase. In cul-2(ek1) (c, d), the leftward cell is in anaphase, while the rightward cell is in prometaphase. Arrowhead in (d) indicates chromosomal fragment that is not co-segregated. e, f, g, h, Genomic DNA from wild-type (e), cul-2(ek1) (g), and mei-1(b284) (h) zygotes that were fixed immediately upon fusion of maternal and paternal pronuclei and then stained with propidium iodide (PI) to visualize DNA. A projection of the three-dimensional confocal serial sections were collapsed onto two dimensions. Note that several of the 30 mei-1(b284) chromosomes overlap due to projection of the three-dimensional sections onto two dimensions. (f) Confocal projection of PI-stained genomic DNA of *cul-2* RNAi zygote from a wild-type parent that was fixed five minutes after fusion of maternal and paternal pronuclei. Note that wild-type embryos complete mitosis within five minutes. The projections in (e-h) are representative of 23, 17, 8, and 10 pronuclear fusions followed, respectively. Scale bars, 10 µm.



CHAPTER III

THE IDENTIFICATION OF C. ELEGANS CUL-2 COMPLEXES

BACKGROUND

Members of the cullin family have been demonstrated to form ubiquitin ligases to target specific substrates, such as cell cycle regulators and transcription regulators, for ubiquitin-mediated degradation by the proteasome [71], [122]. Both Cdc53 and CUL1 form multiple ubiquitin ligase complexes, SCF cullin/RING finger complexes, through association with different SRCs that to date have been members of the F-box protein family [83]. The previous chapter described the characterization of CUL-2 functions in *C. elegans*. Yet the complexes by which CUL-2 functions to regulate aspects of the cell cycle. are not known Identification of CUL-2 interacting proteins will provide insight into the functional mechanisms this cullin undertakes, which is likely to involve functions in multiple ubiquitin ligase complexes.

Multiple CUL2/RING finger complexes

In humans, CUL2 has been observed to form at least three ubiquitin ligase complexes, CUL2/VCB complex, CUL2/mMED8-CB complex, and CUL2/SOCS1-CB complex [92], [94]. The first CUL2/RING finger complex identified was the human CUL2/VCB complex that employs the von Hippel-Lindau tumor suppressor protein (VHL) as the SRC [102], [111], [73]. CUL2/RING finger complexes are structurally

similar to the SCF cullin/RING finger complexes. They contain a cullin, the same Rbx1 protein, Elongin C (a SKP1-like protein), and a SRC subunit (member of BC-box protein family). SKP1 in the core SCF complex can bind different F-box proteins (SRCs), thus allowing the formation of different SCF complexes. Similar to SKP1, Elongin C can also bind multiple proteins containing a BC-box instead of a F-box, such as the Suppressors of Cytokine Signaling, to form stable complexes [96], [97]. The large pool of BC-box proteins that Elongin C binds indicates the existence of multiple CUL2/RING finger complexes.

Human CUL2/VCB complex

1. The von Hippel-Lindau tumor suppressor (VHL)

VHL is a tumor suppressor gene. Germline mutations of the *VHL* gene are responsible for inherited VHL disease, which is characterized by a predisposition to develop tumors of the central nervous system, retina, pancreas, adrenal gland, and kidney [198]. Further, *VHL* is inactivated in ~80% of sporadic clear-cell renal carcinoma (RCC), which is the most common form of kidney cancer [199], [200], [201], [202]. The reintroduction of wild-type *VHL* into *VHL* (-/-) clear cell RCC cell lines suppresses the ability of RCC cells to form tumors in a nude mouse xenograft assay [198]. Therefore, VHL is believed to play a "gate-keeper" role with respect to the development of RCC.

The tumors caused by inactivation of VHL are highly vascular. These tumors produce high levels of vascular endothelial growth factor (VEGF) and platelet-derived growth factor- β (PDGF) [203]. VEGF and PDGF mRNA are normally induced under hypoxic condition, but in VHL tumors they are present at high levels under both hypoxic

and normoxic conditions [203]. Re-expression of wild type VHL down regulates hypoxia induced mRNA and this regulation is post-transcriptional [203], [204].

2. Human CUL2/VCB complex possesses ubiquitin ligase activity

The discovery that VHL is a component of a ubiquitin ligase provided important insights into how VHL down regulates hypoxia inducible mRNA. VHL was first found to form a trimeric complex with Elongin B and C by the Klausner lab [205]. Elongin B and C are components of RNA polymerase II elongation complex SIII [206]. Both proteins bind the transcription elongation factor Elongin A to form a stable and active SIII complex [206]. Because of this initial observation, VHL had long been suspected of having a role in transcription inhibition by sequestering Elongin B and C from Elongin A; this view was predominant until the finding that CUL2 was another component in the VCB complex [102]. The binding of CUL2 to VHL strictly depends on the integrity of the stable VCB complex. About 70% of naturally occurring VHL mutations disrupt the interaction between VHL and Elongin BC, which subsequently disrupts the interaction between CUL2 and VHL [198]. The interaction domain was mapped to a 10 amino acid region, which is conserved between VHL and Elongin A and was later named the BCbox motif [205].

The CUL2/VCB complex was suspected of functioning as an E3 ubiquitin ligase because of its structural similarity to SCF complexes. The evidence supporting this hypothesis came from the finding that the interaction between VHL and CUL2 is essential for negatively regulating the levels of the α subunits of hypoxia-inducible factor-1 (HIF1) by ubiquitin-mediated proteolysis under normoxic condition [111], [207].

The CUL2/VCB complex was subsequently shown to function as an E3 *in vitro* by two groups [208], [74]. This ubiquitin ligase activity is lost in tumor-derived VHL mutants including ones containing missense mutations that don't disrupt the CUL2/VHL interaction, such as Y98 mutation [209], [210]. Instead of using Cdc34 as its E2, the CUL2/VCB complex works together with E2s UBC5Ha, b, and c [74]. A *D. melanogaster* CUL2/VCB complex was found to contain E3 ubiquitin activity similar to the human complex [211].

HIF is a transcription complex, which controls the transcription of multiple genes in response to hypoxia. The CUL2/VCB complex is required for the degradation of HIF subunits, HIF1 α and HIF2 α , under normal oxygen conditions [112], [212], [76]. Both HIF1 α and HIF2 α contain an extensive transferable oxygen-dependent degradation domain (ODDD) [207]. The binding of α subunits of HIF1 to VHL relies on oxygen-dependent posttranscriptional hydroxylation on specific prolyl residues within this ODDD domain [213], [214], [215]. The enzyme dioxygenase, which is required for hydroxyl proline modification was first identified as EGL-9 in *C. elegans* [216]. This enzyme family is conserved among species. Besides HIF1 α and HIF2 α , VHL was also shown to target another substrate, activated atypical protein kinase C, for ubiquitin-mediated proteolysis through the CUL2/VCB ubiquitin ligase [217]. Although the identified substrates can explain some of the VHL tumor phenotypes, such as angiogenesis, due to a failure to degrade HIF1 α and HIF2 α , the mechanism of VHL-mediated tumor suppression still remains unclear.

3. The crystal structure reveals the mechanism of action

The crystal structure of the VCB complex provides an overview of the protein interaction within the complex [218]. According to the crystal structure, Elongin C binds Elongin B and VHL across two distinct interfaces, whereas VHL and Elongin B don't interact. VHL has two domains, an N-terminal β -domain that binds substrates HIF1 α and HIF2 α and a C-terminal α -domain that mainly binds Elongin C [212], [218]. Upon hydroxylation of proline 564, HIF1 α binds a VHL hydrophobic pocket through the critical contact of hydroxyproline and an extended β strand-like conformation [91], [90]. VHL mutations map to β - and α - domains, with about half on each. This indicates that both macromolecule binding domains are required for VHL tumor suppression. Elongin C binds VHL through helices and loops at its C-terminus [218]. The Elongin B-C binding is through an intermolecular interface dominated by the juxtaposition of two β sheets [218].

The crystal structure study also helps us to understand the function of the CUL2/VCB complex. The H1 helix of VHL α -domain defines one of the most important VHL-Elongin C interacting interfaces, which is almost completely hydrophobic. VHL mutations are found within 10 amino-acids of the H1 helix, which form the so-called BC-box motif [218]. Interestingly, the whole VHL α -domain is structurally similar to the SOCS box (a 40-amino acid degenerate sequence). Also, the overall hydrophobic pattern in the VHL α -domain matches that in the F-box motif as well [218]. In addition, Elongin C and Skp1 share 30% identity at the N-terminus, and the hydrophobic concave surface in the Elongin C-VHL binding region is maintained in Skp1 as well [218]. The structural similarity to the SCF complex provides further support for the ubiquitin ligase function of

CUL2/VCB complex. It also indicates that, like Skp1, Elongin C may bind multiple BCbox/SOCS box proteins to form multiple ubiquitin ligases with CUL2.

Human CUL2/mMED8-CB complex

1. Mediator complexes

Mediators are a class of proteins that form complexes and load onto the promoter region to mediate the interaction of transcription factors with RNA polymerase II [219]. Mediators were first identified in *S. cerevisiae*. Most Mediator proteins are needed for cell viability [219]. Biochemically, they are shown to form multiple protein complexes of more than 20 members that are required for transcriptional activation *in vitro* and *in vivo*. The yeast Mediator complex contains the products of the *srb2*, *srb4*, *srb5*, *srb6*, *srb7*, *srb8*, *srb9*, *srb10/Ssn3*, *Srb11/Ssn8*, *Med1*, *Med2*, *MEd4*, *Med6*, *Med7*, *Med8*, *Med11*, *Rox3*, *Cse2*, *Nut1*, *Nut2/Med10*, *Pgd1/Hrs1*, *Gal11*, *Rgr1*, and *Sin4 genes* [220]. This class of protein complex is conserved from yeast to man [219]. The mammalian Mediator subunits have been identified. mMED8 is a component of the mammalian Mediator complex.

The crystal structure of purified yeast MED/POL II holoenzyme complex revealed three major Mediator domains, head (h), middle (m), and tail (t), that wrap around the globular polymerase [219]. In contrast, the Mediator complex alone conforms to a different compact structure. This indicates that Mediator complex must undergo an extensive conformational change to be able to wrap around POL II. The MED8 subunit is in the head domain and partners with eight other subunits. The head domain is thought to act mainly as a signal processor that directly modulates POL II activity.

2. Identification of a human CUL2/mMED8-CB complex

Recently, the MED8 subunit was found to form a ubiquitin ligase with CUL2, Elongin B, C, and Rbx1 in humans [94]. This Mediator subunit contains a BC-box motif, and mutating the conserved leucine and cysteine in this BC-box causes mMED8 to lose the ability to interact with the Elongin BC complex. This suggests that mMED8 interacts with Elongin BC through its BC-box. mMED8 can also physically interact with CUL2 and RBX1 [94]. In addition, this CUL2/mMED8-CB complex was co-fractioned with other Mediator subunits, indicating the *in vivo* existence of the complex and the association of this complex with other Mediator subunits [94]. The isolated complex had ubiquitin ligase activity. However, it is not known which substrates this CUL2/mMED8-CB complex targets. A hypothesis was proposed that the Mediator subunit may bring the ubiquitin ligase into the promoter region, thereby allowing the targeting of other Mediator subunits or transcription factors for ubiquitination to regulate transcription. The discovery that CUL2 forms a ubiquitin ligase with a BC-box containing protein, mMED8, also confirms the older hypothesis that BC-box proteins other than VHL would bind to Elongin BC to form ubiquitin ligases with CUL2.

Human CUL2/SOCS1-CB complex

1. SOCS box proteins

SOCS box proteins were initially identified as suppressors of cytokine signaling. In humans, the SOCS box family contains eight members, cytokine-inducible SH2containing protein (CIS) and SOCS1-7, all of which harbor two protein interacting domains: an SH2 domain at the N-terminus and a SOCS box at the C-terminus [95]. Upon cytokine stimulation, SOCS1-3 and CIS are involved in suppressing the cytokine signaling pathway by binding and inhibiting the activity of Janus kinase (JAK), which functions in the signaling pathway to activate STAT proteins that upregulate the transcription of multiple genes including SOCS members [221]. Besides the canonical SOCS proteins, additional proteins containing C-terminal SOCS boxes were identified by homology search. To date, more than 40 such proteins are identified in nine different families conserved among species [95]. Instead of a SH2 domain at the N-terminus, these proteins contain different motifs, including WD40-repeat in WSB proteins, Ankyrin-repeat in ASB proteins, SPRY-domain in SSB proteins, and GTPase-domain in RAR-like proteins [221]. The functions of most SOCS-box proteins are not known [222].

The finding that multiple SOCS box proteins are capable of binding the Elongin BC complex sheds light on their possible functions in ubiquitination. Two groups initially demonstrated that Elongin BC complex interacts with multiple SOCS box proteins, such as SOCS1, ASB2, WSB1, and Rar1, *in vitro* and *in vivo* [96], [97]. The interaction is dependent on the presence of the BC-box that is a subdomain of the SOCS box. Mutating the BC-box abolishes the SOCS box protein-Elongin BC interaction [96].

2. A potential human CUL2/SOCS1-CB complex

The first indication for the existence of a CUL2/SOCS1-CB complex was from the study of SOCS1 function. SOCS1 was found to inhibit the kinase activity of JAK type 2. Elongin BC binding to SOCS1 can enhance the inhibition of JAK2 activity [96]. With a translocation-derived chimeric JAK2, TEL-JAK2 which constitutively activates the tyrosine kinase activity, the SOCS1 inhibition is evident upon overexpression of both SOCS1 and TEL-JAK2 [92]. Kamizono et al [92] provided evidence that this inhibition depended on SOCS box-mediated proteasomal degradation of TEL-JAK2 upon phosphorylation. They also showed that SOCS1 can physically interact with CUL2 and that a dominant negative CUL2 mutant can suppress SOCS1-dependent TEL-JAK2 degradation. Therefore, it is possible that SOCS1 associates with CUL2, Elongin C, and B to form a ubiquitin ligase to target JAK2 for ubiquitin-mediated proteolysis.

3. Other Cullin/BC-box protein complexes

Over 40 vertebrate proteins contain a SOCS box, which includes a BC-box [95]. This finding indicates that it is very possible that all these SOCS box proteins and other BC-box proteins can form ubiquitin ligases with members of cullin family including CUL2. Indeed, vertebrate CUL5 was also found to complex with Elongin BC [93]. MUF1, a SOCS box protein, can associate with CUL5, Elongin B, C, and Rbx1 to assemble a ubiquitin ligase. In addition, several other SOCS box proteins, Elongin A, WSB1, and SOCS1, were found to assemble into possible ubiquitin ligases with CUL5 in a manner similar to MUF1 [93]. Therefore, it would not be surprising to discover that different BC-box proteins partner with CUL-2 to form multiple ubiquitin ligases in *C. elegans* or other species.

Ubiquitin and transcription

The abundance of cellular proteins is determined at two levels, degradation through proteolysis and synthesis through transcription and translation. Transcriptional regulation includes the control of both chromatin structure and initiation by RNA Polymerase II. The initiation of gene transcription in eukaryotic cells involves the loading of RNA Polymerase II, general transcription factors (GTFs), and other transcription factors, such as activators, co-activators, and repressors, to promoter regions [223]. Recently, emerging evidence, summarized below, indicates the involvement of ubiquitin, a critical player in the ubiquitin-mediated proteolysis pathway, in transcription control [59]. The functional role of ubiquitin in transcription unites the two major regulatory pathways for determining protein abundance, proteolysis and gene transcription control.

