EVOLUTION OF CYCLIN DEPENDENT KINASES AND THE REGULATION OF MEIOSIS AND POLARITY IN *C. ELEGANS*

by

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(Under the Direction of Edward T. Kipreos)

ABSTRACT

The CDK-cyclin complex is the major regulator of cell cycle. Multiple mechanisms control the CDK-cyclin complex activity, among them there are two important mechanisms: the activating phosphorylation of CDKs by CDK-activating kinases (CAKs) and proteolysis of CDK regulators through the ubiquitination-26s proteasome pathway. This dissertation consists of two projects. In the first study, genome-wide phylogenomic analysis were performed to study the evolutionary relationship of the CDK extended family in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis*, *C. elegans*, *Drosophila*, and humans. The 4x sequenced *Giardia lamblia* genome was also scanned by BLAST, PROFILE, and HMM in search for ortholog of one of the CDK gene, the CAK *Cak1*. We were able to assign orthology to the majority of the CDK genes, and our results allowed us to make the following new observations: 1) CDK-related genes from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *C. elegans*, *Drosophila*, and humans can be put into ten

clades. Among them, seven clades appear to be ancestral, because they contain both yeast and metazoan orthologs. 2) 13 of the *Arabidopsis* CDKs form an exclusive clade with no ortholog from any other organism, suggesting a gene divergence event happening only in plants. 3) The divergent CDK family member, Cak1, has orthologs only in yeasts but not in any other organisms, suggesting that the Cak1 clade is highly divergent and might have not been conserved in eukaryotes. 4) In metazoa, there are divergent CDKs whose orthology could not be assigned. These divergent metazoan CDKs could serve as candidates for functional analysis in search of unidentified metazoan CAK(s).

In the second study, insights into meiotic cell cycle control as well as anterior-posterior polarity in *C. elegans* were gained by studying animals that are depleted of the CUL-2 cullin/RING finger ubiquitin ligase complex, which has been shown to regulate protein degradation. We found that in *cul-2* mutant, the anaphase II chromosomes movement is severely delayed or abolished, and the early embryo polarity cannot be established correctly. Our result showed that the CUL-2 cullin ring-finger complex is required for anaphase II-specific chromosome movement but not for chromosome separation. This study provides the first evidence that cullin/RING finger complex-dependent protein degradation is needed to regulate meiotic anaphase and polarity.

INDEX WORDS: CDK, CAK, Phylogenomics, Phylogeny, Evolution, Protein ubiquitination, Protein degradation, CUL-2, Cullin RING-finger complex, Meiosis, Anaphase chromosome movement, Polarity, Embryo, *C. elegans*

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DEDICATION

This dissertation is dedicated to my dear Mom and Dad, without whom this dissertation would be impossible.

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TABLE OF CONTENTS

Page

CHAPTER 1

LITERATURE REVIEW

One of the major discoveries during the past 20 years is that cell cycle progression is largely regulated by the activity of a class of kinase complexes that are conserved in all eukaryotes. CDK-cyclin complexes are composed of a kinase called cyclin-dependent kinase (CDK) and an unstable regulatory protein called cyclin. CDKs are proline-directed kinases phosphorylating serine (S) or threonine (T) residues within a consensus (S/T)P sequence. CDKs are not active as monomers and require binding to a cyclin to become active. By binding to different cyclins at different cell cycle stages, CDKs are able to promote cell cycle progression in response to developmental and environmental cues.

Regulation of the cell cycle by CDKs

In both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, a single CDK (Cdc28 and Cdc2, respectively) is responsible for catalyzing all major cell cycle transitions (Morgan 1997). In higher eukaryotes, there has been an expansion in the number of CDKs that regulate the cell cycle. This expansion allows a specialization of CDKs for particular cell cycle phases.

G1 and S phase CDKs: CDK4, CDK6, CDK3 and CDK2

In mammalian tissue culture cells, the addition of growth factors induces quiescent cells to enter the cell cycle. The G1 phase cyclins D1, D2, and D3 are among the first transcribed genes after growth factor addition (Matsushime et al., 1991; Sherr, 1995; Won et al., 1992). The three cyclin D proteins bind and activate CDK4 or CDK6. These complexes are responsible for phosphorylating the major G1 phase regulator retinoblastoma protein (Rb). Rb family members negatively regulate G1 phase progression. When unphosphorylated, Rb binds to the E2F family transcription factors and inhibits their function. The phosphorylation of Rb releases E2F from Rb and enables it to transcribe downstream genes (Ikeda et al., 1996, Moberg et al., 1996). One of the E2F target genes is cyclin E, a G1-S phase cyclin (Botz et al., 1996; Geng et al., 1996). Once expressed, cyclin E binds to CDK2 to promote S phase entry and DNA replication (Dulic et al., 1992; Koff et al., 1992).

The function of CDK3 in cell cycle regulation is not well understood. CDK3 expression level is very low, rendering *in vivo* detection difficult (Meyerson et al., 1992, van den Heuvel and Harlow, 1993). As a result, neither a CDK3-associated cyclin nor an activation pattern has been reported. CDK3 has been proposed to be required for G1 phase progression because dominant negative *cdk3* mutants block cells in S phase (van den Heuvel and Harlow, 1993) and overexpressed CDK3 can assemble with E2F *in vivo* (Hofmann and Livingston, 1996). However, a mouse *cdk3* nonsense mutant, which is likely a functional null, is healthy without any obvious cell cycle defects, indicating that CDK3 activity, if essential, must be redundant with other CDKs (Ye et al., 2001).

The G2 and M phase CDK: CDK1

The activation of the CDK1/cyclin B1 complex at late G2 phase triggers entry into mitosis. In contrast to other CDKs that have more regulatory roles, CDK1/cyclin B activity directly induces many of the dramatic cellular rearrangements that occur during mitosis. Its substrates include: nuclear lamins, whose phosphorylation is required for nuclear envelope breakdown (Dessev et al., 1991); Eg5, whose phosphorylation is required for bipolar spindle formation (Blangy et al., 1995); the general transcription factor TFIIIH, whose mitotic phosphorylation inhibits transcription (Long et al., 1998); and the ribosomal S6 protein kinase, whose M phase phosphorylation inhibits translation (Papst et al., 1998).

***Arabidopsis* CDKs and the cell cycle**

Plant development is unique among multicellular organisms in that its cell cycle is generally restricted to a specific tissue, the meristems. Cells that have grown out of the meristems usually only elongate but do not divide anymore. Nevertheless, the major cell cycle machinery is still conserved between plants and other eukaryotes (Huntley and Murray, 1999).

Plant CDKs have been loosely classified based on their sequence features, expression patterns, and functions. A-type CDKs all contain a PSTAIRE sequence, which is the canonic cyclin-interacting motif on CDKs. A-type CDKs are expressed constitutively and have been shown to regulate various cell cycle stages (Fobert et al., 1996, Mironov et al., 1999). Other plant CDKs have variant sequences at the equivalent position of PSTAIRE. Among them, B-type CDKs are expressed during S-to-M phase

and have been proposed to function as mitotic CDKs (Segers et al., 1996, Mironov et al., 1999). C-type CDKs share homology to human CDK9 and CHED kinases and are expressed in specific tissues such as flowers (Lessard et al., 1999).

Regulation of transcription by CDKs

CDKs are not merely cell cycle regulators. They also regulate transcription by phosphorylating the carboxyl-terminal domain (CTD) of RNA polymerase II (RNAP II) as well as a range of general and regulatory transcription factors (Oelgeschlager, 2002, Inamoto et al., 1997, Ko et al., 1997).

Transcription initiation requires an assembly of a transcription preinitiation complex (PIC) on the core promoter region of the gene. PIC consists of RNAP II and six general transcription factors TFIIA, -B, -D, -E, -F, and -H (for a review, see Lee and Young, 2000). The CTD is located on the largest subunit of RNAP II. It contains a heptapeptide sequence, YSPTSPS that repeats multiple times. The sequence is conserved among eukaryotes, but the number of repeats varies (Allison et al., 1988). The phosphorylation of CTD plays an important role in transcription. CTD has to be in a hypophosphorylated state for RNAP II to form the PIC (Chesnut et al., 1992, Usheva et al., 1992), and it has been suggested that CTD phosphorylation facilitates promoter clearance by destabilizing the PIC and allowing RNAP II to proceed away from the promoter (Zhang and Corden, 1991).

Four budding yeast CDKs, Kin28, Srb10, Sgv1, and Ctk1 have been found to phosphorylate the RNAP II CTD. In metazoa, Kin28's ortholog CDK7, Srb10's ortholog CDK8, and Sgv1's ortholog CDK9 were also shown to be CTD kinases.

CDK7, TFIIH and CTD phosphorylation

Both CDK7 and its budding yeast ortholog Kin28 are components of TFIIH. TFIIH is one of the general transcription factors that are required for PIC assembly. TFIIH exists in two forms. The "core TFIIH" is composed of two ATP-dependent DNA helicases and functions in nucleotide excision repair. The "holo TFIIH" consists of the core and a CDK7-cyclin H-Mat1 complex that provides a CTD kinase activity (for a review, see Svejstrup et al., 1995).

TFIIH-mediated CTD phosphorylation has been shown to stimulate RNAP II transcription *in vitro* (Orphanides et al., 1996). *In vivo* experiments showed that the transcription of the majority of yeast genes is dependent on Kin28 (Valay et al., 1995, Cismowski et al., 1995). However, the exact role of TFIIH in CTD phosphorylation is not well understood (Oelgeschlager, 2002).

Other CDK functions

A unique CDK, CDK5, functions to regulate neither the cell cycle nor transcription. It is only detectable in non-cycling cells and functions mainly in neuron development (for a review, see Smith and Tsai, 2002). CDK5 does not associate with a cyclin although one of its binding partners, p35, has a cyclin fold (Tang et al., 1997). The phosphorylation pattern of CDK5 is also unique: structural analysis predicts that T-loop phosphorylation inhibits CDK5 activity instead of activating it (see discussions below, Tarricone et al., 2001).

Many CDKs do not contain the signature PSTAIRE sequence. Instead, they have PCTAIRE, PISSLRE or other sequences at the equivalent position. The exact biological functions of these CDKs are not clear (Morgan, 1997).

Regulation of CDK activity

CDK activity is tightly regulated through four mechanisms: 1) binding to activating cyclins; 2) binding to inhibitory cyclin-dependent kinase inhibitors (CKIs); 3) inhibitory phosphorylation of the CDK by Ser/Thr and Tyr dual function kinases; and 4) activating phosphorylation of the CDK by CDK-activating kinases (CAKs).

Binding to cyclins

Cell cycle CDKs are inactive unless bound to cyclins, and cyclin expression patterns are strictly regulated during the cell cycle. A failure to express cyclins leads to cell cycle delay or arrest, and a failure to degrade cyclins at the appropriate time can cause severe cell cycle defects. For example, cyclin E expression has to be turned on in late G1 phase to allow S phase entry (Botz et al., 1996; Geng et al., 1996). Cyclin E then has to be degraded in S phase to ensure a proper cell cycle progression: In mice, failure to degrade cyclin E leads to cell over proliferation and embryonic lethality (Dealy et al., 1999; Wang et al., 1999).

Binding by inhibitory cyclin-dependent kinase inhibitors (CKIs)

CKIs can respond to a broad range of anti-growth signals such as senescence (Alcorta et al., 1996), contact inhibition (Polyak et al., 1994), and DNA damage checkpoint controls

(el-Deiry et al., 1993) to negatively regulate the cell cycle. Two categories of CKIs have been identified in mammalian cells, CIP/KIP and INK4. CIP/KIP family members such as $p21^{Cip1}$ and $p27^{Kip1}$ can bind many kinds of CDK-cyclin complexes and inhibit their functions. In contrast, INK4 family members such as $p16^{INK4a}$ and $p19^{INK4d}$ are specific for the G1 phase CDKs, CDK4 and CDK6. They can either bind CDK monomers and prevent them from binding to cyclins, or directly inhibit CDK-cyclin complexes.