Ubiquitin ligases target transcription factors for degradation

One way to control gene transcription through ubiquitin is to target specific transcription factors for proteolysis by various ubiquitin ligases. For instance, HIF transcription factor subunits, HIF1 α and HIF2 α , are degraded by the CUL2/VCB complex [112]. Multiple SCF Cullin/RING finger complexes also control the stability of transcription factors: Met4 by SCF^{Met30}; Gcn4 by SCF^{Cdc4}; and IKB, β -catenin, ATF4, and Smad3 by SCF^{β -TRCP} [224], [225], [226], [59]. In addition to these gene-specific transcription factors, the RNA Polymerase II large subunit is also regulated by ubiquitin-mediated proteolysis [59]. However, so far, there is no evidence that GTFs are targeted for proteolysis by ubiquitin ligases. In addition, there is no direct evidence to show that ubiquitin ligases are recruited to the promoter region to degrade transcription factors. The identification of CUL2/mMED8-CB ubiquitin ligase provides an indication that one or more Mediator subunits may function to bring ubiquitin ligases to promoter regions. Two subunits of Mediator complex, Srb10 and Srb11, can assemble into a CDK/cyclin

complex, with Srb10 as a CDK and Srb11 as a cyclin [219]. This raises the possibility that some transcription factors are phosphorylated and hence marked for ubiquitination by an E3 at the promoter region.

Regulation of transcription by ubiquitin modification

The adding of ubiquitin to transcription factors not only functions to mark the ubiquitinated transcription factors for proteolysis, it can also serve as a modifier of transcriptional activity [59]. This modification can either activate or repress transcription factors to regulate multiple cellular processes. In addition to transcription factors, histones can also be ubiquitinated [227]. The modification of histones can change the chromatin structure, which then regulates gene transcription in general [228]. Histone H1, H2A, H2B, and even H3 were found to be modified by either mono-ubiquitin or polyubiquitin [227]. The ubiquitination of histones requires a E1 and a E2. Yeast employs the same E1 for the ubiquitin-mediated proteolysis pathway to activate ubiquitin for histone modification [227]. Ubiquitin is subsequently transferred to a E2, such as Rad6/Ubc2 and Cdc34/Ubc3 in yeast, which directly transfers ubiquitin to histones without the need for the presence of E3s [227].

Recruitment of proteasomal subunits to promoter region

The finding that proteasome subunits localize in the promoter region intensifies the drama of ubiquitin transcription control. Several AAA ATPases that function within the 19S regulatory particle are recruited to promoters by the transcriptional activator Gal4 [229]. These 19S subunits were demonstrated to regulate transcription. For example, the subunit Sug1 is required for gene transcription activation [230]. Currently it is not known how the gene transcription activation is achieved by 19S proteasomal subunits. One possibility is that these proteasomal subunits recognize ubiquitinated transcriptional repressors within the promoter region and facilitate their folding, thus promoting their degradation by 20S core particle.

C. elegans CUL-2 functions

C. elegans CUL-2 has multiple functions. In addition to the functions described in the previous chapter, another three functions have also been characterized by Weiwei Zhong and Ji Liu in our lab [197], (Liu et al., personal communication). Taken together, five aspects of *C. elegans* CUL-2 function have been described. First, CUL-2 is required to negatively regulate the levels of the inhibitory subunit of CDK, CKI-1, and a failure to do so contributes to a G1 phase cell cycle arrest. Second, CUL-2 is required for mitotic chromosome condensation, and in *cul-2* mutants chromosomes fail to condense. Third, in the absence of CUL-2, mitotic progression is greatly delayed, indicating an additional mitotic function for CUL-2. Fourth, CUL-2 is required for proper cytoskeletal movements. And finally, *cul-2* mutants are defective in meiotic anaphase II progression.

We hypothesize that *C. elegans* CUL-2 forms multiple CUL-2/RING finger complexes that contain different SRCs, possibly BC-box proteins. The goal for the research described in this Chapter is to test this hypothesis, to identify such SRCs if they exist, and to answer the following questions. Is the human CUL2/VCB complex conserved in *C. elegans*? Is VHL responsible for any of the *C. elegans* CUL-2 functions? If not, what other SRC is? Both genetic and molecular approaches were employed to

uncover CUL-2 complexes in *C. elegans*: homology searches and RNAi phenotypic screening to define potential CUL-2 complex components, and affinity purification and mass spectrometry (MS) to identify CUL-2 interactors.

RESULTS

Components of human CUL2/VCB complex are conserved in *C. elegans*

Using protein sequence from human, mouse, yeast, and/or fly, BLAST searches were performed to identify components of human CUL2/VCB complex in *C. elegans*. The orthologs of all five human CUL2/VCB components, CUL-2, Elongin C, Elongin B, RBX-1, and VHL, were identified (Fig 3.1a; fig 3.2a; table 3.1). Interestingly, there are two Elongin C homologs, ELC-1 and ELC-2, in *C. elegans* (Fig 3.1a,b). Phylogenetic analysis suggests that ELC-1 is much more similar to human and *Drosophila* Elongin C, compared to ELC-2, which groups together with *S. cerevisiae* Elongin C (Fig 3.1b). In addition, we also identified the orthologs of human UBCH5 (an E2 for human CUL2/VCB E3 ubiquitin ligase) and HIF1a and HIF2a (substrates of human CUL2/VCB complex) (Table 3.1). The identities between different human counterparts and *C. elegans* ortholog proteins were compared, ranging from 6.9% to 94.6% (Fig 3.1a; fig 3.2a; table 3.1). Among them, UBC-5 and RBX-1 are most similar to their human counterparts.

RNAi of components of CUL-2/VCB complex other than vhl phenocopies cul-2

By RNA-mediated gene inactivation (RNAi) technique in *C. elegans* [124], the functions of the orthologs of the human CUL2/VCB components were checked. *elc-2*

RNAi produces normal progeny (data not shown). Both *elc-1* and *elb-1* RNAi generate embryos and larva which completely phenocope *cul-2* mutants although some of the meiotic *cul-2* pheotypes are not apparent for *elb-1* RNAi (Fig 3.3a,d,i,k,g,h,j; data not shown). Over 60% of *elc-1*(RNAi) embryos and over 30% of *elb-1*(RNAi) embryos are arrested. In these embryos, all of the *cul-2* embryonic phenotypes, multiple nuclei, chromosome uncondensation, chromosome bridges, unequal DNA segregation, delayed mitoses, extensive cytoskeleton movement, meiosis II defect, and polarity defects, were observed (Fig. 3.3a,d,k,g,h; data not shown) (Liu et al., personal communication). In addition, the G1 arrest phenotypes of *cul-2* mutants, big and few germ cells were observed in *elc-1*(RNAi) or *elb-1*(RNAi) F1 progeny that escaped the embryonic arrest and developed into adults (Fig 3.3i,k,j). These adults appear to be Unc (worms that are uncoordinated and can't move), which is the only phenotype different from *cul-2* observed to date.

rbx-1 RNAi produces 100% arrested embryos which completely phenocopies the *cul-2* embryonic phenotypes (Fig 3.3b,e). RNAi of *ubc2*, which is the ortholog of human UBC5H, causes two phenotypes. Some embryos are arrested at an early stage with multiple nuclei (Fig 3.3c,f), which is similar to some of the *cul-2* embryonic phenotype. However, the extensive cytoskeleton movement is not obvious in these embryos. In addition, *ubc-2* RNAi also manifests a more severe one cell stage arrest phenotype in over 30% percent of the animals, suggesting *cul-2* independent functions (data not shown). The *ubc-2*(RNAi) F1 larva that hatch arrest at the L2 stage with large hypodermal cells (data not shown). This further suggests that *C. elegans* UBC-5 may not only function with CUL-2 but probably also with other ubiquitin ligases. Inactivation of

the *C. elegans UBC3* ortholog, *Cdc-34*, which is an E2 for SCF complexes in yeast and vertebrates, generates wild type progeny (data not shown).

If the CUL-2/VCB complex is responsible for CUL-2 cell cycle functions, partial or complete *cul-2* phenotypes would be expected by knocking out the SRC of this complex, the VHL ortholog. However, when we analyzed the *C. elegans vhl* ortholog, *F08G12.4*, either inactivated by RNAi or by deletion (the mutant allele *ok161* was obtained from the Robert Barstead laboratory), there was no phenocopy of *cul-2*. Animals homozygous for the mutant allele *ok161*, in which the exon 1 and exon 2 of *vhl* are removed by deletion, appears to be wild type (Fig 3.2b).

FLAG-tagged CUL-2 complex purification

To isolate CUL-2 interacting proteins, especially SRCs, another approach was employed to affinity purify the FLAG-tagged CUL-2 associating proteins. The *cul-*2/unc64 heterozygous mutants were transformed by *cul-2-FLAG* construct, *PFCA* or *PCFA*, with FLAG either inserted in the N-terminus or C-terminus of CUL-2. Both *cul-2-FLAG* constructs can rescue *cul-2* mutants. Compared to non-transgenic *cul-2* homozygous mutants, the number of embryos produced by *PCFA* or *PFCA* transgenic mutants increased 10-20 fold (123 compared to 17 on average; n=7). *cul-2(ek1)* homozygous embryos arrest with about 24 cells, however the cell number increases in transgenic mutant embryos with an average over 100 cells (data not shown). Some embryos from *PCFA* but not *PFCA* transformation can even hatch and develop to the adult stage. These embryos exhibit good CUL-2-FLAG protein expression, as determined by immunofluorescence staining with anti-FLAG antibody (data not shown). However, none of these transgenic *cul-2* mutants can produce viable progeny to maintain the strain. This may be due to the dramatically decrease of the CUL-2-FLAG protein level because the expression of CUL-2-FLAG drops as transgenic generations increase. The germ cells recognize the multiple repeat DNA sequences then shut down their transcription [231].

Because the *PCFA* rescues *cul-2* better, the affinity purification was performed with the *PCFA* transgenic *cul-2/unc64* worms, with the non-transgenic *cul-2/unc64* strain as a control. CUL-2-FLAG and control eluates were analyzed by SDS PAGE (Fig 3.4a). Compared to control, CUL-2-FLAG eluate has 13 extra bands at the following size: 191, 188, 148, 90, 85, 76, 66, 39, 29, 15.5, 15.0, 14.5, and 14.2 kDa. These are potential CUL-2 associating proteins. The 85 and 90kDa protein bands were confirmed to be CUL-2-FLAG by western blot (Fig 3.4b). The following protein bands, 191, 148, 90, 85, 76, 39, 29, 15.5, and 14.5kDa, are reproducible in different purifications (Fig 3.4a and Fig 3.5). In addition, similar results were obtained from different *PCFA* transgenic lines (data not shown). Therefore, these protein bands are potentially real CUL-2 interactors.

MS Identification of CUL-2 interactors separated by 1-D gel

When separated on a 1-D gel and compared to the control eluate, some protein bands are clearly present only in the CUL-2-FLAG eluate (Fig. 3.4a and Fig 3.5). These protein bands and the corresponding control bands were cut out, trypsinized, and analyzed by mass spectrometry. Both the 85kDa and 90kDa proteins were identified as CUL-2-FLAG, consistent with the western result (Fig 3.4b and Fig 3.5). Additionally, the following five proteins were identified: a 148kDa protein *C. elegans* TIP120; a 110kDa protein APT-5, which is the *C. elegans* Adaptin δ subunit; a 50 kDa protein band as a mixture of EF-1 α (Elongation factor-1 α ; 50.6kDa) and a HMT ortholog (histone methyltransferase; 44.8kDa); and a 12kDa protein that is histone H4 (Fig 3.5 and Table 3.2). Among these proteins, three contain BC-box motifs: TIP120, APT-5, and HMT. For the 110kDa protein band, in addition to APT-5, a second protein, VIT-6, was identified by MS (Table 3.2). The molecular weight of VIT-6 (193kDa) doesn't correspond to the protein size (around 110kDa). Later, a 190 kDa protein was identified as VIT-6 (data not shown). However, the same peptides were also present in the control sample, indicating that VIT-6 non-specifically binds beads and is not a CUL-2 interactor. It also suggests that a degradation product of VIT-6 is present in the 110kDa band.

MS identification of CUL-2 interactors separated by 2-D gel

Most proteins with relatively low molecular weight in the CUL-2-FLAG eluate were not well-separated. Similar sized protein bands appear to be present in both the CUL-2-FLAG and control gels, although the intensity of certain control bands is low compared to that in the CUL-2-FLAG eluate. This is potentially due to multiple proteins with the same or similar molecular weight co-migrating. To identify the specific proteins from a 1-D gel can be frustrating and inefficient as data analysis for a mixture of tryptic peptides from several proteins is difficult. Two dimensional gel electrophoresis (2-D) was used to resolve the proteins. Proteins were separated according to their pI in the first dimension then according to their molecular weight in the second dimension. On the 2D gels there were at least fourteen protein spots that were unique to the CUL-2-FLAG eluate (Fig 3.6). These 14 spots plus a control blank spot (Spot 15) were picked, trypsinized in gel, and analyzed by mass spectrometry. An additional four proteins were identified through this method (Table 3.2).

Among the total of 14 spots, three spots, 5, 6, and 14, which ran next to each other on the 2-D gel (Fig 3.6), were identified as the same protein, *C. elegans* transcription activator Pur α -like protein 1 (PLP-1) (Fig 3.6; table 3.2). The second protein identified was spot 1 as CLP-7 (cysteine endopeptidase) (Fig 3.6; table 3.2). In addition, spot 2 was identified as RFA (replication factor A) (Fig 3.6; table 3.2). Finally, both MS and MS/MS, spot 13 was identified and confirmed as VHL (Fig 3.6; table 3.2). Interestingly, the MS search picked up ELC-1 but not ELC-2 as top candidate for spot 10 (a molecular weight 10-15kDa and a pI 3.0-5.0) although the score is too low to be sure of the identification of the protein (Fig 3.6; data not shown). The MS/MS for spot 10 was not successful. Nevertheless, the molecular weight and pI of ELC-1 (13.8kDa and pI 4.8) correspond to those of spot 10 (Fig 3.6). Therefore, it is likely that spot 10 is indeed ELC-1.

From a different 2-D preparation, APC-11 was identified by MS/MS as a CUL-2 interactor (Table 3.2; data not shown).