Inhibitory phosphorylation of the CDK by Ser/Thr kinases

Phosphorylation of CDKs on the conserved Tyr15 and/or Thr 14 sites inhibits kinase activity (Parker and Piwnica-Worms, 1992). Two Ser/Thr kinases, Wee1 and Myt1, are responsible for the phosphorylation (Lundgren et al., 1991). In yeasts and animals, the inhibitory phosphorylation mainly functions to inhibit the G2-M phase transition of the cell cycle by negatively regulating the activity of mitotic CDKs. The inhibitory phosphorylation is part of the G2 checkpoint machinery and delays the initiation of M phase when morphological defects or DNA damage arise (Tourret and McKeon, 1996, Wells et al., 1999). A phosphatase, Cdc25, is required to remove the phosphates from the CDK and thereby trigger M phase entry.

Activating phosphorylation of the CDK

Most CDKs require activating phosphorylation by a CDK-activating kinase (CAK). A detailed description of CAK activity is presented below.

Structural analyses shed light on CDK regulatory mechanisms

Several studies focusing on the crystal structure of CDK2 and CDK2-cyclin A complexes have shed a great deal of light on how CDK activity is regulated by the four mechanisms listed above (Brotherton et al., 1998; De Bondt et al., 1993; Jeffrey et al., 1995; Russo et al., 1996; Russo et al., 1998). Although the studies focused on CDK2, the information is very likely to be applicable to other CDK-cyclin complexes because of the high level of sequence conservation among CDKs.

CDK2 is composed of two lobes (De Bondt et al., 1993). The amino-terminal lobe (residues 1-85) is small and consists of a single α helix, α1, and a sheet of five antiparallel β-sheets, β1-β5. α1 has a highly conserved PSTAIRE segment, which is the cyclin binding site. PSTAIRE also has a catalytic role because it carries the catalytic residue Glu51. Between β1 and β2 the amino-terminal lobe also has a conserved glycine-rich segment which contains residues Thr14 and Tyr15, both of which are target sites for the inhibitory phosphorylation. The carboxy-terminal lobe is bigger than the N-terminal lobe and is rich in α-helixes. It contains a regulatory T-loop, which includes residues that lie between the conserved DFG and APE motifs (residues 145-147 and 170-172, respectively), and contains the activating phosphorylation site Thr160. A deep cleft is located between the two lobes to bind to ATP and to serve as the catalytic center.

Monomeric CDK2 is inactive. Two major factors in its structure contribute to its inactivity. First, PSTAIRE is positioned in a way that it interferes with interactions between the residues Glu51 (the E in PSTAIRE) and Lys33. As a result, although ATP can still bind to CDK2, its alignment is not correct for the chemical reaction. Second, the T-loop is blocking the substrate access to ATP.

The binding of cyclin A to CDK2 does not markedly change the confomation of the cyclin, but it has a big impact on CDK2 conformation (Jeffrey et al., 1995). Cyclin binding alters the conformation of the PSTAIRE helix and the T-loop on CDK2, and also changes the relative orientation of N- and C-terminal lobes. Upon cyclin binding, the PSTAIRE helix moves and brings the enzymatically important Glu51 side-chain into the catalytic cleft and allows Glu51 to interact with Lys33. As a result, ATP phosphate atoms can now be correctly aligned for catalysis (Jeffrey et al., 1995). Further, cyclin binding also largely relieves the T-loop block and exposes Thr160 for phosphorylation (Jeffrey et al., 1995). Consistent with the structural data, it is worthwhile to mention that cyclin binding is a requirement for activating phosphorylation by CAK in many cases. For example, studies using a starfish CAK showed that CAK can only phosphorylate CDK1 when it is bound to a cyclin (Devault et al., 1995).

An unphosphorylated CDK2-cyclin A complex only exhibits 0.2% of the activity of the fully activated complex (Brown et al., 1999). Activating phosphorylation brings more conformational changes. Basically, Thr160 acts as an organizing center that helps to align catalytic residues inside the cleft. Upon phosphorylation, it contacts three arginine residues: Arg50 of the PSTAIRE helix, Arg126 adjacent to the catalytic residue Arg127, and Arg150 at the start of the T-loop. As a result, a protein-substrate catalytic site is modified to allow for maximum activity.

In contrast to a detailed understanding of the structural significance of the activating phosphorylation, the structural significance of Thr14 and/or Tyr15 phosphorylation is not as clear. Thr14 and Tyr15 are both located on the roof of the ATP-binding pocket. It is therefore possible that phosphorylation could interfere with

ATP or protein substrate binding, or it could prevent ATP from adopting a correct conformation.

The crystal structure of $p27^{Kip1}$ bound to the phosphorylated cyclin A-CDK2 complex, and the crystal structure of $p16^{INK4a}$ or $p19^{INK4d}$ bound to CDK6 have been reported (Russo et al., 1996, Brotherton et al., 1998; Russo et al., 1998). $p27^{Kip1}$ interacts with both CDK2 and cyclin A (Russo et al., 1996). It mimics ATP and inserts a small helix into the CDK2 catalytic cleft to form van der Waals and hydrogen bonds with active site residues. $p27^{Kip1}$ also changes the shape of the catalytic cleft to make many of the residues in the cleft incapable of interacting with ATP. As a result, ATP binding is blocked by the presence of $p27^{Kip1}$.

INK4 inhibits CDK6 through a different mechanism. In the $p16^{INK4a-}$ or $p19^{INK4d-}$ bound CDK6 structure, the inhibitor interacts with both lobes of the CDK by binding next to the catalytic cleft, opposite to the cyclin binding site (Brotherton et al., 1998, Russo et al., 1998). The interaction leads to a conformational change in CDK6, making it unable to bind cyclin. Another impact of INK4 binding is that the ATP binding site is also distorted. This might either reduce ATP affinity for the cleft or disorient bound ATP.

Activating phosphorylation by CDK-activating kinase (CAK)

The activating phosphorylation of CDKs is catalyzed by a CDK-activating kinase (CAK). CAK phosphorylation can lead to a 500-fold increase of activity for the CDK2-cyclin A complex (Brown et al., 1999). CAK is an essential gene for cell cycle progression. In budding yeast, CAK is indispensable for Cdc28 and Kin28 activation (Kaldis et al., 1996,

Thuret et al., 1996, Espinoza et al., 1998, Kimmelman et al., 1999). In *Drosophila*, a lack of CAK activity leads to cell cycle arrest and lethality (Larochelle et al., 1998). CAK has also been shown to be required for meiosis in budding yeast (Sutton and Freiman, 1997, Wagner et al., 1997).

CDK7 is a metazoan CAK

The first CAK was identified biochemically in starfish and Xenopus when researchers fractionated cell extract and looked for activities that could phosphorylate and activate either CDK1 or CDK2 (Fesquet et al., 1993, Connell-Crowley et al., 1993). Both groups of researchers found that their CAK activities were provided by CDK7 and its partner cyclin H. Later the same complex was found to be a CAK in a number of other organisms such as *Drosophila*, mice, and humans (Kaldis, 1999). As discussed above, CDK7 has a major role in transcription activation as a TFIIH component, where the CDK7-cyclin H-Mat1 trimer acts as a CTD kinase. In fact mammalian CDK7-cyclin H-Mat1 complex can exist either inside TFIIH or independent of TFIIH (Drapkin et al., 1996, Roy et al., 1994, Shiekhattar et al., 1995).

Mcs6 and Csk1 are identified in fission yeast as redundant CAKs

The CDK7 ortholog in fission yeast, Mcs6, has also been identified as a CAK (Damagnez et al., 1995). *In vitro* experiments showed that when in complex with its cyclin Mcs2, Mcs6 is able to phosphorylate Cdc2 on the conserved Thr160 equivalent site (Damagnez et al., 1995). Mcs6 is also able to phosphorylate the CTD of RNAP II (Damagnez et al., 1995, Buck et al., 1995). However, cells depleted of Mcs6 can still enter mitosis and

segregate their chromosomes, suggesting that Cdc2 was still active without Mcs6 (Molz et al., 1989, Lee et al., 1999). Later it was found that a non-conventional CDK, Csk1, acts as a CAK to redundantly activate Cdc2 (Lee et al., 1999). Inactivation of both Mcs6 and Csk1 in fission yeast led to Cdc2 inactivation and cell cycle arrest, indicating that there were no other redundant CAKs for Cdc2 activation (Lee et al., 1999). In addition to phosphorylating Cdc2, Csk1 is also a CAK for Mcs6 and is therefore called CAK-activating kinase (CAKAK). Csk1 phosphorylates the Thr160 equivalent site on Mcs6 *in vitro*, and it is depletion led to a 2-3 fold reduction of the Mcs6-Mcs2 kinases activity (Molz and Beach, 1993).

Compared to CDK7/Mcs7, Csk1 is a much more unique CDK for four reasons: the sequence of Csk1 is divergent from other CDKs; it works as a monomer without the requirement for cyclin binding; it activates both cyclin-complexed Mcs6 and monomeric Mcs6 with equal efficiency; and it does not need activating phosphorylation to be active (Hermand et al., 1998).

Cak1 can account for all *in vivo* CAK activities in budding yeast

In contrast to vertebrate or fission yeast CDK7, the CDK7 ortholog in budding yeast, Kin28, only exists in the TFIIH complex and does not show any CAK activity *in vitro* (Valay et al., 1995). The *in vivo* CAK for both Cdc28 and Kin28 in budding yeast was found to be Cak1, another divergent CDK (Thuret et al., 1996, Espinoza et al., 1998). Like Csk1, Cak1 has a divergent sequence and is fully active without a cyclin partner or activating phosphorylation (Kaldis, Sutton, and Solomon 1996; Thuret et al. 1996). No *CAK1* ortholog had been reported in any other organism before our study (see Chapter 2).

The orthology of an *Arabidopsis* CAK is not clear

A CDK7 homolog in rice, R2, has been proposed to be a CAK based on *in vitro* kinase assay and complementation experiments. R2 was found to rescue a budding yeast *cak1* mutant and can phosphorylate human CDK2 at Thr160 (Hata, 1991). However, the *in vivo* function of R2 has not been established.

A similar CAK complementation experiment was carried out in the plant *Arabidopsis,* and Cat1At was identified as a high copy suppressor of one of the budding yeast *cak1* mutations (Umeda, 2002; Umeda et al., 1998). Cak1At also suppresses the fission yeast *mcs6* mutation (Umeda et al., 1998). Cak1At exists in a 180 kDa complex in Arabidopsis, but its binding partner has not been identified (Umeda et al., 1998). Since it is able to complement the yeast mutants without the introduction of additional genes, it is possible that Cat1At works as a monomer. No CTD phosphorylation activity has been observed for Cat1At, and biochemical fractionation shows that Cak1At is in a complex different from the complex containing CTD-kinase activity in Arabidopsis extract. Again, the *in vivo* significance of Cak1At is not clear.

Both R2 and Cat1At proteins have a plant-specific C-terminal region of 60-70 residues that has significant sequence similarity between the two genes. However, although R2 is a CDK7 ortholog, blast searches have failed to find an orthologous gene for *Cat1At* in other organisms (Hata, 1991, Umeda, 2002; Umeda et al., 1998).

CAK regulation

The dual function of CDK7 in both the cell cycle and transcription prompts an exciting proposal that the cell cycle and transcription are coupled through the regulation of CDK7.

However, cell cycle-dependent regulation of CAK activity has not been established. Many studies have reported that CDK7 and Cak1 protein levels as well as their CAK activities do not fluctuate throughout the cell cycle (Tassan et al., 1994; Darbon et al., 1994; Matsuoka et al., 1994; Tassan et al., 1995; Poon et al., 1994; Espinoza et al., 1996, Sutton and Freiman, 1997).

Despite constant CAK activity, the Thr160 phosphorylation on certain CDKs does seem to be regulated. In HeLa cells, CDK2 phosphorylation on Thr160 was found to increase during S and G2 phases (Gu et al., 1992). In *Drosophila*, CDK7 phosphorylation does not change appreciably in a cell cycle-specific manner but instead varies between different developmental stages and tissues (Larochelle et al., 2001). Four mechanisms could contribute to this regulation. First, a physical meeting of CAK and its CDK substrate could be regulated by cell cycle-dependent changes in their subcellular locations. CDK7 has been found to localized in the nucleus (Tassan et al., 1994, Jordan et al., 1997), while Cak1 is mainly cytoplasmic (Kaldis, 1999, Kaldis et al., 1998a). CDKs have been known to travel between nucleus and cytosol in a cell cycle-dependent fashion. For example, CDK1 is in the cytosol during G1, S, and G2 phases but enters the nucleus during early M phase (Ookata et al., 1992, Pines and Hunter, 1991). Second, structural data predicts and experimental data confirms that CDK phosphorylation by CAKs depends on cyclin binding, and the availability of cyclin is cell cycle-dependent (Jeffrey et al., 1995; Morris et al., 2002; Solomon et al., 1993; Kaldis et al., 1998b). Therefore, the cell cycle-dependent fluctuation of cyclins will affect CAK activity towards CDKs. Third, phosphorylation of CDKs by CAK can be blocked by CKIs (Kaldis et al., 1998b, Aprelikova et al., 1995), and the presence of CKIs is cell cycle-

dependent (Morgan, 1997). Finally, the phosphorylation on Thr160 can be removed by the kinase-associated phosphatase, KAP (Hannon et al., 1994). KAP only interacts with monomeric cdk2 and after cell cycle-dependent dissociation of cyclins (Gyuris et al., 1993, Hannon et al., 1994). Therefore, by removing the phosphate from Thr160 in a cell cycle-dependent manner, the phosphorylation state of CDKs can be differentially regulated during different cell cycle phases.

Regulation of TFIIH transcriptional activity

Both the protein level and the activity of the CDK7 CAK activity do not seem to change during the cell cycle (Kaldis et al., 1998a). Then is the TFIIH CDK7 complex regulated in a cell cycle-dependent manner? A few recent reports suggested that this is the case. TFIIH's transcription activity decreases during M phase, and this decrease is mediated by phosphorylation of CDK7 at Ser164, which is near the conserved activating phosphorylation site, Thr170 (Oelgeschlager, 2002). Phosphorylation of either Ser164 or Thr170 has been shown to increase CDK7's CAK activity in *Drosophila*, suggesting that CDK7 in TFIIH and CDK7 in CAK can be differentially regulated during the cell cycle (Larochelle et al., 2001).

Besides phosphorylation, the transcriptional activity of CDK7 can also be regulated by CKI binding. Recent evidence suggests that the INK4a CKI is able to inhibit the CTD kinase activity of CDK7 *in vivo*, thus providing another route to cell cycle-dependent regulation of TFIIH (Nishiwaki et al., 2000; Serizawa, 1998).

Evidence for unidentified CAK in metazoa

In budding yeast, Cak1 is able to account for all *in vivo* CAK activities as it has been shown to be the CAK for both the cell cycle CDK, Cdc28, and the transcription CDK, Kin28 (Espinoza et al., 1998; Kimmelman et al., 1999). In metazoa, however, a number of studies suggest that CDK7 is not able to account for all of the observed CAK activity in cells. In the *Drosophila cdk7* mutant, although CDK1 lost its activating phosphorylation, CDK2 was still phosphorylated at Thr160, suggesting that an unidentified CAK activity for CDK2 was still intact in the *cdk7* mutant (Larochelle et al., 1998). Further, although in *Xenopus* and starfish oocyte extracts CAK activity fractionated as a single peak (Solomon et al., 1993), in vertebrate somatic cells a second CAK activity was detected in addition to the CDK7 complex (Kaldis and Solomon, 2000). This second complex was able to react with an antibody against budding yeast Cak1 (Kaldis and Solomon, 2000), and furthermore, its down-regulation in response to TGF-beta led to decreased CDK2 Thr160 phosphorylation as well as decreased CDK2 kinase activity (Nagahara et al., 1999).

Another puzzle comes from CDK7's own activation. In fission yeast, the CAK for the CDK7 ortholog Mcs6 is Csk1; and without Csk1, Mcs6 activity decreases significantly (Hermand et al., 1998). In metazoa, a CAK for CDK7 has not been identified. However, it has been reported that CDK7 can become active without being phosphorylated on its Thr160: a Thr160Ala CDK2 mutant is fully active when complexed with cyclin H and Mat1 *in vitro*, suggesting that binding to Mat1 can also activate CDK7 (Fisher et al., 1995). Could binding to MAT1 be sufficient to activate CDK7 *in vivo*? A clear answer came from *Drosophila*, where researchers mutated both

phosphorylatable sites Ser164 and Thr170 into alanine residues and asked if the mutated genes could rescue a *cdk7* null mutant (Larochelle et al., 2001). The result was that all mutations, S164A, T170A, or S164A/T170A double mutants were able to rescue the null mutant to some degree. This result suggested that CDK7 phosphorylation on its T-loop was not an absolute necessity for its kinase activity, and that presumably MAT1 binding was able to activate CDK7 *in vivo*. However, the researchers found that the CDK7 T-loop is normally phosphorylated *in vivo* and that phosphorylation is required to form a stable CDK7-cyclin-MAT1 trimer both *in vitro* and *in vivo*. Therefore, the question of what kinase is responsible for CDK7's T-loop phosphorylation still remains. CDK7 self-phosphorylation is not likely because *in vitro* evidence showed that CDK7 was not able to phosphorylate itself (Fisher and Morgan, 1994, Fisher et al., 1995, Labbe et al., 1994). Therefore, it appears that additional metazoan CAKs are waiting to be identified.

The machinery of ubiquitin-dependent proteolysis

Proteins that control cell cycle transitions must be accurately regulated to ensure proper cell cycle progression. The regulators of CDK activity, cyclins and CKIs, are degraded through the ubiquitin-26S proteasome system (King et al., 1996a). Protein degradation not only indirectly regulates cell cycle progression through modulating CDK activities, it also directly triggers one of the most important cell cycle transitions: the metaphase-to-anaphase transition (King et al., 1996a).

The regulation of the cell cycle by cullin/RING finger and APC ubiquitin-ligase complexes

Ubiquitin

Ubiquitin is a highly conserved 76 amino acid protein (for a review, see Hershko and Ciechanover, 1998). Attachment of a single or a few ubiquitin proteins can modify target protein activity or mark target proteins for lysosomal degradation (Hicke, 1999), while attachment of a ubiquitin chain with more than four ubiquitin molecules marks a protein for cytoplasmic degradation by the 26S proteasome (Thrower et al., 2000).

The mechanics of ubiquitination: E1, E2, and E3 enzymes

The first step of ubiquitination is the activation of ubiquitin at its C-terminus. The action is catalyzed by a ubiquitin activating enzyme (E1), which forms a thio lester bond between the C-terminus of ubiquitin and a Cys residue of E1 (Haas et al., 1982). In many organisms including humans and budding yeast, there is only a single E1, called Uba1 (Pickart, 2001).

The activated ubiquitin is then transferred from the E1 to a ubiquitin conjugating enzyme (E2). Again a thio lester bond is formed. There are 11 E2s in budding yeast (Pickart, 2001). Their structures are similar to one another, with highly conserved residues surrounding the catalytic center Cys residue (Pickart, 2001), suggesting a conserved catalytic mechanism.

Ubiquitin-protein ligase enzymes (E3) facilitate the transfer of ubiquitin from the E2 to substrates by binding both the substrates and the E2 and positioning them to catalyze the reaction (Pickart, 2001). The C-terminal Gly76 on ubiquitin forms an

isopeptide bond with a Lys residue either on the substrate or on another already attached ubiquitin molecule (Pickart, 2001).

The E2, generally in combination with an E3, is capable of both attaching the first ubiquitin onto the substrate and elongating the ubiquitin chain on the substrate (Pickart, 2001). However, it has recently been discovered that a novel ubiquitin conjugation factor, E4, specifically elongates the ubiquitin chains on the substrate without interacting with the substrate itself (Koegl et al., 1999). In yeast, inactivation of an E4, *ufd-2*, produced yeast that had poor growth and formed very few colonies under a variety of stress conditions such as heavy metal and ethanol exposure, suggesting that this E4 is involved in stress tolerance (Koegl et al., 1999).

26S proteasome

The 26S proteasome is the major protease for non-lysosomal protein degradation (Pickart, 2001). It is composed of a cylinder-shaped 20S core subunit and two 19S regulatory subunits attached to each end of the cylinder (Conaway et al., 2002). The 20S core is a cylinder composed of four stacked rings, and proteolysis occurs inside the cylinder, separated from the general intracellular environment. The two 19S complexes, each of which has 15-20 subunits comprising a lid layer and a base layer, function to recognize the ubiquitin chain, unfold the substrate, and aid their transport into the narrow openings of the 20S core (Glickman et al., 1998).

Regulation of ubiquitination is largely dependent on the E3

The substrate specificity of the ubiquitination system is mainly decided by E3s (Pickart, 2001). Based on their sequence and structural features, E3s can be categorized into two types: the HECT (homologous to E6-AP carboxy-terminus) domain E3s and the RING (really interesting new gene) finger domain E3s (Pickart, 2001). The HECT domain contains about 350 amino acids, among which is a conserved Cys residue that is essential for forming a thio lester bond with ubiquitin (Huibregtse et al., 1995). HECT E3s are involved in a broad range of processes. For example, p53 and Notch are both HECT E3 substrates (Pickart, 2001). However, HECT E3s have not been shown to directly function in cell cycle regulation. The RING finger domain is ~70 amino acids in length with Cys and His residues surrounding two zinc ions (Lorick et al., 1999). The zinc ions and their ligands (the Cys and the His) are catalytically inert, suggesting that the RING domain is mainly acting as a scaffold to mediate protein interactions and not a catalyzing center (Borden, 2000). Two classes of RING finger E3 complexes have been shown to regulate cell cycle progression: the cullin/RING finger family, and the anaphase-promoting complex or cyclosome (APC, Peters, 1998).

Cullin/RING finger complexes

Cullin/RING finger complexes are composed of a cullin, a RING finger protein, and several other proteins. They do not form thio lester bonds with ubiquitin but instead serve as scaffolds to bring the E2-ubiquitin and the substrate together (Zheng et al., 2002).

One subset of cullin/RING finger complexes, SCF complexes, are essential for the G1-to-S phase cell cycle transition in budding yeast by degrading G1 regulators such as cyclins and CKIs (Peters, 1998). The SCF complex has four components: the cullin family member CUL1 (metazoa) or Cdc53 (budding yeast); the RING H2 finger protein RBX1/ROC1/Hrt1; Skp1; and an F-box protein, which recognizes and binds phosphorylated proteins as substrates (Peters, 1998). The core cullin/RING finger complex (the cullin, Skp1, and Rbx1) can bind to different F-box proteins to expand the repertoire of substrates that can be recognized. The functional significance of the SCF complex was first recognized in budding yeast where two of its major substrates are key G1-to-S phase regulators. First, when complexed with the F-box protein Cdc4, the SCF complex directs the degradation of the G1 phase CKI, Sic1, to allow activation of the Clb5/6-Cdc28 CDK-cyclin complexes that promote S phase entry (Feldman et al., 1997). Second, when complexed with the F-box protein Grr1, the SCF complex directs the degradation of the G1 phase cyclins, Cln1, 2, and 3, preventing them from interfering with downstream events (Skowyra et al., 1999). In metazoa, SCF complexes have been shown to direct the degradation of the G1 phase cyclin E and the CKIs p21 and p27 (Wang et al., 1999, Dealy et al., 1999, Yu et al., 1998, Tsvetkov et al., 1999), suggesting a functional conservation.