RNAi characterization of potential CUL-2 interactors

To gain insight into the functions of the potential CUL-2 interactors, RNAi was performed. RNAi of *tip120* produces various phenotypes. Five phenotypes are apparent in F1 progeny: arrested embryos with multiple nuclei (Fig 3.7d), developmental arrest as larva (data not shown), abnormal tail shape (Fig 3.7e), slow development, and everted vulva (Fig 3.7f). Most of the F1 progeny develop slower than wild type; they usually

reach L1-L2 stage when the wild type progeny become adults already. When they finally become adults four or five days later, eversion of the vulva is observed. The abnormal tails exist in F1 progeny, which either develop more slowly than normal or arrest at L1-L2 stage. RNAi of another gene, *ef-1* α , also produces multiple phenotypes. In the injected hermaphrodites, germ cells becomes large and few 2-3 days post injection (Fig 3.7a). While the control wild type are still making a lot of eggs, these hermaphrodites cease to produce eggs. The few F1 progeny from these injected hermaphrodites either are arrested as embryos with multiple nuclei and uncondensed DNA or as L1-L2 stage larva (data not shown). Most *apt-5*(RNAi) progeny appear normal, but there are a small number of progeny that arrest at the L1 and L2 stages (Fig 3.7b). These larvae have a dumpy (Dpy) phenotype. The *C. elegans med*8 ortholog, *Y62F5A.1*, is also essential for embryogenesis since RNAi of gene *Y62F5A.1* causes 100% embryonic arrest (Fig 3.7c). Finally, RNAi of HMT gene, *K12H6.11*, didn't generate any obvious phenotypes (data not shown).

Phenotypic screening for potential SRCs of CUL-2 ubiquitin ligases

Proteins containing a BC-box motif have been found to interact with Elongin BC to form ubiquitin ligases [94], [93], [74], [96], [97]. The finding that *elc-1* or *elb-1* RNAi phenocopies *cul-2* mutants suggests that *C. elegans* Elongin B and C function with CUL-2, potentially by forming multiple CUL-2/RING finger ubiquitin ligases. Therefore, SRCs for CUL-2 ubiquitin ligases are expected to be BC-box proteins, which would interact with Elongin BC. We performed a bioinformatics search of the C. elegans genome and found 1058 proteins that contained the BC-box motif,

(A,P,S,T)LxxxCxxx(A,I,L,V). We then searched for secondary motifs in these proteins using RGS-BLAST [159]. We analyzed the secondary motifs and were particularly interested in proteins containing a secondary protein interaction domain because a potential SRC has to have at least two protein interaction domains: one to bind to the substrate and another to interact with Elongin BC. We only chose 31 genes encoding BCbox proteins for analysis, most of which contain another protein interaction domain besides BC-box, such as WD-40 repeats, an SH2 domain, or leucine-rich repeats. We also included genes that had been reported to have embryonic or larval arrest phenotypes in large-scale RNAi screens [232], [233], [234], [235], [236] (Table 3.3).

dsRNA was injected into a transgenic strain containing histone:GFP to allow visualization of genomic DNA in the RNAi animals. Of the genes chosen for study, the microinjection of fourteen genes was not successful or needed to be repeated (Table 3.3). There is no observable phenotype for RNAi of six genes (Table 3.3). Eleven of the other genes produced phenotypes (Table 3.3). For eight of the latter, the RNAi progeny arrested as embryos (Table 3.3). Multiple nuclei were observed in the arrested embryos for gene *F34D10.2*, *F57B10.2*, *ZC376.6*, *R10E4.4*, and *Y110A7A.1*. Further characterization is needed to distinguish if they have *cul-2* phenotype(s) or not (Table 3.3).

Interestingly, RNAi of gene *F28D1.1* or *F55F8.3* produced a G1 arrest phenotype, which is one of the *cul-2* phenotypes. 100% of the progeny from hermaphrodites injected with dsRNA of gene *F28D1.1* arrest at the L1 stage. In these L1 larvae, cell cycle arrest was observed with a smaller number of large hypodermal cells compared to wild type L1 larvae (Fig 3.8a,b). Similarly, RNAi of gene *F55F8.3*

generated over 90% L1 arrested F1 progeny. Although there appear to be similar numbers of hypodermal cells compared to wild type, the cell size is about twice as large as the wild type cells (Fig 3.8a,c). To characterize the large cells, these larvae were stained with DAPI to visualize their DNA. Compared to wild type or other normal size cells in these larvae, the DNA intensity in these big cells is lower (Fig 3.9a,b,c). This indicates that they have a only 2n DNA content spreading over a nucleus which is bigger than normal, while other cells contain either 2n (G1 phase) or 4n (S-G2 phases). Therefore, these big larval cells are arrested in the G1 phase. In addition, *F55F8.3*(RNAi) larvae that escaped from the L1 arrest became adults that laid arrested embryos with multiple nuclei (Fig 3.9d,e).

DISCUSSION

Genetic analysis of conserved CUL-2 interactors

By homology search, components of a potential *C. elegans* CUL-2/VCB complex were identified (Fig 3.1; fig 3.2a). By RNAi, their *in vivo* functions were characterized. Inactivation of different critical components of a protein complex would be expected to produce the same mutant phenotype, that is, the mutants should phenocopy one another. While the observation of a phenocopy does not prove that two components function together, it strengthens the conviction that a protein complex that is found in humans is conserved in *C. elegans*. RNAi of all orthologs of the human CUL2/VCB complex, except for VHL, produce embryonic phenotypes similar to *cul-2* mutants (Fig 3.3a-h). While F1 larva from RNAi of *elc-1* or *elb-1* show a *cul-2*-independent phenotype, Unc, they also exhibit a similar *cul-2* germ cell G1 arrest phenotype (Fig 3.3i-k).

From the above results, three inferences can be drawn. First, components of the human CUL2/VCB complex are conserved and are likely to function together in a complex in *C. elegans*. Second, the *C. elegans* CUL-2/VCB complex (which uses VHL as SRC) is not responsible for CUL-2 cell cycle functions, as inactivating *vhl* does not phenocopy *cul-2* mutants. Third, there are likely to be other CUL-2 complexes that exist in *C. elegans*. These complexes may use CUL-2/RBX-1/Elongin C-B as the core complex but include SRCs other than VHL to perform CUL-2 cell cycle functions.

Identification of potential CUL-2 interactors using a biochemical approach

In light of our hypothesis, immunoaffinity isolation of FLAG-tagged CUL-2 was employed and associating proteins were subsequently identified by mass spectrometry. A FLAG-tagged affinity purification method has been used by labs in other fields with great success since the small size of the FLAG moiety and its hydrophilic nature usually don't affect the fusion protein and its association with interacting proteins [237]. Mass spectrometry can identify proteins at the femtomole levels, which greatly increases the chance of identification compared to conventional protein sequencing methods [238]. Currently, ten proteins have been successfully identified as CUL-2 interactors, with both 1-D and 2-D separation techniques.

The reliability of this approach is supported by the following three reasons. First, the *cul-2-FLAG* construct can partially rescue the *cul-2(ek1)* mutant, suggesting that the CUL2-FLAG protein can function in *vivo*. Second, the MS and MS/MS identification of CUL-2-FLAG protein (Fig 3.5), whose presence was confirmed by Western blot with anti-FLAG antibody (Fig 3.4b), suggests the reliability of the mass spectrometry. Third,

the CUL-2 complex component VHL was identified by both MS and MS/MS (Table 3.2). In addition, ELC-1 was found as a potential protein for spot 10 by MS (data not shown). This latter result is consistent with our genetic data that indicates that ELC-1 functions in a CUL-2 complex. The following discussion of the identified CUL-2 interactors poses three questions. What is the possibility that the identified protein is a real CUL-2 interactor? In what respect does it interact with CUL-2? How is it likely to carry out CUL-2-related functions?

TIP120

In three different preparations, the same 148kDa protein was identified as TIP120 by MS and MS/MS (Table 3.2). In addition, this band was present in different purifications reproducibly and abundantly (Fig 3.4a; Fig 3.5; and data not shown). In the control eluate, no protein was present at the same molecular weight. The reproducibility and level of the interaction strongly suggests that TIP120 is a real CUL-2 interactor.

Vertebrate TIP120 was identified as a TATA-box binding protein (TBP) interacting protein [239]. TBP is a central component for transcriptional regulation and is a target for various transcriptional regulators. TIP120 was found to stimulate eukaryotic transcription of RNA Polymerases I, II, and III, all of which interact with TBP [240]. Recombinant TIP120 can activate the basal transcription level of various promoters. Interestingly, vertebrate TIP120 contains both a BC-box and HEAT repeats, the latter of which presents in condensins, cohesins, and proteins with chromosome-related functions [241]. In addition, TIP120 was found in a complex with TBP and the 19S proteasomal subunits, which are recruited to the promoter region [242].

The *C. elegans* TIP120 is essential for both embryogenesis and larval development as inactivation of *tip120* produces arrested embryos, developmentally arrested larvae, and larvae with a slow development rate (Fig 3.7d; data not shown). This suggests that TIP120 is essential for *C. elegans* growth and development and may regulate transcription as does its vertebrate counterpart. The finding that *C. elegans* TIP120 is a BC-box protein makes it a possible Elongin BC interacting protein. Therefore, we propose that CUL-2 may form a ubiquitin ligase using TIP120 as its SRC to regulate gene transcription.

Histone H4 and HMT (histone methyltransferase)

Unexpectedly, histone H4 protein (12.7kDa) was identified from 1-D gel at the size where ELC-1 is expected (13.8kDa). However, by MS/MS, histone H4 was identified repetitively in different experiments (Table 3.2). This indicates that it could be a real CUL-2 interactor. In 2-D gels, more than five unique spots are present at the size between 10-15kDa for CUL-2-FLAG eluate (Fig 3.6). This suggests that Histone H4 can be present together with other proteins such as ELC-1 at the same molecular weight range. The identification of HMT is particularly interesting in light of the association between histone H4 and CUL-2. Although HMT was identified together with EF-1 α in a single protein band, it is probably a real CUL-2 interactor because multiple proteins with a similar molecular weight are likely to co-migrate on 1-D gel (Table 3.2; fig 3.5).

In other species, HMT specifically methylates histones H3 and H4 [228]. There are three types of HMT identified to date: K-HMT that modifies lysine; R-HMT that target arginine; and DOT1 that recognizes the body of histone H3 instead of targeting the

tail of histones as do other HMTs [243]. The identified *C. elegans* HMT belongs to the K-HMT family. Members of K-HMT family have been shown to be involved in heterochromatic gene silencing. Suvar3-9, a K-HMT, is recruited to chromatin by corepressors and represses transcription in a gene specific manner [243]. In addition to transcriptional repression, methylation of histone H3 can also activate transcription if the methylation takes place at K4 instead of K9 [244], [245]. The methylation on K20 of histone H4 is associated with silent chromatin [246]. This methylation and its K-HMT level are cell-cycle regulated [247]. Rice et al. found that entry into mitosis promotes H4 methylation, and the relevent K-HMT is associated with mitotic chromosomes [247].

Identification of both histone H4 and HMT as CUL-2 interactors suggests that CUL-2 regulates gene transcription. Perhaps, CUL-2, HMT, and histone H4 are part of a large complex that affects chromatin structure by multiple means including ubiquitination and methylation. The finding that HMT contains a BC-box also suggested another hypothesis that CUL-2 forms a complex with HMT and Elongin BC to modify histone H4 or H3 with methylation.

PLP-1

Three spots, 5, 6, and 14, which are next to each other (Fig 3.6), were all identified as the same protein, PLP-1. This is not a picking contamination but more likely a real identification since this didn't happen with any other adjacent spots. In addition, the molecular weight of PLP-1 corresponds to the cut spots (Table 3.2; Fig 3.6). A modification, such as phosphorylation, can shift the pI of a protein but only rarely changes the molecular weight. PLP-1 is a transcription activator of *end-1* and *end-3*

promoters and was found to regulate endoderm development in *C. elegans* (Witze et al., personal communication). PLP-1 can be phosphorylated *in vitro* (Witze et al., personal communication). And only when PLP-1 is phosphorylated can it bind to DNA (Witze et al., personal communication). This suggests that these three spots, 5, 6, and 14, corresponds to different phosphorylated forms of PLP-1. This raises the possibility that it could be a substrate for CUL-2 ubiquitin ligase since phosphorylation marks proteins for ubiquitination by CUL-1/RING finger complexes. For instance, phosphorylated Cln1-2 and Sic1 are targeted by SCF complexes for proteolysis in yeast [5].

VHL and ELC-1

The identification of VHL by both MS and MS/MS suggests that spot 13 is indeed VHL (Table 3.2). The approximate mass of spot 13 is smaller than 20kDa suggesting that the spot picked for identification is partially degraded VHL product (Fig 3.6). In addition, ELC-1 was considered to be the protein for spot 10 by MS (data not shown). The above results suggest that a physical interaction among *C. elegans* CUL-2, ELC-1, and VHL exists *in vivo*.

Other proteins identified

Interestingly, APC11 is indicated to be a CUL-2 interactor by mass spectrometry analysis. APC11 is a RING finger protein, which was found in APC complex. In yeast and *Xenopus*, the APC complex degrades B type cyclin to allow exit from mitosis [66], [128]. Embryos produced by *cul-2* mutants are defective in mitosis exit [197]. In addition, maternal cyclin B remains constant in *cul-2* mutants, while it is rapidly

degraded in wild type (Liu and Kipreos, personal communication). Therefore, it is likely that CUL-2 degrades cyclin B by regulating APC activity or independently forming a complex with APC11 in *C. elegans*.

Four other proteins, EF-1 α , APT-5, RFA, and CLP-7, were also identified as CUL-2 interactors. EF-1 α is the nucleotide-binding subunit of the EF-1 multi-protein complex, which regulates translational elongation [248]. In addition to its role in translation, EF-1 α may contribute to cytoskeleton assembly [249]. EF-1 is highly conserved among species, suggesting that its functions may be conserved as well. *C. elegans* EF-1 α is required for embryogenesis and postembryogenesis. Inactivation of *ef-1\alpha* generates arrested embryos with multiple nuclei and uncondensed DNA (data not shown), which are similar to some of *cul-2* phenotypes. Interestingly, EF-2, which functions together with EF-1 in translation regulation, was found to have an increased protein level in *cul-2*(RNAi) worms (Dhingra and Kipreos, personal communication). Even though these data suggests there might be a link between CUL-2 and EF-1 α , further experiments are needed to rule out the possibility that the association of CUL-2 with EF-1 α is a non-specific binding of *in vivo* abundant proteins.

APT-5 is the δ subunit of the adaptin complex, which is involved in vesicle transportation [250]. Inactivation of *apt-5* generates *dpy* like worms (Fig 3.7b). APT-5 is a BC-box protein, suggesting an interaction with CUL-2 through the Elongin BC complex. In both human and yeast, RFA, replication factor A, binds single-stranded-DNA [251]. At the G1-to-S phase transition, RFA is phosphorylated. This phosphorylation remains through out G2 [251]. Therefore, replication factor A is

suggested to have a role in DNA replication. CLP-7 is a cysteine endopeptidase. The *C*. *elegans* functions of RFA and CLP-7 remain to be characterized.

Identification of potential CUL-2 SRCs by bioinformatic and genetic approaches

The C. elegans genome is completely sequenced [125], which facilitates the use of a bioinformatic approach to discovering complex components. We have taken advantage of the sequenced genome and the RNAi technique to screen for potential SRCs for CUL-2/RING finger complex. After finishing the first round of screening, we have identified two candidate genes, F28D1.1 and F55F8.3, that have a particularly promising RNAi phenotype, a G1 cell cycle arrest, which was also observed in the *cul-2* mutants (Fig 3.8a,b,c; fig 3.9a,b,c). Both F28D1.1 and F55F8.3 encode proteins containing a BCbox and WD-40 repeats (Table 3.3). WD-40 repeats mediate protein-protein interactions [252]. For instance, many SRCs of SCF complexes contain this motif in addition to the Fbox [66]. RNAi of F28D1.1 or F55F8.3 generated almost 100% arrested L1 larvae in which G1 arrest was observed in some skin cells (Fig 3.8a,b,c; fig 3.9a,b,c). These two genes are included as potential SRCs for CUL-2 ubiquitin ligases because they phenocopy cul-2. Experiments are underway to test whether either of these proteins interact directly with Elongin C using the yeast two-hybrid system and coimmunoprecipitation.