Another subset of metazoan cullin/RING finger complexes containing the cullin CUL-2 has been implicated in cell cycle regulation as well. In mammals, CUL-2 forms a VCB complex consisting of 5 proteins: CUL2; RBX1; elongin C, which shares homology with Skp1; elongin B, which shares homology with ubiquitin; and the von Hippel-Lindau (VHL) tumor suppressor protein (Kamura et al., 1999; Pause et al., 1997;

Lonergan et al., 1998). VHL functions as a substrate recognition factor for the degradation of hypoxia-inducible factor-1α and -2α (HIF-1α, HIF-2α), atypical protein kinase C, and the VDU1 and VDU2 deubiquitinating enzymes (Maxwell et al., 1999; Okuda and Kimura, 1988; Li et al., 2002). Similar to what occurs with the SCF complex, It is likely that the core CUL-2 complex will interact with other substrate recognition factors, as human SOCS-1 can replace VHL to target the degradation of the chromosomal translocation fusion protein TEL-JAK2 (Kamizono et al., 2001). In *C. elegans*, CUL-2 is required for multiple cellular functions including cell cycle regulatory events (Feng et al., 1999). It negatively regulates the protein level of a CDK inhibitor, CKI-1, to allow the G1-S phase cell cycle transition. CUL-2 is also required to promote proper cytoskeletal movements, mitotic progression, and mitotic chromosome condensation (Feng et al., 1999).

Anaphase promoting complex (APC) or cyclosome

The finding that cyclin levels fluctuate in a cell cycle-dependent manner in *Xenopus* and clam egg extracts led to the discovery of the APC (cyclosome). The APC (cyclosome) was initially identified as an activity that promoted the ubiquitin-mediated proteolysis of cyclins at the metaphase-to-anaphase transition (King et al., 1995; Sudakin et al., 1995). Yeast genetics identified specific APC components in screens for mutants that failed to degrade cyclin B and had M phase defects (Irniger et al., 1995).

APC is a large protein complex. Currently 12 subunits have been discovered in budding yeast, but the binding of substrate-binding adaptors such as Cdc20 or Cdh1 is also required for APC activity (Page and Hieter, 1999). Some of the APC subunits share

homology with cullin/RING finger complexes. For example, one of the essential APC subunits, APC11, is a RING-finger protein (Page and Hieter, 1999). Another subunit, APC2, has a cullin-homology domain (Zachariae et al., 1998).

APC substrates have been found to contain a Destruction Box motif or a Ken Box motif, or both (Pfleger and Kirschner, 2000). Mutagenizing conserved residues in these motifs leads to a failure of degradation, suggesting that these sequences function as degradation signals (King et al., 1996b, Pfleger and Kirschner, 2000) . These sequences are loosely conserved. The Destruction Box usually follows the pattern RXXLXXXXN and can be recognized by APC complexes containing the substrate recognition subunits Cdc20 or Cdh1 (Jackson et al., 2000). The consensus sequence for the Ken Box is KENXXXN/D and this motif is only recognized by APC/Cdh1 (Page and Hieter, 1999).

The regulation of the metaphase-anaphase transition by APC

In mitosis, the initiation of anaphase is marked by the separation of sister chromatids resulting from a loss of cohesion between them. Sister chromatid cohesion is mainly mediated by cohesin (for a review, see Nasmyth, 2001). Mitotic cohesin contains four subunits: Smc1, Smc3, Scc1, and Scc3. They form a complex to produce cohesion, and mutational inactivation of any one of them leads to premature sister chromatid separation before anaphase (Nasmyth, 2001). In budding yeast, chromatin immunoprecipitation using antibodies against cohesin subunits showed that cohesin is localized at the centromeric region and on specific chromosome arm loci (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999). The mechanism of cohesin-mediated chromosome cohesion is not well understood. Smc1 and Smc3 are SMC (structural

maintenance of chromosomes) proteins and have two characteristic long stretches of coiled-coiled structure (Lowe et al., 2001; Melby et al., 1998). Theoretically, they could form flexible V-shape molecules that clamp chromosomes together. They also are capable of aggregating DNA molecules by facilitating intermolecular catenations (Nasmyth, 2001). Scc1 has been speculated to act as a lynchpin to lock the SMCs in position with the help of Scc3 (Nasmyth, 2001).

In mammalian cells, cohesin first binds chromatin during telophase (Sumara et al., 2000). In S phase, cohesion between sister chromatin is thought to be established during the passage of the replication fork (Nasmyth, 2001). In the metaphase-to-anaphase transition, Scc1 is cleaved by a novel cysteine protease called separase, leading to sister chromatid separation (Uhlmann et al., 2000). APC is the key player in Scc1 cleavage: before anaphase, separase is held inactive by its inhibitory binding partner securin. At the onset of anaphase, securin is ubiquitinated by APC and then degraded by the 26S proteasome (for a review, see Peters, 2002). Upon securin degradation, separase is freed to degrade Scc1. If chromosomes are not aligned correctly at the metaphase plate or if appropriate spindle-chromosome connections have not yet been established, then a checkpoint protein Mad2 will inhibit APC activity by binding to Cdc20, the substrate-binding APC adaptor. Without the activation of APC, securin will not be degraded, separase will not be active, and tsister chromatids will not separate (for a review, see Hoyt, 2001).

Securin degradation is not the only role of APC in promoting the metaphase-to-anaphase transition. Mutational inactivation of securin in various organisms such as yeast, mice, and humans did not result in a premature sister chromatid separation,

indicating that in the absence of securin other mechanisms are still working to inhibit separase activity prior to anaphase (Alexandru et al., 1999; Jallepalli et al., 2001; Mei et al., 2001; Wang et al., 2001). One of these mechanisms could be CDK-mediated inhibition. In *Xenopus* egg extracts, it was found that high CDK1 activity was able to inhibit separase activity but not securin degradation (Stemmann et al., 2001). Mutational inactivation of a phosphorylation site on separase relieved the inhibition and led to chromosome separation, suggesting that CDK1 phosphorylates separase to inhibit its activity (Stemmann et al., 2001). APC has been shown to partially degrade M phase cyclins at the metaphase-to-anaphase transition. In yeast, at the metaphase-to-anaphase transition, Clb2 is partially degraded by Cdc20-APC, and this degradation leads to a partial inactivation of Cdc28 activity (Yeong et al., 2000). Therefore, it is very likely that APC is required at the metaphase-to-anaphase transition both to reduce CDK1 activity by partially degrading cyclins, and to completely degrade securin. This model can also explain the finding that in *Drosophila,* cyclin A has to be degraded in metaphase, and a nondegradable cyclin A mutant leads to anaphase delay (Peters, 2002).

Meiotic Chromosome Separation

The APC is also required for cohesin degradation in meiosis. Meiosis is a specialized cell cycle in which two rounds of cell divisions follow one round of DNA duplication. In meiosis I, chromosome homologs segregate. In meiosis II, sister chromatids segregate. Just as for mitosis, meiotic chromosome separation is also mediated by APC and separase-dependent cohesin cleavage, although Scc1, Smc1, and Scc3 are replaced by the meiotic-specific homologs Rec8, Smc1β, and STAG3, respectively (Klein et al., 1999,

Pezzi et al., 2000; Prieto et al., 2002). Meiotic cohesins mediate not only sister chromatid cohesion, but also chromosome homolog cohesion. In *C. elegans*, depletion of REC-8 by dsRNA-mediated interference (RNAi) led to a premature separation of both chromosome homologs and sister chromatids (Pasierbek et al., 2001). During meiosis I, chromosome homologs are initially held together by the synaptonemal complex (SC, Nasmyth, 2001). The REC-8 cohesin complex is believed to function as a critical SC component, since it is located within the SC and is necessary for SC formation (Nasmyth, 2001). After DNA recombination the SC is dissolved, and chiasmata, or DNA crossovers, remain between homologs. It is believed that the cohesion between homologs, which requires chiasmata formation, is supported by cohesin complexes distal to the crossover (Maguire, 1974, Klein et al., 1999, Buonomo et al., 2000, Watanabe and Nurse, 1999). Therefore, homolog separation in meiotic anaphase I is due to a partial REC-8 degradation from the arms of sister chromatids, while at the same time the centromeric REC-8 is protected from degradation and is responsible for holding sister chromatids together. In anaphase II, REC-8 is completely degraded from sister chromatids (Klein et al., 1999; Buonomo et al., 2000; Watanabe and Nurse, 1999). The mechanism underlying the differential degradation of REC-8 during anaphase I and II is not well understood. A recent paper has shown that the aurora-B kinase AIR-2 is required for REC-8 degradation during meiosis, and the selective phosphorylation of REC-8 by AIR-2 has been proposed to allow the differential degradation of REC-8 in its two distinct chromosomal locations (chromosome arms versus the centromere region; Rogers et al., 2002).

Other players involved in anaphase chromatid separation

After cohesin degradation, processes such as DNA decatenation and DNA condensation are required to fully dissolve the two separating DNA molecules. Topoisomerase II (Topo II) acts to decatenate intertwined DNA molecules by making a double-stranded break in DNA and allowing other DNA strands to migrate through the break. In fission yeast *top2* (Topo II) mutants, anaphase chromatids are pulled towards the poles but fail to fully resolve from each other. As a result, chromosomes are stretched and appear "streaked" (Uemura et al., 1987).

The condensin complex is important for chromosome condensation and segregation. In *Xenopus* egg extract, condensin was purified as a 13S protein complex consisting of two SMC subunits, XCAP-C and XCAP-E, and three non-SMC subunits, XCAP-D2, XCAP-G, and XCAP-H (Hirano and Mitchison, 1994). Condensin has DNA-stimulated ATPase activity and is able to introduce global positive writhe into circular DNA in an ATP-dependent manner (Kimura et al., 1998). In yeast, the condensin complex was shown to recruit Topo II to the chromosomes and was hypothesized to help decatenate DNAs by facilitating Topo II action (for a review, see Hirano, 2000). Mutational inactivation of condensin leads to chromosome segregation defects. A budding yeast condensin mutant displays a DNA segregation phenotype similar to that of the *top2* mutant (Bhalla et al., 2002). In *C. elegans*, depletion of a condensin, SMC-4, led to chromosome bridges at both meiotic and mitotic anaphases, confirming that it is required to fully resolve separating anaphase chromosomes (Hagstrom et al., 2002).

Chromosome movement in mitosis and meiosis is directed by microtubule dynamics and microtubule-chromosome interactions

Along with chromosome cohesion, spindle-directed chromosome movement is another important aspect of mitosis. After the chromosomes have been captured by microtubules (MTs) emitted from both spindle poles, they oscillate between the poles until finally aligned at the metaphase plate. This process is called chromosome congregation, and it includes poleward movements and anti-poleward movements (Rieder and Salmon, 1994). In anaphase, after chromosome cohesion has been dissolved, the separating chromosomes undergo a rapid poleward movement to segregate from each other. Both poleward and anti-poleward movements are dynamic processes directed by at least two kinds of force-generating mechanisms: MT polymerization/depolymerization and MT motors.

In most eukaryotic organisms, MTs are attached to chromosomes at kinetochores. Electron microscopy of mitotic chromosomes in vertebrate somatic cells reveals that the kinetochore has two electron-dense plates with an electron-lucent middle layer in between. The inner plate is the closest to the centromeric heterochromatin and contains proteins like CENP-A, a histone H3 variant (Maney et al., 2000). The corona-shaped outer plate has MTs directly inserting into it and contains a variety of proteins such as the motors proteins CENP-E and cytoplamic dynein, and the checkpoint protein Bub1 (Taylor and McKeon, 1997). The centromeric region between the inner plates of the two opposing sister chromatids is not part of a kinetochore but contains passenger proteins,

such as the aurora B-INCEP-survivin complex, that transiently associate with the kinetochore in a cell cycle-dependent fashion (Maney et al., 2000; Oegema et al., 2001).

MT polymerization/depolymerization to drive poleward chromosome movement can happen at two locations, the plus end (kinetochore) and the minus end (poles). One of the oldest models that attempts to explain poleward chromosome movement is the poleward flux/treadmill model (for a review, see Rieder and Salmon, 1994). In this model, MT depolymerization occurs at the poles and generates a force to pull chromosomes poleward. Poleward flux has been observed in many organisms but its functional significance varies between different systems. In vertebrate somatic cells, the rate of poleward flux, measured by fluorescence photoactivating experiments, is too slow to account for poleward chromosome movement, especially during anaphase (Mitchison and Salmon, 1992, Waters et al., 1996). On the contrary, in Xenupos egg extract and insect meiocytes, the rate of poleward flux is similar to the total anaphase chromosome movement (Desai et al., 1998). Therefore, it is proposed that poleward flux might be predominant in some systems, such as meiosis, but is not a major mechanism in other systems, such as vertebrate somatic cells (Desai et al., 1998).

What mechanism could then account for poleward chromosome movement in vertebrate somatic cells? Many studies showed that the kinetochore is the primary site of MT disassembly in vertebrate somatic cells, suggesting that MT disassembly at the kinetochore could provide a poleward force (Mitchison and Salmon, 1992, Waters et al., 1996, Zhai et al., 1995, Gorbsky et al., 1987). More importantly, a number of kinetochore motor proteins have been identified to function in MT disassembly or to couple chromosomes to the shrinking MTs. Inactivation of these kinetochore motor

proteins, MCAK and CENP-E, has been shown to lead to defects in poleward chromosome movement. MCAK is a kinesin-related plus end-directed motor that is localized to the centromere region (Maney et al., 1998; Wordeman and Mitchison, 1995). It belongs to the Kin I kinesin family, members of which have been shown to function as MT-destabilizing enzymes instead of as motor proteins (Desai et al., 1999). In CHO cells, depletion of MCAK resulted in an anaphase segregation defect: some chromosomes had difficulty reaching the poles and often lagged, suggesting that MCAK-mediated MT disassembly activity is required for anaphase chromosome movement (Maney et al., 1998). Another kinesin-related plus end-directed motor, centromere protein-E (CENP-E), localizes to the kinetochore corona at the onset of mitosis and then gradually relocates to the spindle midzone after anaphase A (Brown et al., 1996*;* Yen et al., 1991*;* Yen et al., 1992). Based on *in vitro* experiments in which poleward chromosome movement is reconstituted, CENP-E has been proposed to couple chromosomes to the depolymerizing MTs at the kinetochore and is required for anaphase A movement (Lombillo et al., 1995a; Lombillo et al., 1995b).

Both plus-end directed and minus-end directed MT motors have roles in chromosome movement. Cytoplasmic dynein is the only minus end-directed motor that has been found on the kinetochores, suggesting that dynein might function to promote poleward chromosome movements. However, dynein is also required for multiple mitotic and meiotic events including spindle assembly and spindle pole organization, making it difficult to define dynein's function in chromosome movement. Insight into dynein's function in chromosome movement came from the study of another gene, ZW10/ROD. ZW10 functions in targeting the dynein/dynactin complex to the

kinetochore (Starr et al., 1998, Scaerou et al., 1999). Loss of ZW10 leads to a "lagging chromosome" phenotype in anaphase, suggesting that ZW10, and by inference dynein, are required for poleward chromosome movement (Savoian et al., 2000). Further, inactivation of dynein by injecting anti-dynein antibodies or the dynein inhibitor dynamitin into *Drosophila* embryos produced defects in anaphase movement (Sharp et al., 2000). These studies suggest that dynein is indeed involved in directing anaphase chromosome poleward movement.

Chromokinesins are a type of kinesin-like motor that doesn't specifically associate with the kinetochore but rather is found along the length of chromosome arms (Maney et al., 2000). Chromokinesins are important for spindle assembly and chromosome congression. They are proposed to be the major mechanism for producing the polar ejection force that propels chromosome arms away from the spindle pole when chromosomes are congregating. Depletion of the *Xenopus* chromokinesin Xkid from *Xenopus* extract led to a congregation defect but not a spindle assembly defect, suggesting that Xkid is specifically required for anti-pole chromosome movement (Antonio et al., 2000; Funabiki and Murray, 2000). However, Xkid's function seems not to be conserved among organisms. The Xkid ortholog in humans, Kid, is necessary for chromosome arm orientation and oscillation, but not congression, on mitotic spindles (Levesque and Compton, 2001). Interestingly, Xkid must be degraded to allow anaphase chromosome movement in *Xenopus* egg extract (Funabiki and Murray, 2000). Adding nondegradable Xkid to the *Xenopus* extract completely inhibited anaphase chromosome movement but not separation (Funabiki and Murray, 2000). Xkid degradation has been suggested to depend on APC, since adding an APC competitive inhibitor, a cyclin B

fragment with a destruction box, into the extract efficiently blocked Xkid degradation, while adding a cyclin B fragment with a mutated and nonfunctional destruction box failed to block Xkid degradation (Funabiki and Murray, 2000). Whether Xkid degradation is necessary for segregation in organisms other than *Xenopus* remains to be determined.

Polarity in *C. elegans*

The anterior-posterior (A-P) polarity of *C. elegans* is established as early as the zygotic stage. Among the first polarity signs visible under differential interference contrast (DIC) microscopy in the *C. elegans* zygote is a polarized cytoplasmic flow, generated after the oocyte DNA has finished two meiotic divisions, and the maternal and paternal pronuclei have formed (Hird and White, 1993). The cytoplasmic flux is generated at the sperm pronucleus/centrosomes location and flows with a fixed pattern: the internal cytoplasm flows towards the sperm pronucleus/centrosomes, while the cortical cytoplasm is flowing away from the sperm pronucleus/centrosomes. Later, the position of the sperm pronucleus/centrosomes will become the posterior end of the embryo, and the opposite end will become the anterior end. The same kind of polarized cytoplasmic flow also happens in later blastomeres (Hird and White, 1993). Interestingly, this phenomenon happens only in blastomeres that are about to undergo an asymmetric division but never in blastomeres that have a symmetrical division. Furthermore, the initial point of the cytoplasmic flow always correlates with the sperm/mitotic aster position: when nocodazole was used to disrupt the MT structures, the sperm DNA and the asters were attenuated and often misplaced (Hird and White, 1993). Importantly, the cytoplasmic

flow was still generated near the sperm/mitotic aster position. Based on these observations, the authors suggested that the initial polarized pattern of the cytoplasmic flow is directed by sperm/centromere cues and not by oocyte components (Hird and White, 1993).

When a sperm fertilizes an oocyte, it brings in two obvious components: the sperm DNA and the centrosomes. This leads to the question of which component is the source of polarity? It was found that anucleate sperm can fertilize the oocyte and establish a correctly polarized zygote, suggesting that sperm DNA is dispensable for polarity (Sadler and Shakes, 2000). Because the polarity is not established until the sperm asters have formed from of the centrosome, the sperm asters became the primary suspect for the polarity cue.

Two recent studies further suggested that MT structures indeed direct the establishment of polarity. In the first study, a *spd-2* mutant was found to be incapable of establishing polarity: the asymmetric cytoplasmic flow was absent, and the polarity marker proteins PAR-2 and PAR-3 lost their asymmetric localizations (O'Connell et al., 2000). SPD-2 functions in sperm aster formation. The primary defect of the spd-2 mutant is that sperm aster formation is delayed, and the asters when formed are attenuated. This suggests that the spd-2 polarity defect is due to an inability to establish proper sperm asters (O'Connell et al., 2000). However, this research could not exclude the possibility that the SPD-2 protein might be directly involved in establishing polarity. The second study gave a more definitive answer about the relationship between polarity and MT structures such as sperm asters (Wallenfang and Seydoux, 2000). In this work, the authors asked what would happen if sperm asters were disrupted. Previous

experiments with nocodazole had failed to give a clear answer because nocodazole was not able to completely eliminate MT structures and failed to change the polarity pattern (Wallenfang and Seydoux, 2000). In this research, the authors collected a set of mutants that had defective APC component genes and were arrested at metaphase of meiosis I. As a result, the meiotic spindle lasted for a much longer time than in wild-type, and sperm asters failed to form. The cytoplasmic flow did not occur in those mutants. However, the mutant zygote did show some aspects of polarity, because they asymmetrically localized PAR-3 and PAR-2 on the cortex. Importantly, the localization of these PAR proteins was reversed compared to that seen in wild-type, suggesting that the prolonged meiotic spindle, located opposite of the sperm, was able to direct the establishment of polarity on the opposite side of the embryo (Wallenfang and Seydoux, 2000).

How do MT structures direct PAR proteins to be asymmetrically localized in the wild-type? The actin cytoskeleton might be one of the mediators, as pointed out in a review written by Gotta and Ahringer (Gotta and Ahringer, 2001a). Both actin foci and myosin are uniformally distributed throughout the egg cortex at meiosis. When cytoplasmic flow initiates, actin and myosin start to move towards the anterior cortex and accumulate there relative to the posterior end, suggesting that actin and myosin might be directing some polarization processes (Strome and Wood, 1983, Hird and White, 1993). Disrupting the cortical actin network by treatment with cytochalasin D completely blocked cytoplasmic flow in the zygote (Hird and White, 1993). Further, mutations in a non-muscle myosin II heavy chain gene, *nmy-2*, or a myosin light chain gene, *mlc-2*, resulted in loss of polarity; cytoplasmic flow was lacking, and the polarity marker

protein PAR-3 was symmetrically distributed (Guo and Kemphues, 1996, Shelton et al., 1999). Intriguingly, in the *mlc-2* mutant, a small cortical PAR-2 patch was still observed in the posterior half of the embryo (Shelton et al., 1999). One of the more exciting explanations for this is that in *mlc-2*, the sperm asters are still able to give polarity cues even though the actin cytoskeleton has been damaged and is only able to partially convey the cue and localize PAR-2 imperfectly in the posterior. If the actin cytoskeleton is downstream of MT signals but upstream of the Par genes, then we would expect that *par* mutants would have a normally polarized actin/myosin structure and dynamics, including a normal cytoplasmic stream. It was observed that cytoplasmic streaming was normal in *par-1* and *par-4* mutants, weak in *par-2* mutants, but absent in *par-3* mutants (Kirby et al., 1990). Taking together, these data suggest that 1) the actin cytoskeleton is likely a link between the MT polarity cue and some of the PAR proteins, and 2) PAR-3 and the actin cytoskeleton are dependent on each other for their polarity pattern.

What are the PAR proteins? Currently six *par* genes have been reported, *par-1* through *par-6* (Kemphues, 2000*;* Morton et al., 2002). PAR-1 and PAR-4 are Ser/Thr kinases. PAR-2 is a RING finger protein. PAR-3 and PAR-6 are PDZ domain proteins that have been found in a trimeric complex with an atypical protein kinase C (aPKC). PAR proteins seem to function in a directed polarity pathway but oftentimes are inter-dependent on one another. The genetic interactions between *par* genes currently are not well sorted out (Kemphues, 2000).

One of the most important functions of PAR proteins is to unequally place the mitotic spindle poles. In the wild-type zygote, the first mitotic spindle is shifted posteriorly, producing a smaller P cell after the first mitotic division. If the spindle

midzone of the *C. elegans* zygote is severed by a laser beam during anaphase, then the posterior pole and associated chromosomes travel towards the posterior end with increased speed relative to the anerior pole, suggesting that increased force is being exerted on the posterior pole to pull it towards posterior cortex, relative to the force operating on the anterior pole (Grill et al., 2001). These results also suggest that the sliding midzone MTs do not produce a dominant force for the poleward movement of chromosomes or spindles in the first mitosis, because poleward movements do not cease when the midzone MTs are ablated, but rather increase in speed (Grill et al., 2001). To examine how PAR proteins influence the spindle pulling force, a similar midzone MT-ablation experiment was conducted in *par-2* and *par-3* mutants. In *par-2* mutants, after ablation of the midzone MTs, the rate of poleward migration of the anterior and posterior poles was the same, but was equivalent to the slower anterior movement observed in wild-type (Grill et al., 2001). This result suggests that the normally posterior-located PAR-2 is required to generate force to drag the posterior pole toward the cortex with increased speed relative to the movement of the anterior pole. In *par-3* mutants, PAR-2 is localized uniformly around the cortex (rather than being localized exclusively to the posterior*;* Cheng et al., 1995). After ablating the midzone MTs in par-3 mutants, the rate of poleward migration of the anterior and posterior poles was the same but was equivalent to the faster posterior movement observed in wild-type (Grill et al., 2001). This result suggests that having PAR-2 at both anterior and posterior cortexes caused increased force generation in both directions. Therefore, to generate an unequal mitotic division, PAR-2 is required to drag the spindle towards the posterior cortex, and PAR-3 is required to exclude PAR-2 from the anterior cortex (Grill et al., 2001). The bridge

between PAR genes and the spindle pulling force might be heterotrimeric G proteins. Upon inactivation of redundant Gα proteins, PAR protein locations are normal, but placement of the spindle in the zygote is defective, suggesting that Gα proteins are required to link PARs and spindle placement (Gotta and Ahringer, 2001b).