Possible mechanisms for CUL-2 molecular functions

By the combination of genetic, bioinformatic, and biochemical approaches, a number of candidate proteins have been successfully identified as potential CUL-2

interactors. This current knowledge of the identified proteins promotes conjecture on how CUL-2 may function to regulate aspects of cellular physiology.

Conventional ubiquitin-mediated protein degradation

The RNAi phenotypes of *elc-1*, *elb-1*, *rbx-1*, and *ubc2* suggest that CUL-2 functions with ELC-1, ELB-1, RBX-1, and UBC-5 to regulate cell cycle events. The human counterparts of these proteins form the core ubiquitin ligase complex with CUL-2, using UBCH5 as an E2 [208], [74], [102]. Therefore, it is extremely likely that *C. elegans* CUL-2 also forms ubiquitin ligases with these proteins in the core complex (Fig 5.1). Biochemically, VHL was identified as a CUL-2 interactor. This suggests the existence of a *C. elegans* CUL-2/VCB complex *in vivo*. At the same time, several BC-box proteins were also identified as CUL-2 interactors by CUL-2-FLAG purification: TIP120, HMT, and APT-5. This suggests that CUL-2, like its ortholog human CUL2, forms multiple ubiquitin ligases using different SRCs (Fig 5.1). The protein products of genes *F28D1.1* and *F55F8.3* are potential candidates to be SRCs for CUL-2 ubiquitin ligase complexes that target CKI-1 for ubiquitin-mediated degradation [197].

The regulation of gene transcription

Surprisingly, a number of proteins involved in transcription were identified as CUL-2 interactors: transcriptional activator (PLP-1), co-activator (TIP120), chromosome associating proteins histone H4 and HMT. The identification of these proteins appears to relate CUL-2 E3 ubiquitin ligase complexes to transcriptional regulation. Other E3s, such as SCF^{Met30} , SCF^{Cdc4} , and $SCF^{\beta-TRCP}$, have been found to regulate transcription by targeting
various transcription factors for ubiquitin-mediated proteolysis [224], [225], [226], [59]. PLP-1 is a transcription activator and phosphorylated PLP-1 binds to DNA to activate transcription (Witze et al., personal communication). In *C. elegans* embryos, the PLP-1 protein is distributed asymmetrically in early embryos (Witze et al., personal communication). The asymmetrical distribution of PLP-1 could be the consequence of targeted protein degradation in certain cells but not in others. Therefore, it is possible that a CUL-2/RING finger ubiquitin ligase targets the phosphorylated PLP-1 for degradation.

The fact that TIP120 is both a BC-box protein and a transcription co-activator makes us suspect that the CUL-2 ubiquitin ligase may target transcription factors for degradation or modification by using another transcription factor, such as TIP120, as an SRC. Consistent with this hypothesis, the Conaway lab discovered that mMED8, a subunit of the mammalian transcriptional Mediator complex, binds Elongin BC and forms a ubiquitin ligase with CUL2 [94]. This ubiquitin ligase was co-purified with other components of the Mediator complex, indicating that this class of CUL-2 ubiquitin ligase may dock on or near the promoter region. [94].

E3s not only target transcription factors for degradation but also modify them with ubiquitin to either activate or repress their transcriptional activity [59]. In addition, histone methylation has been found to be involved in gene transcriptional regulation [228]. HMTs can specifically methylate histones H3 and H4, which either activate transcription or repress transcription based on the modification site and the targeted amino acid residues [244], [245], [243]. *C. elegans* HMT and histone H4 are potential CUL-2 interactors (Table 3.2). This suggests that CUL-2 may regulate gene transcription through the interaction with histone H4 and HMT in *C. elegans*.

MATERIALS AND METHODS

DNA constructs

The *elc-1(Y82E9BR.15)*/PCR4-TOPO and *elc-2(W03H1.2)*/PCR4-TOPO constructs were generated by inserting PCR-amplified genomic *elc-1* (1.96kb) and *elc-2* (0.84kb) DNA into the PCR4-TOPO vector, respectively (Invitrogen). The *cul-2-FLAG* purification constructs, promoter-ATG-*FLAG-cul-2*-stop-polyA (*PFCA*) and promoter-ATG-*cul-2-FLAG*-stop-polyA (*PCFA*), were made by using a two-step PCR technique, as described [253]. The Platinum high fidelity enzyme PCR system (Life Technologies) was used to obtain PCR products with fewer mutations. The *FLAG* sequence was introduced into either the upstream primer to make the N-terminal FLAG tag or the downstream primer to make a C-terminal FLAG tag with the sequence "DYKDADDDK".

C. elegans transformation and integration

The *CUL-2-FLAG* construct, *PFCA* or *PCFA*, was gel-purified. The *rol6*/PRF4 plasmid was used as co-injection marker; this produces progeny with a rolling phenotype (Roller), allowing them to be distinguished from non-transgenic progeny. A final concentration of 2 ng/µl *PFCA* or *PCFA*, 2 ng/µl *rol6*/PRF4, and 100 ng/µl N2 genomic DNA was injected into the distal region of both gonad arms of *cul-2/unc64* heterozygous mutants. A stable line that rescued *cul-2* mutant phenotypes and produced 10-30% rollers was treated with γ -radiation (4500 rad) to integrate the extrachromosomal transgenic

arrays into chromosomes. The integrated worms were selected for the ability to produce 100% of progeny carrying the transgene.

FLAG purification

The first or second generation of the integrated transgenic worms were synchronized then grown to gravid adults at 25°. A 60% (w/w) sucrose gradient was used to clean worms before purification. The FLAG purification was modified from a protocol as described [254]. Worm pellets were resuspended with lysis buffer (100 or 250 mM NaCl, 0.2% Triton X-100, 50 mM Hepes-KOH pH 7.5, 10% glycerol, 0.5 mM PMSF, and 3 mM DTT), containing EDTA-free complete protease inhibitor (Roche). A French press (Amicon) (5000 psi) was used to break worms. The supernatant was incubated with 10 ug/ml anti-FLAG M2 antibody (Sigma) for 2 hr at 4°C after the precleaning incubation with protein G sepharose at 4°C for at least 30 min. The protein G sepharose beads (Amersham Biosciences) were then added to immobilize the complexes. After washes with lysis buffer for at least 5 times (10 min each, at 4°C), the CUL-2-FLAG complexes was eluted by incubation with 0.4 mg/ml FLAG peptide (Sigma).

Gel electrophoresis and staining

The eluted CUL-2-FLAG complexes were concentrated and cleaned-up by using the Plus-one SDS clean-up kit (Amersham Biosciences). Proteins were resolved on 5-15% SDS Tris-glycine gradient gels made with a gradient maker (C.B.S. Scientific Co.). The proteins that were analyzed by two dimensional gel were cleaned-up and concentrated by using the Plus-one 2D clean-up kit (Amersham Biosciences). The first dimension was run on an 18 cm immobilized pH gradient strip gel (pH 3-10, linear, Amersham Biosciences). The active rehydration was applied with a 100 voltage for 12 hr on IPGphor (Amersham Biosciences), then proteins were separated on the first dimension with a 8000 voltage. The second dimension gel was 5-15% SDS Tris-glycine gradient gel made in our lab. The Benchmark protein ladder (Invitrogen) was used as a marker. The separated proteins were visualized by silver staining as described [255]. Sypro Ruby dye (Molecular Probe) was used to stain the proteins gels in preparation for in-gel digestion and mass spectrometry as described [256], [257].

In-gel digestion and mass spectrometry

For 1-D gels, protein bands in both CUL-2-FLAG transgenic and control lanes were subjected to digestion and MS and/or MS/MS analysis. Proteins separated by 2D gel electrophoresis were imaged using the 2D Master Gel Imager in the UGA Proteomics Center. The protein spots unique to the CUL-2-FLAG transgenic line were picked with a spot picker in the Ettan Spot Handling Workstation. The destaining of silver stained proteins was carried out as described [258]. Sypro Ruby stained gel plugs were destained with 3 mM Tris.cl solution (pH 8.8) in 50% acetonitrile (Sigma). The in gel digestion steps were carried out as described [255], [238]. Equal volume of saturated α cyanocinnamic acid (Sigma) was mixed with peptides for MALDI-TOF. At least two peptides unique to the CUL-2-FLAG eluate were chosen for MS/MS analysis. The machines used for mass spectrometry (MS) were the QTOF (Micromass, England), the 4700 Proteomic Analyzer TOF/TOF (Applied Biosystems), and/or API QSTAR Pulsar (Applied Biosystems). The MS and MS/MS data collection was mainly done in the UGA Proteomics Center of the Molecular Genetics Instrumentation Facilities.

Database searches and protein identification

Both ProteinProspector (UCSF) and Profound (Rockefeller University) web-based search engines were used for database searching and identification [259]. The mass tolerance varies on different machines (0.02% MW for Qstar and 0.05% MW for TOF-TOF after calibration). The identification of a protein by MS is based on the satisfaction of all three requirements: the rank (the top match for both Profound and ProteinProspector); the MOUSE score for ProteinProspector and the Z score for Profound; and the percentage of peptides matched for ProteinProspector and the probability (1.0) for Profound. The identification of a protein by MS/MS was based on a combination of the rank (the top match), percentage ions matched, and MS candidate. In addition, MW and pI for sample and its identified protein were compared.

Bioinformatics and phylogenetic analysis

The conserved BC-box motif was used to search BC-box proteins in *C. elegans*. The computer programs used for searching *C. elegans* BC-box motifs were MEME, MOTIFSEARCH, and RGS-BLAST. The GenBank database in which the search was done was housed in the GCG at UGA.

The protein sequences from different species were obtained from the National Center for Biotechnology Information (NCBI) and *C. elegans* genome databases. Homologs were identified by BLAST search [260]. Initial protein sequence alignments

were made by the CLUSTAL X program [261]. The identities of *C. elegans* proteins, compared to its human counterparts, were obtained by pairwise alignment using the CLUSTAL X program. The alignment was then optimized by hand. Spacer regions were excluded for phylogenetic analysis. The neighbor-joining (NJ) [262] and maximum-likelihood (ML) methods were used for phylogenetic analysis as described [263]. Bootstrap values were obtained from 1,000 replicates.

Genetics and RNAi

The following strains of *C. elegans* were used: Bristol N2; CB5602 [*vhl*(ok161)]; ET65 [cul-2(ek1)/unc64(e246)]; AZ212 [unc-119(ed3)], with integrated DNA ruIs32 (pAZ132: pie-1 promoter/GFP::histone H2B)]; RNAi was performed using double stranded RNA (dsRNA) derived from cDNA clones for *elB-1/Y41C4A.10* (cDNA clone yk172c6, GenBank acc. no. C09514), rbx-1/ZK287.5 (cDNA clone yk455h11, acc. no. C48151), ubc5/let-70 (cDNA clone yk478e5, acc. no. C50177), ubc3/Y71G12B.15 (cDNA clone yk103b3 acc. no. D75328 and yk79g2 acc. no. D74338), vhl/F08G12.4 (cDNA clone yk166e4, acc. no. C09301), tip-120/Y102A5A.1 (cDNA clone yk530b2, acc. no. AV188919, and cDNA clone yk563g2, acc. no. AV189886), apt-5/W09G10.4 (cDNA clone yk442h10, acc. no. C71251), med8/Y62F5A.1 (cDNA clone yk512b10, acc. no. AV187457), K12H6.11 (cDNA clone yk289g9, acc. no. C42169), and ef-1α/F31E3.5 (cDNA clone yk46b10, acc. no. D37514). The yk clones for the BC-box screening are listed in Table 3.3. *elc-1* and *elc-2* RNA were made from the *elc-1(Y82E9BR.15)*/PCR4-TOPO and *elc-2(W03H1.2)*/PCR4-TOPO constructs, respectively. Sense and antisense RNA were made using the T3 and T7 MegaScript kits (Ambion), respectively. dsRNA

was annealed and injected at a concentration of 0.5-1 mg/ml into young adults as described [197].

Western blot

Western blots were performed by separating proteins on SDS PAGE then transferring them to PVDF membrane, as described (Molecular Cloning). Membrane was blocked with 5% dry milk power in PBS. The primary antibody, anti-FLAG M2 (Sigma), and the secondary antibody, anti-mouse-IgG horseradish peroxidase (Sigma), were used. The ECL western blot was carried out according to the ECL kit instruction provided by the manufacturer (Amersham Bioscience). Chemiluminescent Hypersensitive film (Kodak) were used for protein band visualization.

Microscopy

Embryos and animals were observed by differential contrast interference (DIC) and immunofluorescence microscopy using a Zeiss Axioskop microscope. Images were taken with a Hamamatsu ORCA-ER digital camera with Openlab 3.0.8 software (Improvision). All images were processed with Adobe Photoshop 6.0. Matched images were taken with the same exposure time and processed identically.

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mutant; M. Warren and T. Andacht for helpful discussions and data collections of MS and MS/MS; V. Dhingra for helpful discussion; R. Santurri for technical work. We thank the *Caenorhabditis* Genetics Center for *C. elegans* strains and the Genome Sequencing Consortium for *C. elegans* genomic sequence and cosmids. This project was supported by RF ACS grant RSG-251/01 and HFSPO grant RG-229/98 to ETK.

Human CUL2/VCB complex component	C.elegans orthologs (Cosmid)	Protein identity (%)
CUL2	CUL-2 (ZK520.4a)	42.17
Elongin C	ELC-1 (Y82E9BR.15)	45.9
	ELC-2 (W03H1.2)	25.7
Elongin B	ELB-1 (<i>Y41C4A.10</i>)	6.9
RBX1	RBX-1 (<i>ZK</i> 287.5)	81.5
VHL	VHL (F08G12.4)	17
UBCH5	UBC-5 (<i>M</i> 7.1)	90.5; 94.6#
HIF1a; HIF2a	HIF-1a (<i>F38A6.3</i>)	28; 29*

Table 3.1: CUL2/VCB components in human and C. elegans

"/" denotes that protein identity is not compared between *C. elegans* and humans.

shows the identities of *C. elegans* UBC-5, compared to human UBCH5A and UBC5HB, respectively.

* represents the identities of *C. elegans* HIF1 α subunit, compared to human HIF1 α and HIF2 α , respectively.