PAR proteins also function to partition maternal proteins asymmetrically. In the wild-type, germline proteins such as PIE-1 and POS-1 are moved into the posterior P cell and degraded in the anterior AB cell during the 1st mitosis (for a review, see Gotta and Ahringer, 2001a). In *par* mutants, asymmetrical PIE-1 and POS-1 distribution is lost (for a review, see Kemphues, 2000). Two genes *mex-5* and *mex-6* have been proposed in linking PARs with germline protein targeting. In *mex-5* and *mex-6* mutants, PAR locations are normal, the spindle position is normal, but germline proteins are symmetrically located (Schubert et al., 2000). How MEX-5 and MEX-6 direct the protein targeting is not understood at this stage (Gotta and Ahringer, 2001a).

This dissertation consists of six chapters. In the literature review chapter, Chapter 2, I will provide a brief overview of four areas of biology that have relevance to my thesis work: 1) the function and regulation of CDK-cyclin complexes, with particular emphasis on their regulation by CDK-activating kinases (CAKs); 2) the regulation of the cell cycle by cullin/RING finger and APC ubiquitin-ligase complexes; 3) the regulation of meiotic chromosome movement and 4) the establishment of anterior-posterior polarity in *C. elegans* and its connection to meiosis. Chapter 3 and Chapter 4 describe our studies on the evolution of CDKs and CDK-activating kinases. Chapter 5 describe our studies on the role of CUL-2, a ubiquitin ligase, in meiosis and polarity regulation in *C. elegans*.

The final chapter, Chapter 6, is a general discussion of my dissertation work. Results are summarized, implications are discussed, and further studies are proposed.

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CHAPTER 2

EVOLUTION OF CYCLIN-DEPENDENT KINASES (CDKS) AND CDK-ACTIVATING KINASES (CAKS): DIFFERENTIAL CONSERVATION OF CAKS IN YEAST AND METAZOA[1]

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ABSTRACT

Cyclin-dependent kinases (CDKs) function as central regulators of both the cell cycle and transcription. CDK activation depends on phosphorylation by a CDK-activating kinase (CAK). Different CAKs have been identified in budding yeast, fission yeast, and metazoa. All known CAKs belong to the extended CDK family. The sole budding yeast CAK, *CAK1*, and one of the two CAKs in fission yeast, *csk1*, have diverged considerably from other CDKs. Cell cycle regulatory components have been largely conserved in eukaryotes, however, orthologs of neither *CAK1* nor *csk1* have been identified in other species to date. To determine the evolutionary relationships of yeast and metazoan CAKs, we performed a phylogenetic analysis of the extended CDK family in budding yeast, fission yeast, humans, the fruit fly *Drosophila melanogaster*, and the nematode *Caenorhabditis elegans*. We observed that there are ten clades for CDK-related genes, of which seven appear ancestral, containing both yeast and metazoan genes. The four clades that contain CDKs that regulate transcription by phosphorylating the carboxyl-terminal domain (CTD) of RNA Polymerase II have generally only a single orthologous gene in each species of yeast and metazoa. In contrast, the ancestral cell cycle CDK (analogous to budding yeast CDC28) gave rise to a number of genes in metazoa, as did the ancestor of budding yeast PHO85. One ancestral clade is unique in that there are fission yeast and metazoan members, but there is no budding yeast ortholog, suggesting that it was lost subsequent to evolutionary divergence. Interestingly, *CAK1* and *csk1* branch together with high bootstrap support values. We used both the Relative Apparent Synapomorphy Analysis (RASA) method in combination with the S-F method of sampling reduced character sets and gamma-corrected distance methods to confirm that

the *CAK1*/*csk1* association was not an artifact of long-branch attraction. This result suggests that *CAK1* and *csk1* are orthologs and that a central aspect of CAK regulation has been conserved in budding and fission yeast. Although there are metazoan CDK-family members for which we could not define ancestral lineage, our analysis failed to identify metazoan *CAK1*/*csk1* orthologs, suggesting that if the *CAK1*/*csk1* gene existed in the metazoan ancestor it has not been conserved.

INTRODUCTION

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that must bind to cyclin proteins to become active (Pines 1995). They were originally identified as essential regulators of cell cycle progression. CDKs are required for the G1-to-S phase cell cycle transition, initiation of DNA replication, the G2-to-M phase cell cycle transition, and initiation of multiple mitotic events (Sherr 1994; Stillman 1996; King, Jackson, and Kirschner 1994). The first CDKs to be identified were the budding yeast cell cycle regulator *CDC28* and the orthologous fission yeast cell cycle regulator *cdc2* (Nasmyth and Reed 1980; Beach, Durkacz, and Nurse 1982; Lorincz and Reed 1984; Hindley and Phear 1984). There is an extended eukaryotic family of CDKs that share homology with *CDC28* and *cdc2*. While certain CDK-family members function to regulate the cell cycle, other CDKs have been found to function in other cellular pathways, most notably as central regulators of transcription (Morgan 1997). The functions of many CDKs are still unknown (Morgan 1997).

In both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, a single CDK (Cdc28 and Cdc2, respectively), is responsible for catalyzing all major cell cycle transitions (Morgan 1997). In higher eukaryotes, there has been an expansion in the number of CDKs that regulate the cell cycle, with five cell cycle CDKs in mammals. This expansion allowed the specialization of CDKs for particular cell cycle transitions/functions: CDK4, CDK6, and CDK3 regulate G1 phase progression and entry into S phase; CDK2 is required for entry into S phase and DNA replication; and CDK1 (CDC2) is required for mitosis (Morgan 1997;

Sherr 1994; Stillman 1996; King, Jackson, and Kirschner 1994; van den Heuvel and Harlow 1993).

In *S. cerevisiae*, five CDKs function to regulate transcription. Three of these CDKs, Kin28, Srb10, and Ctk1, regulate mRNA synthesis by phosphorylating the carboxyl-terminal domain (CTD) of RNA Polymerase II (Valay et al. 1995; Liao et al. 1995; Lee 1991; Sterner et al. 1995) Sgv1 regulates transcription, potentially also as a CTD kinase, as its ortholog CDK9 functions as a CTD kinase (Prelich and Winston 1993; Reines, Conaway, and Conaway 1999). Finally, Pho85 functions to inhibit gene transcription in response to phosphate (Lenburg and O'Shea 1996). Pho85 also has a secondary role in promoting cell cycle progression, as it is required for G1-to-S phase progression when the G1 cyclins Cln1 and Cln2 are missing (Measday et al. 1994; Espinoza et al. 1994).

CDK activity is tightly regulated through four mechanisms: 1) binding by activating cyclins; 2) binding by inhibitory cyclin-dependent kinase inhibitors (CKIs); 3) inhibitory phosphorylation of the CDK; and 4) activating phosphorylation of the CDK. The activating phosphorylation is catalyzed by a CDK-activating kinase (CAK) (Kaldis 1999). CAK phosphorylates a conserved threonine residue in CDKs (throughout this paper this site will be referred to as Thr 160, which corresponds to the position in CDK2, although the exact amino acid position varies among the kinases). This phosphorylation stabilizes cyclin-CDK interaction and enhances substrate binding (Russo, Jeffrey, and Pavletich 1996). CDKs depend on CAK phosphorylation for activation, and a loss of CAK activity causes cell cycle arrest (Lee et al. 1999; Larochelle et al. 1998; Thuret et al. 1996; Kaldis, Sutton, and Solomon 1996; Espinoza et al. 1996).

In *S. cerevisiae*, Cak1/Civ1 is the sole essential CAK for both the cell cycle CDK Cdc28, and the transcriptional CDK Kin28 (Thuret et al. 1996; Kaldis, Sutton, and Solomon 1996; Espinoza et al. 1998). It is a distant member of the CDK family and has diverged considerably not only from other CDKs but also from other kinases. Cak1 is fully active without a cyclin partner or activating phosphorylation (Kaldis, Sutton, and Solomon 1996; Thuret et al. 1996). Furthermore, it lacks the consensus GXGXXG motif that is implicated in nucleotide binding for all classes of protein kinase (Hanks and Hunter 1995). Currently only two other kinases have been found that also lack the entire GXGXXG motif, Mik1 and Vps15 (Kaldis 1999). No *CAK1* ortholog has been reported in any other organism to date.

The budding yeast CDK Kin28 possesses no CAK activity *in vitro* or *in vivo* (Valay et al. 1995; Feaver et al. 1994; Cismowski et al. 1995). However, in metazoans, the Kin28 ortholog CDK7/p40^{MO15} was identified as a CAK based on its ability to phosphorylate and activate CDK1 *in vitro* (Poon et al. 1993; Solomon, Harper, and Shuttleworth 1993; Tassan et al. 1994; Darbon et al. 1994). In *Drosophila melanogaster* , a *Cdk7* temperature sensitive mutant was found to be defective for CAK activity for the CDK1/Cyclin A and CDK1/Cyclin B complexes (Larochelle et al. 1998). The conclusion that CDK7 was the *in vivo* CAK for CDK1 was tempered by the fact that CDK7 also functions as a transcriptional activator and therefore the loss of CAK activity could have resulted from an inability to synthesize the true CAK. The *D. melanogaster Cdk7* mutations did not affect the activating phosphorylation of CDK2 *in vivo*, suggesting that there are additional CAKs in metazoans (Larochelle et al. 1998). CDK7 binds Cyclin H

and requires activating phosphorylation. The *in vivo* CAK for CDK7 is currently not known.

In *S. pombe*, the Kin28 ortholog Mcs6/Mop1/Crk1 is a CAK for the cell cycle CDK, Cdc2 (Buck, Russell, and Millar 1995; Damagnez, Makela, and Cottarel 1995). A divergent CDK family member, Csk1, works redundantly with Mcs6 to activate Cdc2 (Lee et al. 1999). Csk1 also phosphorylates Mcs6 on the consensus activating site S165 (Hermand et al. 1998; Molz and Beach 1993).

Cell cycle regulators are largely conserved among eukaryotes (Pines 1995). As Cak1 is an essential cell cycle regulator in budding yeast, it is perplexing that no *CAK1* ortholog has been found in any other organisms. Similarly, no ortholog of fission yeast *csk1* has been reported. Since the metazoan CAK CDK7 does not appear to be sufficient for the activation of all CDKs (Larochelle et al. 1998), and CDK7 itself needs activating phosphorylation, it is likely that there exists unidentified metazoan CAK(s).

The genome sequence of *Caenorhabditis elegans* is essentially complete (The C.Elegans Sequencing Consortium 1998), allowing a comprehensive analysis of a metazoan genome. Further, the genome of *D. melanogaster* has also been sequenced (Adams et. al. 2000). We identified 13 *D. melanogaster* and 14 *C. elegans* extended CDK family members. We undertook a phylogenetic analysis of the extended CDK family in budding yeast, fission yeast, and metazoa in order to provide insight into the evolution of the CDK family and to address whether an ortholog of *CAK1* could be identified in metazoans.

MATERIALS AND METHODS

Identification and Alignment of Protein Sequences

Yeast, human, *D. melanogaster*, and *C. elegans* CDK protein sequences were obtained from the National Center for Biotechnology Information (NCBI), the Institute for Genomic Research (TIGR), and *C. elegans* genome databases. CDKs were identified with BLAST (Altschul et al. 1997) and PROFILE (Gribskov, Luthy, and Eisenberg 1990) searches of the databases. The exon structure of three *D. melanogaster* sequences that are not represented in the EST databases, *AC017581*, *AC014407,* and *AC018104*, were predicted from the genomic sequence based on kinase domain homology and splice site consensus. The predicted exons for the *AC017581* kinase domain are at nucleotide positions (96,285-96,040), (95,976-95,588), and (95,296-94,819); the predicted exons for *AC014407* are (22,352-22,122), (22,098-21714), (21,651-21,501), and (21,448-21,277); and the predicted exons for *AC018104* are (100,085-99,918) and (99,860-98,946). Table 2.1 shows the sequences used in this study.

Initial protein sequence alignments were made with the CLUSTAL X program (Thompson, Higgins, and Gibson 1994). The alignment was then optimized by hand to minimize insertions/deletions, using the kinase alignment of Hanks and Hunter, 1995, as a guide. Spacer regions were excluded from the phylogenetic analysis, as was the region between helix 5 and helix 6 that is not conserved among kinases (Hanks, and Hunter 1995) (Fig. 2.1).

Based on the apparent orthology of the *C. elegans* CDK-family members *Y39G10AL*, *F39H11.3*, and *H25P06.2* with human CDKs, we have assigned them the names *cdk-7*, *cdk-8*, and *cdk-9*, respectively. We have refrained from naming *C. elegans*

or *D. melanogaster* genes that are not orthologous to known cyclin-binding proteins, which are given the permanent designate CDK as per convention (Myerson et al. 1992).

Phylogenetic Analysis

The Neighbor-joining distance method (NJ) (Saitou and Nei 1987) and maximum likelihood (ML) were used to create phylogenies. For NJ analyses, a distance matrix of the data set, using the JTT model, was created with the ProtML program of MOLPHY version 2.3 (Adachi and Hasegawa 1996). The matrix was used to create trees with the NEIGHBOR program of the PHYLIP package (Felsenstein 1993), using random order of addition of taxa. Bootstrap values were obtained from 1000 replicates.

Another JTT distance matrix was calculated using a model of rate heterogeneity among sites with eight rates following a gamma distribution plus one invariable rate using the PUZZLE program (Strimmer and von Haeseler 1996) with exact parameter estimates of the gamma distribution parameter alpha and the fraction of invariable sites. The distance matrix was analyzed with the NEIGHBOR program (Felsenstein 1993). Bootstrap values were obtained from 100 replicates.

A maximum likelihood analysis was performed with the ProtML program of MOLPHY version 2.3 (Adachi, and Hasegawa 1996). Input trees derived from Star Decomposition searches (ProtML program) or NJ trees were optimized by Local Branch Rearrangement (LBR) analysis using the JTT model (Adachi, and Hasegawa 1996). We observed that LBR of NJ input trees always gave higher likelihood phylogenies than those obtained by using Star Decomposition input trees. Bootstrap probabilities were

created by ProtML LBR analysis of 100 input data set resamplings created with the SeqBoot program (Felsenstein 1993) using their associated NJ trees as input.

Relative Apparent Synapomorphy Analysis (RASA) was applied using the RASA 2.3 package (Lyons-Weiler 1999; Lyons-Weiler and Hoelzer 1997; Lyons-Weiler, Hoelzer, and Tausch 1996). After signal content analysis, a taxon variance analysis was used to detect taxa that manifested the hallmarks of long branch attraction (Lyons-Weiler 1999).

The removal of fast-evolving characters was accomplished according to the S-F method as described by Brinkmann, et al., 1999. The substitution step was calculated with PAUP 4.0.b2a (Swofford 1993) as described (Brinkmann and Philippe 1999).

Molecular Experiments

An *H01G02.2* cDNA was isolated by PCR from a cDNA library (provided by R. Barstead) using primers designed to amplify the coding region of *H01G02.2*, predicted by the *C. elegans* Genome Consortium (the Sanger Centre, Cambridge, and the Genome Sequencing Center at the Washington University School of Medicine, St. Louis). The *H01G02.2* cDNA was cloned into the pBluescript SKII+ plasmid (Stratagene), and sequenced to confirm the absence of PCR-induced mutations. Sense and antisense *H01G02.2* RNAs were synthesized using the Megascript T7 and T3 kits according to manufacturer instructions (Ambion). Sense and antisense RNAs were annealed to create double-stranded RNA by heating to 93°C for 30 seconds followed by twenty five 30-second incubations at 2° lower increments for each incubation. *H01G02.2* dsRNA was

injected into young adult *C. elegans* hermaphrodites at a concentration of 0.5 to 1 mg/ml. F1 progeny from injected mothers were scored for abnormalities.

To test whether *H01G02.2* could complement *cak1/civ1* mutants, an *H01G02.2* cDNA was subcloned into the *GAL1* promoter yeast expression plasmid pYes2 (Invitrogen). The *H01G02.2*/pYes2 plasmid and the pYes2 plasmid vector control were transformed by electroporation into the *cak1-22* mutant strain SY143: *cak1*::*HIS3* (*LEU2*/cen-*cak1-22*),*ade2*,*his3*,*leu2*,*can1*,*ura3*,*trp1*,*ssd1* (Kaldis, Sutton, and Solomon 1996), and the *civ1-4* mutant strain GF2351: *civ1-4*, *ura3*,*leu2*,*trp1*,*lys2*,*ade2*,*ade3* (Thuret et al. 1996). The growth of strain SY143 (*cak1-22*) and strain GF2351(*civ1-4*) was scored at 25°C (permissive temperature) and 37°C (nonpermissive temperature) on minimal media plates supplemented with 20 mg/ml raffinose (to induce the GAL1 promoter).

RESULTS

Using *S. cerevisiae* and human CDKs and CAKs as probes, BLAST and PROFILE searches were used to identify CDK-family members in budding yeast, fission yeast, *C. elegans*, *D. melanogaster*, and humans. We identified 14 CDK-family members for *C. elegans*, 13 for *D. melanogaster*, and 8 for *S. pombe*. We also included the previously identified 7 *S. cerevisiae* CDK-family members and 21 of the known 22 human CDK-family members in our analysis (human PITSLRE A, which is virtually identical to PITSLRE B, was not included). The CDK family members used in the study are listed in Table 2.1.

Phylogenetic analysis

The evolutionary rate of the yeast, *D. melanogaster* (*D.m.*), *C. elegans* (*C.e.*)and human (*H.s.*) CDK family members was expected to be different. We therefore chose to perform our phylogenetic analysis with the maximum likelihood (ML) method and the Neighbor-joining (NJ) distance method (Felsenstein 1981; Saitou, and Nei 1987). These methods are less likely to be misled than parsimony or compatibility methods when the evolutionary rate differs among taxa (Felsenstein 1978; Saitou and Imanishi 1989). When taxa have dissimilar rates of substitutions, incorporating the differential rate of character changes at given sites by fitting the amino acid data set to gamma-rate distributions facilitates a more accurate phylogeny (Yang 1996). We therefore used gamma-corrected distances for the NJ phylogeny.

The ML and gamma-corrected NJ methods both produced 10 clades for the CDKs (Figs. 2). Seven of the clades for both methods contain both yeast and metazoan members and therefore represent ancestral clades. We will refer to these clades by their *S. cerevisiae* members, except for the clade containing only fission yeast *BC18H10.15*. Two of the clades contain only metazoan members, the CDK4/6 clade and the KKIALRE clade.

Although both Cak1 and Csk1 function as CAKs to activate both cell cycle and transcription CDKs, it has not been recognized that *CAK1* and *csk1* may be orthologs (Hermand et al. 1998; Lee et al. 1999), perhaps because both genes have diverged considerably from other CDKs and from each other. All metazoan and yeast CDK family members (with the exception of *CAK1* and *csk1*) are more similar to all other yeast CDK family members than they are to *CAK1* and *csk1* (Table 2.2, data not shown). In contrast to the bottom ordinal ranking of similarity of other CDKs with *CAK1* and *csk1*, *CAK1* is

the second most similar gene to *csk1* among the seven budding yeast CDK-family members and *csk1* is the fourth most similar gene to *CAK1* among the eight fission yeast CDK-family members (Table 2.2; data not shown). Further, *CAK1* and *csk1* branch together with significant bootstrap values (Fig. 2.2).

A potential source of misleading convergence in trees is the problem of long branch attraction. Long branch attraction is the phenomenon in which taxa that have evolved at a relatively higher rate than other taxa converge in trees due to their shared dissimilarity relative to slow evolving taxa (Felsenstein 1978). To determine if our data set included taxa that could potentially produce long-branch attraction, we employed the RASA method (Relative Apparent Synapomorphy Analysis) (Lyons-Weiler, and Hoelzer 1997; Lyons-Weiler, Hoelzer, and Tausch 1996). RASA compares the cladistic similarity of taxa (RAS; relative apparent synapomorphies) to their phenetic similarity (E; overall similarity). Taxa with long branch attraction problems have abnormally high ratios of RAS to E (Ftvs), because while the overall similarity (E) between these and other taxa is low, the fast-evolving taxa have characters that have changed to the extent that their current state is essentially random and can by chance become identical to the characters of other taxa (homoplasy), thereby generating an abnormally high RAS value. In contrast, slow-evolving taxa diverge from ancestral states slowly and are unlikely to suffer from severe homoplasy. Their shared character traits are more likely to be true synapomorphies (evolutionarily shared character traits) and will be present as a function of overall similarity. Using the RASA program (Lyons-Weiler 1999; Lyons-Weiler, and Hoelzer 1997, Lyons-Weiler, Hoelzer, and Tausch 1996), we found that budding yeast *CAK1* and fission yeast *csk1* have significantly high Ftv scores (25.2 and 32.4,

respectively, relative to the alpha=0.05 significance value of 9.8), suggesting the potential to produce long-branch attractions.

The use of gamma-corrected distances can minimize or negate long-branch attraction (Swofford et al. 1996). However, the RASA program is currently only capable of analyzing unweighted data sets (Lyons-Weiler 1999) and therefore we could not determine if our use of gamma-corrected distances sufficiently minimized the potential for long-branch attraction. An alternative method to minimize potential long-branch attraction is to employ the theoretical framework of the S-F method of Brinkmann and Philippe, in which fast-evolving sites are removed from the data set and phylogenetic relationships are determined based on slow-evolving sites (Brinkmann, and Philippe 1999). Fast-evolving sites with multiple substitutions that occurred only within a given taxa are essentially devoid of phylogenetic content and can act to obscure the true phylogeny.

To perform the SF analysis, we created 17 data sets, S1 through S17, reflecting the sequential addition of faster-evolving character groups onto the slowest evolving (S1) data set. RASA analysis indicated that all of the data sets still had significant Ftvs scores for *CAK1* and *csk1* (data not shown). Both *CAK1* and *csk1* are notable for having changes in sites that are largely invariant. These changes are concentrated in the S1 and S2 data sets, which have 16 of 34 positions in which either Cak1 or Csk1 have differences relative to an invariant amino acid in the rest of the CDK-family taxa. The effect of these differences from a nearly invariant consensus is to make *CAK1* and *csk1* more dissimilar relative to other taxa and contribute to the potential problem of long-branch attraction. The S1 and S2 groups were removed from the S3 through S17 data

sets, to produce S3-3 through S3-17 data sets. Data sets S3-8 through S3-17 still had significant *CAK1* and *csk1* Ftv scores, while data sets S3-3 through S3-7 did not have significant Ftv scores for *CAK1* or *csk1*. Data sets S3-4 through S3-7 retained significant association between *CAK1* and *csk1*, with bootstrap support values of 90 to 99% (Fig. 2.3). Data set S3-3 did not have sufficient phylogenetic information to resolve the clades (data not shown). Therefore, the results of both the gamma-corrected phylogenies and the S-F reduced character phylogenies suggest that the *CAK1* and *csk1* association is not due to the effects of long-branch attraction.