Cut	Μ	MS Top Match			MS/MS Top Match		
band	protein	MOUSE	percent	1^{st} peptide 2^{nd} peptide		ptide	
MW	identified	Score	peptides				
or	(MW/pI)	(Z Score)	matched	protein	percent	protein	percent
Spot #			(%)	identified	ions	identified	ions
				(MW/PI)	matched	(MW/PI)	matched
					(%)		(%)
148	<u>TIP120</u>	1.373e+9	89.5	<u>TIP120</u>	100	<u>TIP120</u>	92.3
kDa	(141.7	(2.01)		(141.7		(141.7	
	kDa/5.5)			kDa/5.5)		kDa/5.5)	
110	VIT-6	6.415e+4	57	<u>APT-5</u>	82.8	Х	Х
kDa	(193	(1.05)		(140			
	kDa/6.8)			kDa/6.2)			
50	EF-1a	7810	37	EF-1a	79.2	<u>HMT</u>	72
kDa	(50.6	(1.57)		(50.6		(44.8	
	kDa/9.1)			kDa/9.1)		kDa/9.5)	
12kDa	Х	Х	Х	Histone	77.7	Histone	75*
				H4		H4	
				(12.7		(12.7	
				kDa/10)		kDa/10)	
Spot	Х	Х	Х	APC11	84.8	Х	Х
F8.4				(15.5			
				kDa/5.0)			
Spot 1	CLP-7	5111	50	\	\	\	\
	(82	(1.88)					
	kDa/4.2)						
Spot 2	RFA	3.215e+5	42	\	\	\	\
	(73	(2.02)					
	kDa/5.7)						
Spot 5	PLP-1	1.353e+5	80	\	\	\	\
	(25.5	(2.37)					
	kDa/9.3)						
Spot 6	PLP-1	3296	53	\	\	\	\
	(25.5	(1.34)					
	kDa/9.3)						
Spot	<u>VHL</u>	567	38	VHL	77.8	Х	X
13	(20.3	(0.18)		(20.3			
	kDa/9.5)			kDa/9.5)			
Spot	PLP-1	1.407e+4	56	\	\	\	\
14	(25.5	(2.38)					
	kDa/9.3)						

 Table 3.2: CUL-2 interactors identified by mass spectrometry

"X" indicates identification unsuccessful. "\" denotes that the MS/MS experiment was not performed due to a good identification with MS only.

*The second peptide used for identifying Histone H4, which corresponds to the 12kDa band cut from the 1-D gel, came from a preparation different from the first peptide. The second peptide, 663.85 Da, was fragmented by Kumar Koli of the UGA CCRC on Q-TOF, and the search result was obtained through Mascot (Matrix Science). The value 75 is the ion score instead of the percentage ion matched (ion score >31 indicates identity or extensive homology). The first peptide, 1465.8 Da, was fragmented in the UGA Proteomics Center on TOF-TOF, and the result was obtained through the MS-TAG search in ProteinProspector.

"_" underlines the BC-box proteins.

Mouse Score is from ProteinProspector search; Z score is from Profound search. The identity is based on when the Mouse Score of the first candidate is greater than 10 times, compared to that of the second candidate.

"Z score: 1.282 corresponds to a 90% percentile match in the searching population; 1.645 corresponds to 95%; 2.326 corresponds to 99%; and 3.090 corresponds to 99.9%."

Gene	Protein name for <i>C. elegans</i> /ortholog of human and fly	yk clone	Other motifs /interesting phenotypes observed by others	RNAi phenotypes observed by us
R13F6.10	Unknown	yk133a10	embryonic lethal	
F53G2.7	MAT1	yk1324f09	embryonic lethal	20% arrested eggs, few L1/L2 arrest with more hypodermal cells
F34D10.2	CDC45	yk1253e03	embryonic lethal	100% embryonic arrest with multinuclei, unequal DNA distribution, and DNA bridge. DNA replication ceases later.
F57B10.2	MEI-2	yk314c6	embryonic lethal	embryonic arrest with unequal DNA distribution and multinuclei
C34D4.14	THR interactor 12	yk1104g04	HECT domain /embryonic lethal	\
ZC376.6	Unknown	yk461c12	embryonic lethal	60% embryos arrest with multinuclei and DNA unequal segregation. some F2 arrest at L1; L2 or L3.
T05C1.6	Unknown	yk1062g03	TIG domain /embryonic lethal	/
R10E4.4	MCM5	yk1065b01	embryonic lethal	100% embryonic arrest with multinuclei, unequal DNA distribution. Mitosis timing normal
F22B5.7	ZYG-9	yk668b5	embryonic lethal	\
Y110A7A.1	Unknown	yk629e2	embryonic lethal	100% embryonic arrest with unequal DNA distribution and multinuclei.

Table 3.3: Phenotypic screening for *C. elegans* BC-box proteins

Gene	Protein name for	yk clone	Other motifs	RNAi phenotypes
	C. elegans		/interesting	observed by us
	/ortholog of		phenotypes	
	human and fly		observed by	
			others	
C01F6.9	Unknown	yk266c4	Other phenotypes	\
F25E5.1	No homologs	yk659f9	Other phenotypes	\
<i>Y39F10B</i> .	No homologs	yk1314b10	Other phenotypes	abnormal germ cell
1				development:
				Germ cells-
				oocytes-germ cells
				again
<i>C13F10.4</i>	Unknown	yk568a10	Other phenotypes	\
R03D7.4	Elongin A	yk1119d11	Other phenotypes	\
F39B2.5	SOCS7	not	SH2 domain	\
		available		
M60.7	ASB15	not	Ankyrin repeats	\
		available		
F23B2.4	\	not	WD40 repeats	\
		available		
F28D1.1	Unknown	yk1193b01	WD40 repeats	100% F1 arrested
				at L1 stage with
				big skin cells.
F52C9.1	No homologs	yk131g12	WD40 repeats	\
F55F8.3	Unknown	yk359a8	WD40 repeats	99% F1 arrested at
				late L1 stage with
				big skin cells. Few
				F2 embryos
				arrested with
		1 1 1 2 11 0 0		multinuclei
VT23B5.2	Beige/FYVE Zine	yk1121h09	WD40 repeats	Normal
	finger protein			
<i>Y23H5B.5</i>	/	Not	WD40 repeats	/
	TT 1	available		N. 1
<i>Y45F10B</i> .	Unknown	yk/6c2	WD40 repeats	Normal
10				
F20D1.7	Unknown (Glioma	yk1036b02	Leucine rich	Normal
	gene)	NT -	repeats	
C05A9.3	/	Not	Ankyrin repeats	
		available		
Y47D3A.2		yk1216b06	Ankyrin repeats	Normal
2				

Gene	Protein name for <i>C.elegans</i> /ortholog of human and fly	yk clone	Other motifs /interesting phenotypes observed by others	RNAi phenotypes observed by us
F32D1.3	Unknown	yk28d9	teratricopeptide repeats	Normal
Y39F10B. 1	\	yk365a2	Small growth defect	embryonic arrest. Need to repeat RNAi
Y46E12BL .3	/	yk78b2	SOCS box with WD40 repeats	Normal
C39F7.2		yk246e12	SPRY domain	few arrested embryos with huge amount of DNA

"\" denotes unsuccessful microinjection or the search/injection not performed.

Fig 3.1: Conservation of Elongin C among species: a, The homology alignment of two *C. elegans* Elongin C proteins (CeELC-1 and CeELC-2) to *S. cerevisiae*, *D. melanogaster*, and human Elongin C proteins. Compared to both fly and human Elongin C proteins, the percentage of identical amino-acids residues among three species are 45.9% for *C. elegans* ELC-1 and 25.7% for *C. elegans* ELC-2. Asterisks denote the identical amino-acids among all Elongin C proteins compared. Dots denote identities among the four species. **b**, Neighbor-joining (NJ) Phylogeny of *C. elegans*, *S. cerevisiae*, *D. melanogaster*, and human Elongin C proteins using the data set presented in Fig 3.1a. Branch lengths are proportional to the estimated number of amino acid replacements; the scale bar indicates amino acid replacement for each site.

ScelC	MSQDFVTLVSKDDKEYE
HsdJ254P11.1	MDGEEKTCGGCEGPDAMYVKLISSDGHEFI
HsFOS39347 1	MNGEEKSCSGYGGPDAMDGKLVSSDDDDFI
DmElonginC	MIAMDEQRG-DKIYGGCEGPDAMYVKLISSDGHEFV
HsElonginC	MDGEEKTYGGCEGPDAMYVKLISSDGHEFI
CeELC-1	MADQNNAIQCDQDAAQPKQYGGIEGPTSQYVKLVSSDDHEFI
CeELC-2	MWWELRKHVYKAFVQIFYQNYNMDKNGSAAGGFQAASNKDDGLEEPDSQKTGAKPQPVPISGLEGPRSKYVKLVSNDDHEFI
	* ** ** *
ScELC	ISRSAAMISPTLKAMIEGPFRESKGRIELKQFDSHILEKAVEYLNYNLKYSGVSEDDDEIPEFEIPT
HsdJ254P11.1	VKREHALTSGTIKTTLGGPGQSAVNETNEVNFREIPSRVLSKVCTYFMYKVRYPNSSTEVPELPIAP
HsFOS39347 1	VKREHTRASGIRKAVLSGPGQSAENETDQVNFREIPPRCYPKHAKACIYFTYKVHYSNSSTEIPEFL
DmElonginC	VKREHALTSGTIRAMLSGPGQFAENEANEVHFREIPSHVLQKVCMYFTYKVRYTNSSTEIPEFPIAP
HsElonginC	VKREHALTSGTIKAMLSGPGQFAENETNEVNFREIPSHVLSKVCMYFTYKVRYTNSSTEIPEFPIAP
CeELC-1	IKRELALTSGTIRAMLSGPGVYAENESNVVYFREIPSHVLQKVCQYFAYKVRYTHAATEIPEFPIPP
CeELC-2	IKREVAMTSKSLRELFANPTVDLAAANNTVYFSDFQSHILQKVCHYLAYKTKYRHSRVAPPFDIPP
	.*** ** ***.
ScELC	EMSLELLLAADYLSI
HsdJ254P11.1	EIALELLMDVSFLDCEIK
HsFOS39347 1	-TALEVLMASDFLDIYIFLFFLFF
DmElonginC	EIALELLMAANFLDC
HsElonginC	EIALELLMAANFLDC
CeELC-1	DVALELLMAANFLDC
CeELC-2	DIAMDLLAAANELEC
	* *

b

а



114

Fig 3.2: *C. elegans* **VHL protein and mutant. a**, Compared to rat and human VHL protein, the identity of *C. elegans* VHL protein is 17.5% and 17.0%, respectively. Asterisks point out identical amino acids among three species. Identical amino-acids between any two species are denoted as a dot. The boxed area is the BC-box motif. **b**, Line drawing of the *vhl* genomic region on chromosome X with exons represented as boxes; and introns and flanking regions as horizontal lines. Shaded areas represent coding regions. The cDNA sequence of *vhl* was sequenced at UGA Molecular Genetic Instrumentation Facility, and the genomic sequence is from cosmid F08G12.4 (GenBank acc. no. C09301). The region deleted in *vhl(ok161)* is represented as the missing region bounded by dashed lines and includes exons 1 and 2. Locations of primers used for the deletion screen are presented below the genomic line drawing.

HumanVHL RatVHL CeVHL	MPRRAENWDEAEVGAEEAGVEEYGPEEDGGEESGAEESGPEESGPEELGAEEEMEAGRPR MPRKAAS AERMPGSEEIEAGRPR
HumanVHL RatVHL CeVHL	PVLRSVNSRE-PSQVIFCNRSPRVVLPVWLNFDGEPQPYPTLPPGTGRRIHSYRGHLWLF PVLRSVNSRE-PSQVIFCNRSPRVVLPLWLNFDGEPQPYPTLPPGTGRRIHSYRGHLWLF PDLGSSTHDNREIRVRFLNRCAYPVDVFWLNPSKQPTKYGTLAQKKYLDIKTFKDHPWVA * * * . : :* * ** * .*** . :* * ** *::::.* *:
HumanVHL RatVHL CeVHL	RDAGTHDGLLVNQTELFVPSLNVDGQPIFANITLPVYTLKERCLQVVRSLVKPENYR RDAGTHDGLLVNQTELFVPSLNVDGQPIFANITLPVYTLKERCLQVVRSLVKPENYR RRSFDGCKVLVNEKEVFWPEPAPRMNLIVRNHCVITMKVQ <u>SLREIAGRSF</u> LRHNPTEVPN *: :***:.*:* *. : *. * **: * :*:*
HumanVHL RatVHL CeVHL	RLDIVRSLYEDLEDHPNVQKDLERLTQERIAHQRMGD RLDIVRSLYEDLEDHPNVRKDIQRLTQEHLENQALGEEPEGVH KIKGLPRELQFEVKHFLDRKQEYSEIVCRSIPPPGPQRPQQ ::.::::::::::::::::::::::::::::::::

b.



1 kb DNA

Fig 3.3: RNAi phenotypes of CUL-2/VCB components. a-h, Embryonic phenotypes of *elc-1* (**a**, **d**), *elb-1* (**g**, **h**), *rbx1* (**b**, **e**), and *ubc2* (**c**, **f**) RNAi. **a-c** and **g** are Differential Interference Contrast (DIC) images of arrested embryos. **d-f** and **h** are histone:GFP images (visualizing DNA) in the arrested embryos. Arrow heads point out the multiple nuclei in one cell. Arrows denote extended cytoskeleton. **i-k**, DIC micrographs of the distal gonad arms of wild-type (**i**), *elb-1* RNAi (**j**), and *elc-1* RNAi (**k**) L4 stage hermaphrodites. The distal tip cell (DTC) is denoted by an arrowhead. Scale bars, 10 µm.



Figure 3.4: CUL2-FLAG purification: **a.** The CUL-2-FLAG affinity purified samples were run on a 5-15% gradient gel. The elution solution from transgenic and control eluate are shown. The transgenic lane (to the right) has 13 extra specific bands, 191, 188, 148, 90 (CUL-2-FLAG), 85, 76, 66, 39, 29, 15.5, 15.0, 14.5, and 14.2 kDa, compared to the control lane (to the left). **b**. Western blot of CUL-2-FLAG affinity-purified eluates from transgenic worms (to the left) and control untransfected worms (to the right) using anti-FLAG antibody. Arrow points to CUL-2-FLAG (85-90kDa), which is only detectable in the transgenic elution.



Fig 3.5: Identification of CUL-2 interactors on 1D gel. Proteins were separated on a 5-15% one dimensional gradient gel. The right lane is CUL-2-FLAG eluate, and the left is control eluate. The extra bands in the CUL-2-FLAG affinity-purified eluate that were identified by mass spectrometry are as follows: 148 kDa band as TIP120 ortholog; 110 kDa band as apt-5; 50 kDa band as Elongation factor-1 α (EF-1 α) and histone methlytransferase (HMT); and 12 kDa band as histone H4.



Fig 3.6: 2-D Sypro Ruby stained CUL-2-FLAG eluate and control eluate. A pH 3-10 IEF strip was used for the first dimension, and a 5-15% gradient gel was run in the second dimension. The gel was stained with Sypro Ruby stain (Molecular Probes), and the image was scanned. Red circles identify spots present only in the CUL-2-FLAG transgenic eluate (upper panel) compared to the control gel (lower panel). The number next to each circle is the spot number. Circle 15 is a blank spot used as a control for digestion and data analysis.



Control Eluate



Fig 3.7: RNAi of CUL-2 interactors. a-c, DIC micrographs of (a) distal gonad arm in hermaphrodites injected with *ef-1* α dsRNA, (b) arrested abnormally shaped L1 produced by hermaphrodites injected with *apt-5* dsRNA, and (c) embryos arrested at late stage from hermaphrodites injected with *Y62F5A.1* (*med* ortholog) dsRNA. An arrow head denotes distal tip cell (DTC). **d-f**, DIC micrographs of F1 progeny from hermaphrodites with gene *Y102A5A.1* (*TIP120* ortholog) depleted by RNAi: (d) arrested embryo in F2 progeny; (e) abnormally shaped tail, and (f) everted vulva in adult. Multiple nuclei in (d) are denoted by arrows. Scale bars, 10 µm.



Fig 3.8: RNAi of gene *F28D1.1* **and** *F55F8.3.* **a-c**, DIC micrographs of (**a**) wild type L1 larvae, (**b**) arrested L1 larvae produced by mothers injected with dsRNA of gene *F28D1.1*, and (**c**) arrested late L1 larvae from mothers injected with dsRNA of gene *F55F8.3*. Lines point out seam cells (Se) and a hyp 7 hypodermal cell (H); Arrows point out the large skin cells in the arrested larvae. Scale bars, 10 μm.



Fig 3.9: DNA content in *F28D1.1*(**RNAi**) **or** *F55F8.3*(**RNAi**) **cells. a-c**, DAPI stained DNA in (**a**) wild type L1 larval cells, (**b**) the arrested L1 larval cells from mothers injected with dsRNA of gene *F28D1.1*, and (**c**) the arrested late L1 larval cells from mothers injected with dsRNA of gene *F55F8.3*. Arrows point out DNA of large skin cells in the arrested larvae. **d-e**, DIC image (**d**) and nuclear DNA visualized by histone:GFP (**e**) of the *F28D1.1* RNAi arrested F2 embryo. Arrow heads point out multiple nuclei. Scale bars, 10 μm.



CHAPTER IV

CUL-4 RESTRAINS DNA REPLICATION LICENSING IN C.ELEGANS

BACKGROUND

Eukaryotic DNA replication regulation

To allow viable cell divisions, genomic DNA, the cellular genetic information, has to be duplicated each cell cycle in an accurate and timely fashion. DNA replication has to be stringently regulated to ensure that the content of the genome is exactly doubled in the course of each cell cycle. The loss of genome stability through over or underreplication can be detrimental to a single cell as well as to the whole organism, causing cell death or diseases including cancer. Cells employ four general mechanisms to achieve accurate DNA replication [251]. First, initiation is triggered at the appropriate times in the cell cycle at thousands of sites spreading along whole chromosomes. Second, initiation is prevented at the same sites in the newly synthesized daughter chromosomes. Third, DNA replication is coordinated with other cell cycle events. Fourth, DNA replication checkpoints block cells with abnormal genome replication from progressing further into the cell cycle, thereby maintaining genome integrity. Below, I will mainly focus on how cells regulate the replication licensing system to license origins of DNA replication and explain how re-replication is prevented.

The process of DNA replication

In eukarytoes, DNA replication factors are conserved among species. The sites required for DNA replication initiation are denoted as DNA replication origins, with unique features for different species [251]. Origin firing, which initiates DNA replication, begins during S phase of the cell cycle. Origin firing requires the assembly of a prereplicative complex (pre-RC) at the replication origins in G1 phase and the activation of the pre-RC in S phase. The binding of the minichromosome maintenance (MCM) protein complex to the replication origin to form the pre-RC is promoted by a number of replication factors. Upon phosphorylation of MCM by CDK and the Dbf4-dependent kinases (DDK), MCM functions as a helicase, melting DNA at the origin and unwinding DNA at the moving replication fork. Three DNA polymerases work together with MCM and other replication factors to synthesize the daughter DNA stand [251].

1. The assembly of the pre-RC

The assembly of the pre-RC at replication origins licenses cells for DNA synthesis. The pre-RC complex includes the origin recognition complex (ORC), CDC6, CDT1, and the MCM complex. The first step in the assembly of the pre-RC is the binding of the initiator, ORC, to replication origins [264]. The ORC is composed of six subunits, ORC1 to ORC6 [251]. Some components of ORC are capable of binding ATP. The ATP association is essential for the high affinity binding of ORC to DNA. Among them, the ATP-binding activity of ORC1 may be the most important as deleting the N-terminal ATP binding region of ORC1 but not other ORCs causes lethality. The replication licensing factors CDC6 and CDT1 are recruited to the origins during G1 phase. The

loading of MCM, composed of MCM2-7 subunits, onto the origins requires the action of both CDC6 and CDT1 [264], [265]. The assembly of the pre-RC mostly occurs during the G1 phase of the cell cycle [266].

2. The activation of the pre-RC

Upon S phase entry, existing pre-RC are activated, thus allowing the loading of additional replication factors, such as CDC45, SLD3, and perhaps DPB11, to mature the pre-RC into a preinitiation complex (pre-IC) [267]. The activation of the pre-RC and assembly of the pre-IC occur in G1 phase for the early-firing origins and in S phase for late-firing origins [267]. CDC45 plays an important role in the subsequent DNA replication initiation as well as elongation [268], [265]. SLD3 recruits CDC45 onto the chromosome [269], [270]. DPB11 interacts with DNA polymerases and has a dual role in both DNA replication and DNA damage checkpoint control [271], [272]. In addition, a number of other proteins, including MCM10 and DRC1, have been implicated in the activation of the pre-RC to initiate DNA replication [267].

The activation of the pre-RC is regulated by CDK and DDK. In *S. cerevisiae*, the CDK Cdc28 acts with two B-type cyclins, Clb5 and Clb6, to drive S phase entry and initiation of DNA replication, while in *S. pombe*, CDK Cdc2 works together with Cig2, a B-type cyclin [251]. In metazoans, CDK2, a S phase-specific CDK, functions with cyclins A and E to promote DNA replication [267]. Although not all the CDK targets are known, it is clear that CDK plays an extremely critical role in regulating DNA replication licensing, such as phosphorylating CDC6, regulating its stability or subcellular localization. Another essential S phase kinase is CDC7, which forms an active complex

with the DBF4 protein. CDC7/DBF4 is recruited to origins through binding the ORC. Biochemical evidence in *X. laevis* suggests that the MCM2-7 complex is an important target of the CDC7/DBF4 kinase complex [267]. Only after being modified by phosphorylation can the MCM complex achieve helicase activity and trigger the initiation of DNA replication by unwinding DNA, thus allowing access of DNA polymerase to the origins. Once activated, the MCM2-7 complex dissociates from DNA, preventing the replication origin re-firing [265]. The loading of the MCM2-7 complex is only achieved again in the G1 phase of the next cell cycle because the high CDK activity in G2 and M phase prevents the association of MCM onto origins [273].

Regulation of DNA replication

CDK, as a master regulator of cell cycle progression, is also important in regulating DNA replication even though the regulatory pathway is not completely understood. Through phosphorylating certain substrates, such as MCM, CDK activates pre-RC to trigger the initiation of DNA replication [267], [251]. Most importantly, CDK also prevents the reinitiation of DNA replication by inhibiting the assembly of new pre-RC on the origins which are already activated. Disruption of CDK activity due to either deletion of cyclin, such as Cdc13 in fission yeast, or overexpression of CDK inhibitors in fission or budding yeast, causes multiple round of DNA replication in the absence of mitosis [251]. The block to pre-RC re-assembly during S phase is achieved through multiple redundant mechanisms in different organisms, which employ unique strategies to restrain the replication licensing system [251], [274].

1. Replication licensing factors

The helicase activity of MCM complex makes it crucial for DNA replication. The binding of the MCM to the ORC is known as "licensing". The cell has to be licensed to replicate DNA. The proteins that recruit MCM onto the replication origin in G1 phase are referred to as replication licensing factors. CDT1, CDC6/CDC18, and ORC are replication licensing factors among eukaryotic species [275], [276], [277], [278], [279]. MCM is suspected to play a regulatory role in replication licensing only in budding yeast, where it is exported from the nucleus after replication in S phase [274]. In other eukaryotes examined, MCM localization does not change and therefore its availability for replication initiation may not be a mechanism to limit the origin re-refiring [274].

2. Regulation of replication licensing factors

To achieve an accurate and timely DNA replication, replication licensing factors have to be loaded onto the origin during G1 phase. To prevent the same origin from firing more than once, these same factors have to be removed from origins either at the beginning of S phase or once the initiation of DNA replication occurs. Although the approaches that organisms undertake to regulate the replication licensing factors are different, they all require the involvement of CDK in this process. CDK phosphorylation has at least three effects on replication licensing factors in different species. First, phosphorylation of certain replication licensing factors by CDK promotes their degradation by the proteasome, such as Cdc6/Cdc18 in yeast. Second, phosphorylation causes other replication licensing factors to be exported out of the nucleus at the onset of S phase, such as CDC6 in humans [280]. Finally, high CDK activity stablilizes an inhibitory subunit Geminin, which binds licensing factors and thus prevents DNA rereplication [146].

2.1. The regulation of CDC6

In budding yeast and fission yeast, Cdc6 and Cdc18, respectively, play a key role in assembling MCM proteins onto the origins of DNA replication [277], [281]. Consistent with its role as a licensing factor, via transcriptional activation, Cdc6/Cdc18 protein levels increase in the late mitosis and G1 phase and accumulate in the nucleus [278]. Immediately before S phase entry, Cdc6/Cdc18 is phosphorylated by CDK and then degraded by ubiquitin-mediated proteolysis, employing SCF^{Cdc4} as the E3 for Cdc6 and SCF^{Pop1/2} for Cdc18 [282], [283], [284], [285], [140]. A failure to degrade Cdc18 causes reinitiation of DNA replication in fission yeast [140], [286]. Further, re-replication phenotypes are observed in fission yeast upon overexpression of Cdc18 and in a Cdc18 mutant that produces a stable Cdc18 protein lacking the N-terminal CDK phosphorylation sites [287], [288], [289]. A Cdc6 gain of function mutant also produces a similar re-replication phenotype in budding yeast [290]. Human and Xenopus CDC6/CDC18 orthologs are also essential for assembly of MCM [291], [292], [293]. However, in contrast to yeast, human CDC6/CDC18 levels remain constant during the cell cycle [294]. Human CDC6 accumulates in the nucleus in mitosis and early G1 phase, but at the onset of S phase most of the CDC6 is exported out of nucleus rather than subjected to proteolysis [294], [295], [296].
2.2 The regulation of CDT1

Cdt1 is also a replication licensing factor and essential for the loading of MCM and the assembly of the pre-RC [297], [274]. Cdt1 was first identified in fission yeast with mRNA and protein levels peaking in late mitosis and early G1 phase [275], [278]. A Cdt1 homolog has recently been identified in budding yeast and also has an important role in DNA replication licensing [298]. Budding yeast Cdt1 is constitutively present through out the cell cycle. Nevertheless, budding yeast Cdt1 accumulates in the nucleus in late mitosis and early G1 phase and is then transported into the cytosol so that it is not present in S phase nuclei. In contrast, both fission yeast and human CDT1 accumulate in G1 nuclei but are then degraded after the initiation of S phase by the proteasome [275], [299], [300]. In addition, in both humans and *Xenopus*, an inhibitory subunit, Geminin, binds to CDT1 through out S, G2, and M phase [146], [301]. At the metaphase to anaphase transition, when CDK activity drops, Geminin is targeted for degradation by APC [302]. This releases CDT1 to load the pre-RC onto the origin in the G1 phase. However, in the human somatic cell cycle, CDT1 is mainly regulated by proteolysis instead of Geminin binding [300].

2.3 The regulation of ORC

Recently, components of the ORC have been recognized as replication licensing factors. In *S. cerevisiae*, the Orc2 subunit is phosphorylated by CDK in late G1, S, G2, and M phase. This phosphorylation occurs in other species including humans and *X. Lavis* and negatively regulates the activity of the ORC complex [303], [304], [305]. Apparently, this negative phosphorylation does not mark Orc2 subunit for degradation in

humans. However, it may still be possible that in other species, Orc2 is degraded. In humans, ORC1 is targeted by SCF^{Skp2} for ubiquitin-mediated degradation beginning at the S phase [279].

The Cullin CUL-4

Vertebrate CUL4

CUL-4 is a member of the cullin family, which is conserved in metazoan [77]. The RING finger protein RBX1 can associate with all cullins including CUL4 to assemble ubiquitin ligases [80]. Similar to other cullins, CUL4 is also modified by Nedd8 [98]. In humans there are two CUL-4 homologs, *CUL4A* and *CUL4B*. *CUL4A* is overexpressed in both breast cancer and hepatocellular carcinomas [306], [307]. Overexpression of *CUL4A* can abrogate the G2/M cell cycle checkpoint in response to DNA damage induced by ionizing radiation [308]. The UV-damaged DNA binding protein (DDB) was found to be ubiquitinated and targeted for proteolysis by CUL4A [309]. This suggests that CUL4A can form ubiquitin ligase(s) at least in humans.

C. elegans CUL-4

CUL-4 is a key cell cycle regulator that has been shown to regulate DNA replication. Inactivation of *cul-4* causes massive increases in the level of genomic DNA due to reinitiation of DNA replication. Below is a summary of *C. elegans* CUL-4 function, which was characterized by Weiwei Zhong, a previous graduate student in our lab.

1. cul-4 RNAi causes massive increases in DNA amount

In *C. elegans*, the orthologous *cul-4* gene is expressed throughout development. *cul-4* functions were probed by inactivating the *cul-4* gene with RNAi. *cul-4* RNAi is very effective since it reduces *cul-4* mRNA to levels not significantly higher than background. The *cul-4(RNAi)* phenotype in the progeny of injected hermaphrodites is predominantly a developmental arrest at the L2 larval stage. In the arrested *cul-4(RNAi)* larvae there is a dramatic increase in the size of blast cell nuclei. Larger cells were observed among the blast cell lineages: V, M, Q, P, and the somatic gonad cell lineages. The amount of genomic DNA in the enlarged *cul-4(RNAi)* blast cells is dramatically elevated, with over 100n DNA content in seam cells of two or three days arrested *cul-4(RNAi)* larvae, compared to the 2n DNA content of wild type seam cells.

2. The increase of DNA in *cul-4(RNAi)* cells is due to re-replication

Zhong et al. used three criteria to distinguish between possible models for increased ploidy: failed mitosis, endoreplication, and re-replication. First, the percentage of mitotic cells was determined with immunofluorescence using antibody against phosphorylated histone H3, which is one of the earliest mitotic markers [186]. Compared to unsynchronized wild type larvae, the percentage of mitotic seam cells in *cul-4(RNAi)* larvae decreased 42-fold. In addition, mitotic entry (as indicated by nuclear envelope breakDaown) was not observed in the seam cell lineage of *cul-4(RNAi)* L1 larvae. Therefore, the lack of mitotic entry in *cul-4(RNAi)* blast cells precludes the failed mitosis mechanism, in which a failure to separate sister chromatids and perform cytokinesis causes cells to enter G1 phase with a doubled DNA content.

Second, the duration of S phase was determined for the *cul-4(RNAi)* V4 seam cells using a ribonucleotide reductase (rnr) promoter::GFP strain. The rnr::GFP expression began in both wild type and *cul-4(RNAi)* seam cells at 2 hr post-hatch. While the wild type rnr::GFP signal ended at approximately 5 hr post-hatch, *cul-4(RNAi)* seam cells expressed rnr::GFP continuously through 15 hr post-hatch. In addition, the extent of DNA synthesis was followed using bromodeoxyuridine (BrdU) incorporation at 5-11 hr post-hatch, a time between seam cell divisions. During this time window, 0% of BrdU incorporation was observed in wild type seam cells, whereas 87.5% of *cul-4(RNAi)* seam cells actively incorporated BrdU. This indicates that *cul-4(RNAi)* cells undergo an S phase arrest in which cells continuously synthesize DNA, thereby suggesting the rereplication model.

Third, the DNA content of cul-4(RNAi) seam cells was measured and found to increase continuously, rather than in doublings of 2n as occurs in wild type intestine cells that undergo endoreplication at each larval molt [310]. This further supports the rereplication model since in both the failed mitosis and endoreplication mechanisms, increases in ploidy occur through doublings of genomic DNA, while in the re-replication mechanism ploidy increases are not quantitized. Taken together, these data suggest that the dramatically increased ploidy in cul-4(RNAi) cells results from DNA re-replication.

RESULTS

CDT-1 is a licensing factor in *C.elegans*

The re-replication phenotype of cul-4(RNAi) cells suggested a defect in the control of DNA replication licensing, which functions to limit the extent of DNA replication in S

phase. Cdt1 is the only protein that has been shown to function as a temporally regulated licensing factor in all yeast and metazoan cells examined [275], [311], [312], [313], [300], [314]. The *C. elegans* replication licensing system has not been characterized. We identified a single *C. elegans* Cdt1 ortholog, *cdt-1*. In pairwise alignments, CDT-1 has 23%, 20%, 19%, and 17% identity with its *H. sapiens*, *D. melanogaster*, *S. pombe*, and *S. cerevisiae* orthologs respectively [275], [312], [313], [146]. Inactivation of *cdt-1* by RNAi produced embryos defective for DNA replication (Fig. 4.1). *cdt-1(RNAi)* embryos arrest with approximately 60 cells that contain only trace amounts of DNA, indicating that there is a virtually complete cessation of DNA replication, although cell division continues (Fig. 4.1). In early mitotic divisions, *cdt-1(RNAi)* embryos have unequal DNA segregation and DNA bridges between dividing cells, which are presumably secondary consequences of the defective DNA replication (data not shown). The above data suggest that *C. elegans* CDT-1 is required for DNA replication.

CDT-1 accumulates in *cul-4(RNAi)* cells

We generated affinity-purified antibodies against the full-length CDT-1 protein and used these to determine the CDT-1 expression pattern by immunofluorescence. In adults, CDT-1 is present in germ cell nuclei and is enriched in oocyte nuclei (data not shown). In early embryos, CDT-1 protein is localized to chromosomes in mitotic cells during late metaphase, anaphase, and early telophase (Fig. 4.2a; data not shown). CDT-1 nuclear staining is not present in S phase, which directly follows mitosis in the early embryo (Fig. 4.2a). The anti-CDT-1 staining in embryos is abolished by *cdt-1* RNAi, indicating the validity of the antibody staining pattern (Fig. 4.2a). *cul-4* RNAi generates blast cells with

massively increased DNA content. We observed bright CDT-1 staining in the enlarged cells which underwent re-replication (Fig 4.2b). Nevertheless, CDT-1 did not accumulate in cells that do not exhibit re-replication (Fig 4.2b).

CDT-1 accumulation correlates with DNA re-replication

To check if the accumulated CDT-1 protein contributes to the increased ploidy in cul-4(RNAi) blast cells, two day old arrested cul-4(RNAi) larvae were fed with BrdU for 6-10 hr before harvesting for immunofluorescence. Approximately half of the enlarged cells were found to be actively synthesizing DNA, as shown by incorporation of BrdU, and of these cells, 98% (97/99) had CDT-1 protein (Fig 4.3), which demonstrates that CDT-1 is present in S phase cells. The CDT-1 protein in re-replicating cells is generally nuclear, but can also be present in both the nucleus and cytoplasm (Fig. 4.2b). The correlation between CDT-1 accumulation and DNA re-replication suggested that CDT-1 contributes to the increased ploidy in cul-4(RNAi) larvae cells.

CDT-1 remains constant in *cul-4(RNAi)* cells

The regulation of CDT1 differs among eukaryotic species. Some eukaryotes, such as fission yeast and humans, regulate CDT1 by proteolysis, while others such as budding yeast export CDT1 out of the nucleus [275], [300], [276]. Interestingly, human and *Xenopus* cells also inactivate CDT1 by an inhibitory subunit, Geminin [315], [146]. To gain insights into the regulation of *C. elegans* CDT-1, the timing of CDT-1 expression was examined in the first cell divisions of the V1-V6 seam cells, which enter S phase at 2 hr post-hatch and undergo mitosis at 5 hr post-hatch [316]. In newly hatched larvae, no

cells have detectable CDT-1 expression. However, CDT-1 levels are transiently increased in the nuclei of all blast cells prior to their mitotic divisions. CDT-1 expression started at approximately 20 min post-hatch in a subset of seam cells and by 2 hr post-hatch all seam cells expressed CDT-1 at high levels (Fig. 4.4; data not shown). At 2 hr 10 min posthatch, the number of seam cells with CDT-1 protein dropped precipitously and by 2 hr 30 min most seam cells no longer had CDT-1 protein (Fig. 4.4; Fig 4.5a). The disappearance of CDT-1 protein coincided with the onset of S phase at 2 hr post-hatch. The rapid drop in CDT-1 levels suggests that CDT-1 protein is being degraded.

In cul-4(RNAi) animals there was faint CDT-1 expression in some seam cells at hatch, but this expression increased in the L1 stage with a time course similar to that of wild type, so that at 2 hr post-hatch all seam cells expressed CDT-1 with a staining intensity similar to wild type (Fig. 4.4; data not shown). However, in marked contrast to wild type, CDT-1 levels did not drop after 2 hr post-hatch but remained constant through S phase (Fig. 4.4; Fig 4.5b). To determine if CDT-1 in wild type cells is exported out of the nucleus instead of being degraded, both wild type and cul-4(RNAi) CDT-1 stained images were scanned by confocal microscope to quantitate the nucleus and cytosol CDT-1 level. At 3 hr 30 min post hatch, CDT-1 dispears from the nuclei of wild type seam cells, while CDT-1 still remains in the nucleus of cul-4(RNAi) cells. The intensity of the cytosol level of CDT-1 in *cul-4*(RNAi) cells is about 2-fold higher than that in wild type cells (data not shown). This demonstrates that the total cellular level of CDT-1 in *cul*-4(RNAi) cells is higher than in wild type and that CDT-1 fails to be degraded upon inactivation of *cul-4*. These results indicate that the rapid decrease of CDT-1 protein levels in S phase cells depends on the presence of CUL-4.

Removal of CDT1 suppresses cul-4 re-replication

If a failure to eliminate CDT-1 from S phase cells were contributing to the cul-4(RNAi) re-replication phenotype then removal of one copy of the *cdt-1* gene might reduce the CDT-1 level sufficiently to suppress the phenotype. We tested for this genetic interaction by analyzing the extent of re-replication upon *cul-4* RNAi in a strain heterozygous for the qDf4 deficiency that removes the cdt-1 gene. Strikingly, the cul-4(RNAi) re-replication phenotype was suppressed five-fold in the heterozygous strain (Fig. 4.6). In contrast, there was no significant suppression of the cul-4(RNAi) rereplication phenotype in a strain heterozygous for a deficiency that deletes the ortholog of the yeast replication licensing factor Cdc6 (Fig. 4.6). Interestingly, depletion of *cul-4* by RNAi in the heterozygous *cdt-1* deficiency strain still produced a penetrant L2 larval arrest, suggesting that the L2 larval arrest and DNA re-replication phenotypes arise from two genetically separable CUL-4 functions. Our results suggest that the requirement of CUL-4 for the maintenance of stable genome ploidy derives in part from CUL-4mediated removal of CDT-1 in S phase cells, which ensures that CDT-1 is not available to initiate additional rounds of replication origin firing.

DISCUSSION

CUL-4 negatively regulates CDT-1 levels

Mammalian CUL4A is overexpressed in breast cancer and this overexpression is correlated with a low level of DDB resulting from targeted ubiquitin-mediated proteolysis [306], [309]. As we have shown, *C. elegans* CUL-4 is required to restrain DNA replication licensing. Inactivation of *cul-4* causes cells to remain in S phase and reinitiate DNA replication. This increased ploidy correlates with accumulated CDT-1 in S phase cells. Further, while CDT-1 is rapidly removed from S phase cells in wild type, this does not occur in *cul-4*(RNAi) cells (Fig 4.4 and Fig 4.5a,b). Our results indicate that CUL-4 is required for the degradation of CDT-1 in S phase. We propose that this is a direct function of CUL-4 ubiquitin-ligase activity. A failure to remove CDT-1 from S phase cells contributes to the DNA re-replication phenotype observed in *cul-4*(RNAi) cells.

In *Xenopus* early embryonic cell divisions, Cdt1 is regulated by binding to the inhibitory protein Geminin [315]. In mammalian tissue culture cells, Cdt1 is degraded in S phase and Geminin is only observed after Cdt1 has been degraded, suggesting that Geminin performs only a secondary role to inhibit Cdt1 that may escape degradation [300]. Our observation that *cul-4(RNAi)* cells fail to remove CDT-1 from S phase cells indicates that other CDT-1 control mechanisms, if they exist, cannot compensate for a failure to degrade CDT-1 in S phase.

CUL-4 may negatively regulate replication factors other than CDT-1

In other eukaryotes, redundant controls operate to restrain DNA replication licensing [274]. In yeast, disruption of multiple redundant licensing controls is required to produce substantial re-replication [317], [278]. In vertebrates, disruption of the replication licensing system has not yet been shown to produce re-replication. That inactivation of only a single gene, *cul-4*, leads to massive re-replication implies that CUL-4 is likely to regulate multiple aspects of DNA replication licensing.

Potential other replication licensing candidates regulated by CUL-4 include CDC-6 and ORC-1. Both CDC6/CDC18 and subunits of ORC are demonstrated to be essential for DNA replication licensing in yeast and vertebrates [274]. Yeast Cdc6/Cdc18 is regulated through ubiquitin-mediated proteolysis [282], [283], [284], [285], [140]. In addition, human ORC1 is targeted for degradation [279]. Therefore, it is possible that CUL-4 regulates CDC-6 and/or ORC-1 by ubiquitin-mediated degradation to prevent reinitiation of DNA replication. C. elegans has one cdc-6 ortholog and one orc-1 ortholog. RNAi of *cdc-6* produces embryos with a shredded DNA phenotype in mitotic cells, indicative of a failure of DNA replication. Overall, the cdc-6(RNAi) defect in DNA replication, while readily apparent, is not as severe as that seen in *cdt-1*(RNAi) embryos (Zhong and Kipreos, personal communication). Similar to CDT-1, CDC-6 protein accumulates in the large re-replicating cul-4(RNAi) cells. It would be interesting to determine the CDC-6 expression pattern during the cell cycle to determine if CDC-6 is degraded upon entry into S phase. For ORC-1, we have already generated polyclonal antibody and are in the process of checking ORC-1 protein level in *cul-4*(RNAi) cells.

Both CUL-4 and the DNA replication licensing factors are conserved among species. Different organisms employs somewhat distinct mechanisms to regulate replication licensing. It will be interesting to learn whether CUL-4 orthologs play similar roles in regulating DNA replication licensing in other metazoans.

MATERIALS AND METHODS

Genetics and RNAi

The following strains of C. elegans were used: KR926 [hDf6 dpy-5(e61) unc-

13(e450)/szT1, unc-3(e151)/szT1]; RE249 [qDf4/szT1 I; +/szT1]; JR667 [unc-119(e2498::Tc1) III, wIs51 (a seam cell::GFP marker)]. RNAi was performed using double stranded RNA (dsRNA) derived from cDNA clones for *cul-4* (cDNA clone yk34c8, GenBank acc. no. D36543) and *cdt-1*/Y54E10.15 (yk10c5, acc. no. D34651). Sense and antisense RNA were made using the T3 and T7 MegaScript kits (Ambion), respectively. dsRNA was annealed and injected at a concentration of 0.5-1 mg/ml into young adults as described [124]. RNAi was also conducted by the feeding method [318], which generated identical phenotypes.

Antibodies and immunofluorescence

Anti-CDT-1 sera were produced in rabbits using purified recombinant His-tagged full-length CDT-1 fusion protein expressed from the pET15b vector (Novagen). Affinity purification was performed with His-CDT-1 fusion protein linked to activated CL4B Fast flow Sepharose (Amersham). Affinity-purified anti-CDT-1 antibodies from two different rabbits gave the same pattern of immunofluorescence staining. Other antibodies used were directed against anti-tubulin (N356, Amersham); anti-nuclear pore (Mab414, BabCo); anti-AJM-1 [319] (MH27, Developmental Studies Hybridoma Bank); and antiphosphorylated histone H3 (Upstate Biotechnology). Secondary antibodies were antirabbit and anti-mouse Alexa Fluor 488, 546, and 633 (Molecular Probes). Immunofluorescence was performed on animals fixed using the "freeze-crack" method as described [197]. DNA was stained with either 1 µg/ml DAPI, or with 50 µg/ml propidium iodide (PI) after 20 µg/ml RNAase A treatment for 1 hr at 37° C. For analysis of CDT-1 expression in L1 larvae at set times post-hatch, pretzel-stage embryos were collected and observed, larvae that hatched were transferred at 10 min increments to plates with $E \ coli$ OP50 as feeding bacteria. For each time point an average of 9.4 L1 larvae were analyzed for CDT-1 expression.

Microscopy

Animals were observed by differential contrast interference (DIC) and immunofluorescence microscopy using a Zeiss Axioskop microscope. Images were taken with a Hamamatsu ORCA-ER digital camera with Openlab 3.0.8 software (Improvision). All images were processed with Adobe Photoshop 6.0. Matched images were taken with the same exposure time and processed identically. Matched images of anti-CDT-1, anti-AJM-1, and DAPI staining for Fig 4.5 were deconvolved to equivalent extents to minimize background fluorescence using the multineighbor deconvolution program of Openlab.

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Fig 4.1: *cdt-1(RNAi)* **phenotype**. DIC, DAPI, and anti-nuclear pore stain of wild type 60-cell stage and arrested *cdt-1(RNAi)* embryo. Scale bars represent 10 μm.



Fig 4.2: Anti-CDT-1 staining. a, Anti-CDT-1 antibody is specific. anti-CDT-1, DAPI, and anti-tubulin stain of wild type and cdt-1(RNAi) 2-cell stage embryos. The exposure of the DAPI image for the cdt-1(RNAi) embryo is twice as long as for wild type to allow visualization of the lower level of DNA. In the wild type embryo, the right cell is in anaphase and the left cell is in late telophase. In the cdt-1(RNAi) embryo, the right cell is in anaphase and the left cell is in metaphase. Arrows indicate anaphase chromosomes. **b**, Enlarged seam cell in arrested cul-4(RNAi) L2 larvae stained with anti-CDT-1 antibody and DAPI (blue). Scale bars represent 10 μ m.





Fig 4.3: Anti-CDT-1, anti-BrdU, and DAPI staining of enlarged seam cell in arrested *cul-4(RNAi)* L2 larvae. Scale bars, 10 μm.



Fig 4.4: Graph of the average number of V1-V6 seam cells that had nuclear CDT-1 staining in L1 wild type and cul-4(RNAi) larvae at the times indicated post-hatch. Black squares denote wild type seam cells and red diamonds denote cul-4(RNAi) seam cells.



Fig 4.5: Wild type (**a**) and *cul-4(RNAi)* (**b**) L1 larvae 3 hr 20 min post-hatch, stained with CDT-1 and DAPI (blue). Anti-AJM-1 [252] staining of adhesion junctions at seam cell boundaries (red) is overlaid to highlight the V1-V6 seam cells, which are labeled. Scale bars, 10 μm.



b

cul-4 RNAi



Fig 4.6: DNA content of *cul-4(RNAi)* seam cells in the genetic background of either wild type or strains heterozygous for deficiencies qDf4 or hDf6, which delete (Δ) *cdt-1* or *cdc-6*, respectively. Heterozygous strains of these deficiencies are wild type and have normal DNA replication (data not shown).



CHAPTER V

GENERAL DISCUSSION

THE CONTRIBUTIONS OF THE RESEARCH

Using the genetically-tractable nematode *C. elegans* as a model system, we have learned the functions of two cullin members, CUL-2 and CUL-4. Both CUL-2 and CUL-4 are cell cycle regulators, similar to the more widely studied CUL-1. Moreover, we have uncovered functional molecular pathways for both CUL-2 and CUL-4. The identification of CUL-2 interactors strongly suggests that CUL-2 functions in multiple cullin/RING finger complexes to target substrates for ubiquitin-mediated proteolysis. Our analysis also indicates that CUL-2 may have a role in transcriptional regulation. CUL-4 functions to restrain DNA replication licensing in the cell cycle by potentially targeting CDT-1 for degradation. By uncovering the functions of *C. elegans* CUL-2 and CUL-4 our research has enriched the understanding of cell cycle control by ubiquitin-mediated proteolysis and provided indications for the functions of their orthologs in other species.

C. elegans CUL-2

What have we learned about CUL-2? By analyzing the phenotype resulting from the inactivation of the *cul-2* gene, via both RNAi and the isolation of a molecular null deletion allele (*ek1*), we discovered that CUL-2 is a positive cell cycle regulator. In *cul-2* mutants, germ cells begin to slow down their cell divisions in the L3 larval stage and undergo a G1 phase cell cycle arrest in the L4 stage. We determined that the G1 arrest is caused, at least in part, by a post-transcriptional increase in the level of the negative cell cycle regulator CKI-1, a member of the CIP/KIP family of CDK inhibitors.

The embryos produced by *cul-2* homozygotes arrest with approximately 24 cells with a phenotype similar to the *cul-2* RNAi. There are four major mutant phenotypes in the *cul-2* mutant embryos. First, there is a defect in chromosome condensation. This primary defect produces a number of secondary defects, including the formation of multiple nuclei, DNA bridges, and unequal DNA segregation. Second, *cul-2* cells are delayed in mitotic progression through prometaphase. Although this delay may be a secondary defect of the uncondensed chromosomes, this would not be expected, as early embryonic metazoan cell divisions, which cycle rapidly between S and M phases, are generally not subject to checkpoints, e.g., defects in DNA replication, mitotic spindle assembly, or DNA condensation have been found not to trigger checkpoints in these cells [320], [321], [322], [323]. Third, there is a basic defect in cytoskeletal organization apparent from three phenotypes. Finally, CUL-2 also functions in meiotic cell cycle regulation as the *cul-2(ek1)* mutant is defective in meiotic anaphase II progression (Liu et al., personal communication).

Currently, besides the characterizations by another two previous graduate students in our lab, five aspects of CUL-2 functions were revealed and promoted another question: through which mechanisms does CUL-2 function to regulate cell cycle events? The indications were obtained from the identification and functional characterization of CUL-2 interactors and potential CUL-2/RING finger complexes. By homology search, we identified the *C. elegans* orthologs of human CUL2/VCB complex components. Using RNAi, we characterized functions of the core complex of potential CUL-2/RING finger complexes, composed of CUL-2, ELC-1, ELB-1, and RBX1. All components of the core CUL-2/RING finger complex phenocopy the *cul-2(ek1)* mutant. However, the potential *C. elegans* CUL-2/VCB complex is not responsible for CUL-2 cell cycle functions since inactivation of the SRC, *vhl*, doesn't phenocopy *cul-2*. We concluded that CUL-2 forms CUL-2/RING finger complexes, using a common core complex and SRCs other than VHL, to regulate aspects of cell cycle progression. The possible E2 enzyme for CUL-2/RING finger complex is UBC-5 rather than Cdc34 since RNAi of *UBC5* but not the *Cdc34* ortholog phenocopies *cul-2*. BC-box proteins are potential SRCs for CUL-2/RING finger complexes the BC-box is essential for the interaction with the core Elongin BC complex [205], [218], [96], [97].

Ten potential CUL-2 interactors were uncovered by CUL-2-FLAG affinity purification and mass spectrometry. Four of them, TIP120, APT-5, VHL, and HMT, are BC-box proteins. We propose that CUL-2 will form at least four CUL-2/RING finger complexes, using these proteins as SRCs respectively. The function of the human CUL2/VCB complex is to target HIF1 α and 2 α for ubiquitin-mediated proteolysis [208], [74], [112], [212], [76]. The function of *C. elegans* CUL-2/VCB complex is probably similar to its human counterpart, targeting HIF1 α for ubiquitin-mediated proteolysis, because in *C. elegans* HIF1 α accumulates in a *vhl* mutant under normoxic conditions [216]. The identification of an ortholog of a mammalian transcription co-activator, TIP120 [240], implicates CUL-2 in transcriptional regulation. In addition, another three CUL-2 interactors, PLP-1 (a transcription activator for genes required in endodermal development) (Witze et al., personal communication), histone H4, and HMT (an ortholog of *Drosophila* suppressor of position-effect variegation 3-9 (Suvar3-9)), were discovered. In humans, mouse, and yeast, this histone methyltransferase specifically methylates lysine 9 of histone H3, inducing gene silencing [228], [243]. This further suggests that CUL-2 regulates transcription potentially by regulating transcription factors or chromatin structures.

Finally, we performed phenotypic screening for *C. elegans* BC-box proteins to look for potential SRCs for CUL-2/RING finger complexes. We expected that inactivation of a SRC of CUL-2/RING finger complexes would produce one or multiple phenotypes similar to that seen in the *cul-2* mutant. Among the 31 candidate BC-box proteins, inactivation of gene *F28D1.1* or *F55F8.3* produced one of the *cul-2* phenotypes, G1 cell cycle arrest. We are very interested if one or both of these BC-box proteins are indeed SRCs of CUL-2/RING finger complexes and if they are responsible for CKI-1 degradation in G1 phase to promote the S phase entry. These results may provide us an opportunity to study the regulation of the G1-to-S phase transition by ubiquitin-mediated proteolysis in *C. elegans*.

C. elegans CUL-4

The function of CUL-4, another member of the cullin family, was characterized by phenotypic analysis upon inactivating the *cul-4* gene with RNAi. *cul-4*(RNAi) predominantly produces progeny arrested at the L2 larval stage. In these arrested larvae, enlarged cells with massively increased DNA were found in multiple tissues. The DNA amount in these enlarged cells, which are arrested at S phase, are not doubled but increased at a random rate, indicating that these cells are undergoing re-replication. In *cul-4*(RNAi) cells, the MCM complex remains on DNA throughout the prolonged S phase, while in wild type, the MCM complex dissociates from the DNA during S phase (data not shown). This suggests that DNA replication licensing is unregulated in the absence of CUL-4 and that CUL-4 is required to restrain DNA replication licensing.

cdt-1, the *C. elegans* ortholog of DNA replication licensing factor, was identified by homology search. Using anti-CDT-1 antibody, we checked the CDT-1 protein expression in wild type and *cul-4*(RNAi) cells. We discovered that CDT-1 accumulates in the *cul-4*(RNAi) enlarged cells, correlating with DNA re-replication. In addition, in wild type, CDT-1 peaks in the nuclei of G1 cells and is removed from nuclei at the onset of S phase. In contrast, CDT-1 remains in *cul-4*(RNAi) cells during S phase. This suggests that CUL-4 is required for the removal of CDT-1 from S phase cells, potentially by ubiquitin-mediated proteolysis. Other mechanisms, if they exist, such as inhibitory subunit binding [146], are not sufficient to restrain CDT-1. A failure to remove CDT-1 in S phase contributes to the reinitiation of DNA replication. In other species, the presence of the CDT1 licensing factor in S phase allows replication origins to be re-licensed due to the re-loading of MCM complex [297], [274]. In *cul-4(RNAi)* cells, MCM complex is reloaded onto the origin, corresponding to the failure to remove CDT-1 in S phase.

Besides CDT-1, there are several other DNA replication licensing orthologs in *C. elegans*, for example, CDC-6 and ORC-1, which are involved in DNA replication licensing in other species [277], [281], [279]. We predict that CUL-4 will also negatively regulate DNA replication licensing factors other than CDT-1 in *C. elegans* to overcome redundant safeguards that ensure proper replication licensing.

A FUTURE PERSPECTIVE OF THE RESEARCH

Our work not only contributes to the understanding of cell cycle regulation by cullin, but also brings out a new research perspective. The identification of CUL-2 interactors generates multiple subunit candidates for CUL-2/RING finger complexes. The indication that CUL-2 may regulate transcription expands a new field for future research. While the G1 arrest in *cul-2* mutant germ cells have been linked to the regulation of CKI-1, other molecular pathways for CUL-2 cell cycle control are still unknown. In addition, in spite of a wonderful start, it is likely that the research for how CUL-4 functions is just at the beginning. Future systematic experiments will need to be done in order to advance our current understanding. The beautiful view of the whole picture awaits many small pieces being fit into place.

The CUL-2/RING finger complexes

Our research gave further support that there are multiple CUL-2/RING finger complexes (Fig 5.1). Beside VHL, three additional SRC candidates, TIP120, APT-5, and HMT (histone methyltransferase), were identified. By bioinformatic approach and RNAi phenotypic screening for CUL-2 interactors among BC-box proteins, at least two BC-box proteins with WD-40 repeats, F28D1.1 and F55F8.3, are included for further checks to determine if they are SRCs for CUL-2/RING finger complexes. Although inactivation of APT-5 or HMT didn't generate any observable *cul-2* phenotypes, we still include them as potential SRCs for the following two reasons. First, in spite of the potency of RNAi in characterizing gene functions, RNAi is not effective for all genes [324]. Second, *cul-2*

phenotypes could be the result of multiple cellular defects and inactivation of a single SRC may not be able to phenocopy any one of the *cul-2* cell cycle phenotypes.

In order to confirm if the above six proteins are indeed SRCs for CUL-2 complexes and to characterize their roles in forming CUL-2/RING finger complexes, a series of questions have to be asked and then addressed. For instance, is any of these SRC candidates a component of CUL-2/RING finger complexes rather than proteins that associate secondarily with the complex? If so, do these SRC candidates directly interact with the Elongin BC complex? Does the interaction require the BC-box motif? What is the composition for each of the CUL-2/RING finger complexes? What are their substrates and their cellular roles? In particular, can any of them be linked to CKI-1 degradation or mitotic chromosome condensation? A variety of molecular and genetic analysis, including co-immunoprecipitation analysis in *C. elegans* and cell culture, the yeast two-hybrid system, and analysis of the loss-of-function and overexpression phenotypes for candidate genes can be used to address some of the above questions.

The transcriptional regulation by CUL-2

Interestingly, our research also suggests that CUL-2 regulates gene transcription. Transcription activators, co-activators, and chromatin associating proteins were identified as CUL-2 interactors. To provide solid evidence for this view, three experimental approaches can be undertaken. First, we can examine the effect on gene expression in the presence or absence of CUL-2. DNA microarray analysis has proven to be a powerful tool to analyze gene expression [325]. If CUL-2 regulates gene transcription, we would expect differences in gene expression by inactivating *cul-2*, compared to wild type and *vhl* mutants. The purpose of using the *vhl* gene expression profile as control is to rule out the possibility that gene expression differences in *cul-2* mutants compared to wild type are solely due to inactivation of the CUL-2/VCB complex, which is likely to target transcription factor HIF1 α for degradation [216]. In addition, we can further check if there is an overlap of gene expression changes upon inactivation of *cul-2*, *tip120*, and *plp-1*.

Second, we can determine if CUL-2 associates with chromatin. Since CUL-2 interacts with chromatin associating proteins, we hypothesize that CUL-2 binds chromatin either directly or indirectly. PLP-1, a transcription activator for *end-1* and *end-3* promoters, was suggested to bind to CUL-2 by our research. Therefore, we can use anti-FLAG antibody to perform CHIP (chromatin immunoprecipitation) assay to pull down CUL-2 associated DNA, then check if *end-1* or *end-3* promoter sequence exists in the pull down or not. In addition, we can detergent extract away CUL-2 that is not chromatin associated then perform immunofluorescence staining to observe if there is any CUL-2 remaining associated with DNA.

Third, we can identify the substrates of CUL-2. The identification of transcription factors or chromatin associating proteins as CUL-2 substrates will provide insight into how CUL-2 regulates transcription. Currently, we suspect that PLP-1 and histone H4 are CUL-2 substrates. We have obtained anti-PLP-1 antibody from the Rothman lab and are in the process of checking the protein level in wild type and *cul-2* mutants. For histone H4, we suspect that CUL-2 modifies histones by participating in a large complex which includes HMT (histone methyltransferase) or other modification enzymes and thus has multiple activities such as methylation and ubiquitination. Human H4 methylation is cell

cycle regulated and carried out by a HMT [326], [247]. It is possible to obtain antihistone antibodies which recognize specific modifications at particular amino acid residues. Using these antibodies, we can compare the histone modification in wild type and *cul-2* to check any possible roles of CUL-2 in modifying histones.

The regulation of DNA replication licensing by CUL-4

We have obtained evidence that *C. elegans* CUL-4 restrains DNA replication licensing in S phase potentially by targeting DNA replication licensing factors for ubiquitin-mediated proteolysis. Our research suggests that CDT-1 is a CUL-4 substrate. However, it is very likely that there are multiple CUL-4 substrates. To further understand how CUL-4 restrains DNA replication licensing, it would be essential to identify additional CUL-4 substrates and to characterize their functions. We would like to ask the following questions: Does CUL-4 negatively regulate multiple DNA replication licensing factors? If so, which factors other than CDT-1 are CUL-4 substrates? Does CUL-4 target their degradation directly or indirectly? If CUL-4/RING finger complex is responsible for targeting DNA replication licensing factors, then which SRC(s) are involved?

Fig 5.1: Multiple *C. elegans* **CUL-2/RING finger complexes. a-c**, Skematic drawing of some of the potential *C. elegans* CUL-2/RING finger complexes. The components for the core complex, CUL-2, ELC-1, ELB-1, and RBX-1, associates with a different BC-box protein, VHL to form a CUL-2/VCB complex (**a**), TIP120 to form a CUL-2/TIP120-CB complex (**b**), or APT-5 to form a CUL-2/APT-5-CB complex (**c**). They may use a UBC-5 as E2 or some other E2s.







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