Are there metazoan orthologs of *CAK1* and *csk1*? There are a number of orphan CDK-family members that do not have apparent yeast orthologs, The KKIALRE clade, CDK4/6 clade, *D.m. AC014407*, *C.e. H01G02.2*, and *H.s.* C*CRK*. Two orphans, *D.m. AC014407*, *C.e. H01G02.2* branched without significant bootstrap support at the base of *CAK1* and *csk1* in the ML and NJ analyses, while *H.s. CCRK* branched nearby (Fig. 2.2). One of the hallmarks of Cak1 and Csk1 is that they are activated without the requirement for phosphorylation at Thr 160 of the T-loop. Both Cak1 and Csk1 lack a phosphorylatable serine (Ser, S) or threonine (Thr, T) residue at this site, and instead contain hydrophobic amino acids (Fig. 2.1). In contrast, the majority of CDKs contain either a Ser or Thr at this site. The exceptions are *C. elegans* H01G02.2 and every member of the SRB10 clade, which contain an aspartic acid (Asp, D) residue at this site, and human CCRK, which has a deletion encompassing most of the T-loop. It is known that placement of an acidic residue (such as aspartic acid) in the place of a Ser or Thr can often mimic the effect of Ser or Thr phosphorylation, therefore the aspartic acid may allow constitutive activation in a manner analogous to phosphorylation of the Thr 160

site (Johnson, Noble, and Owen 1996). Thus, the primary sequences *C.e. H01G02.2* and *H.s. CCRK* suggest that they will be constitutively active, either due to the presence of an acidic amino acid at position 160, or due to the lack of the inhibitory T-loop. In contrast, the CDK-family member that branches the nearest to *CAK1* and *csk1* (although without support), *D.m.* AC014407, has a Ser residue at position 160, suggesting that it will not be constitutively active.

Experiments in our laboratory have failed to support a role for H01G02.2 as a CAK. After inactivating the *H01G02.2* gene in *C. elegans* using double stranded RNA-mediated interference (RNAi) (Fire et al. 1998), we observed variable hyperdermal defects that were not interpretable as cell cycle arrest (data not shown). To test for redundancy of CAK function, we inactivated both *H01G02.2* and *cdk-7* and found that the embryonic arrest phenotype was the same as for RNAi of *cdk-7* alone. Expression of *H01G02.2* in two budding yeast *cak1* mutants, *cak1-22* (Kaldis, Sutton, and Solomon 1996) and *civ1-4* (Thuret et al. 1996), failed to rescue the lethal *cak1* mutant phenotype (data not shown). In contrast, other researchers have expressed *csk1* in the same mutants and found that it complemented *cak1* (Hermand et al. 1998). These results suggest that H01G02.2 is not likely to function as a CAK.

DISCUSSION

We identified 14 CDK-family members in *C. elegans*, 13 in *D. melanogaster*, 8 in *S. pombe*, 7 in *S. cerevisiae* and 21 in humans. There are ten CDK clades, of which seven clades contain yeast and metazoan members. Two other clades, CDK4/6 and KKIALRE, contain only metazoan members. The CAK1 clade contains the budding yeast and fission

yeast CAKs, *CAK1* and *csk1*, which group together with significant bootstrap scores. Our analysis suggests that association of the divergent CDK-family members *CAK1* and *csk1* is not due to the phenomenon of long-branch attraction.

Four of the CDK clades contain members that have been shown to regulate transcription as CTD kinases of RNA Polymerase II. The KIN28 clade contains budding yeast Kin28 and human CDK7, both of which function as part of the general transcription factor TFIIH as CTD kinases to promote transcription (Orphanides, Lagrange, and Reinberg 1996). Kin28 is required for the transcription of 87% of yeast genes (Holstege et al. 1998). The SRB10 clade contains budding yeast Srb10 and human CDK8, both of which function as CTD kinases that negatively regulate transcription by phosphorylating the CTD of RNA Pol II prior to the assembly of the preinitiation complex (Hampsey and Reinberg 1999). Srb10 negatively regulates 3% of yeast genes (Holstege et al. 1998). The SGV1 clade contains *H.s.* CDK9 and *D.m.* Cdk9, both of which function as CTD kinases in pTEFb (positive transcription elongation factor b) to promote productive RNA Pol II elongation (Reines, Conaway, and Conaway 1999; Zhu et al. 1997). An *sgv1* mutant has been found to suppress the effects of a deletion of an upstream activating sequence in yeast, suggesting a function in transcriptional regulation, but the exact mechanism of SGV1 action has not been determined (Prelich, and Winston 1993). The CTK1 clade contains budding yeast CTK1, which was isolated as an *in vitro* CTD kinase (Lee 1991). CTK1 was subsequently found to promote RNA Pol II elongation *in vitro* (Lee and Greenleaf 1997), and both activate and repress a subset of yeast genes *in vivo* (Patturajan et al. 1999). It is interesting that of the seven ancestral CDK clades with both yeast and metazoan members, only the four CTD kinase-containing clades did not have

an expansion in gene number from yeast to metazoa. In each of these clades there is only a single ortholog in each species, with the exception of two closely-related human genes in the CTK1 clade, *CHED* and *KIAA0904*.

The number of CDKs that regulate cell cycle progression has increased considerably from yeast to metazoa. In both budding and fission yeast, a single CDK, Cdc28 or Cdc2, respectively, is largely responsible for initiating all cell cycle progressions. In metazoa, *H.s.* CDK1/*C.e.* NCC-1/*D.m.* Cdc2 functions in mitotic and meiotic progression; *H.s.* CDK4/*H.s.* CDK6/*C.e.* CDK-4/*D.m.* Cdk4 regulate G1 phase progression; and *H.s.* CDK2/*D.m.* Cdc2c functions in S phase progression (Follette and O'Farrell 1997; Boxem, Srinivasan, and van den Heuvel 1999; King, Jackson, and Kirschner 1994; Sherr 1994; Park and Krause 1999; Stillman 1996). In contrast to this conservation of metazoan cell cycle CDKs, there is not a definitive ortholog of the S phase CDK, *CDK2*, in *C. elegans*. A potential *C. elegans CDK2* counterpart is *K03E5.3*. While *K03E5.3* branched at the base of the CDC28 and PHO85 clades (Fig. 2.2), distance matrixes show that *K03E5.3* is most similar to *CDK2* and *CDK3* (Table 2.3). It will be interesting to determine if *K03E5.3* is required for DNA replication in *C. elegans*.

The CDK4/6 clade branches from the base of the CDC28 and PHO85 clades (Fig. 2.2). Our analysis does not allow us to determine whether the ancestral founder of the CDK4/CDK6 clade was the *CDC28* ancestor or the *PHO85* ancestor. Members of the CDK4/6 clade are more similar on average to members of the CDC28 clade than to members of the PHO85 clade (Table 2.2, data not shown). While Cdc28 is the primary CDK for G1 phase progression in budding yeast, Pho85 has a supporting role facilitating

G1 phase progression, and therefore it is possible to envision a conservation of function from either ancestral CDK.

The PHO85 clade has also undergone expansion in metazoans. There are three major metazoan sub-branches, the CDK5 branch, the PFTAIRE branch and the PCTAIRE branch (Fig. 2.2). There is a single orthologous gene in the CDK5 branch for all three metazoa. In vertebrates, *CDK5* is expressed in post-mitotic neuronal cells and is required for central nervous system and brain development (Ohshima et al. 1996; Chae et al. 1997). The PFTAIRE branch also has a single orthologous gene in all three metazoa. The function of the *H.s. PFTK1* is unknown; its murine ortholog, *PFTAIRE-1*, is expressed in brain, testes, and embryos (Besset, Rhee, and Wolgemuth 1998). There are three *PCTAIRE* subfamily members in humans and one in *C. elegans* , *PCTAIRE1, 2, 3* and *pct-1*, respectively. Noticeably absent is a *D. melanogaster* PCTAIRE family member. The three human PCTAIRE proteins have a high percentage of sequence identity among themselves (82-85%), suggesting a recent gene duplication. The functions of the human or *C. elegans PCTAIRE* genes are currently unknown.

The BC18H10.15 clade is unique as an ancestral clade that contains a fission yeast CDK-family member, *BC18H10.15*, but no budding yeast counterpart. This presumably occurred due to the loss of the ancestral gene in budding yeast after the evolutionary divergence of yeast and metazoans. The BC18H10.15 clade contains two sub-branches, PISSLRE and PITSLRE. The PISSLRE branch contains *H.s. PISSLRE* and *D.m. Dcdrk*. The PITSLRE branch contains two tandemly repeated human *PITSLRE* genes, two *C. elegans* genes *B0495.2* and *ZC504.3*, and *D.m. Pitslre*. The two expressed human *PITSLRE* genes, *A* and *B*, have only four amino acid differences in the kinase domain,

and are processed by alternative splicing into multiple isoforms (Lahti, Xiang, and Kidd 1995). PITSLRE proteins are proteolytically processed during apoptosis to produce active truncated proteins (Lahti, Xiang, and Kidd 1995). Expression of a truncated PITSLRE, similar to the proteolytically processed form, can induce apoptosis, and cells lacking PITSLRE have been found to be defective for apoptosis, suggesting that PITSLRE is an integral component of the apoptotic pathway (Lahti et al. 1995; Ariza et al. 1999). The functions of the ubiquitiously expressed non-proteolytically processed forms of PITSLRE are unknown. Inactivation of PISSLRE in mammalian cells with antisense and dominant-negative constructs produced a G2/M phase cell cycle arrest, suggesting a requirement of PISSLRE in this cell cycle transition (Li et al. 1995).

The KKIALRE clade is an orphan clade that contains three human genes, *KKIALRE*, *KKIAMRE*, and *STK9*; one *C. elegans* gene, and one *D. melanogaster* gene. The cellular functions of none of these genes have been determined.

CAK1/csk1

Experimental data indicate that *CAK1* and *csk1* have analogous functions (Hermand et al. 1998; Lee et al. 1999). Our phylogenetic analysis indicates that they are orthologs. While the two taxa are very divergent, we believe that their branching together is indicative of a real orthologous relationship for three reasons. First, we observed significant *CAK1* and *csk1* association with both ML and NJ methods, which are relatively resistant to the effects of long branch attraction (Felsenstein 1978; Saitou, and Imanishi 1989). Furthermore, we have included a comprehensive data set of all available yeast, *C. elegans (C.e.), D. melanogaster (D.m.)* and human (*H.s.*) CDKs as well as a

variety of the closest outgroup taxa to minimize potential long-branch attraction effects (Philippe and Laurent 1998; Lyons-Weiler, and Hoelzer 1997). Second, the *CAK1*/*csk1* association remained after gamma-rate correction of the data set to incorporate unequal rates of evolution at amino acid sites. Third, a reduced data set that no longer had a significant RASA Ftv (indicating the absence/or diminution of a long branch attraction problem) still had significant association of *CAK1* and *csk1*.

The finding of orthologous *CAK1*/*csk1* genes in both budding and fission yeast, which are distantly related to each other, suggests that an ortholog existed in the common ancestor of all eukaryotes, although it cannot be excluded that *CAK1*/*csk1* arose in a fungal ancestor. In both NJ and ML analysis, the metazoan CDK-family members *D.m. AC014407* and *C.e. H01G02.2* branched at the base of the *CAK1* and *csk1* node, while the human gene *CCRK* often branched nearby. These associations were not well supported, with bootstrap values of between 17 and 27%. There is also no significant similarity between the primary sequence of these genes and *CAK1* or *csk1* (Table 2.1, data not shown). Finally, there is no compelling evidence for orthology among the three metazoan genes themselves, which do not associate with high support values (Fig. 2.2). Therefore, while it is possible that these genes arose from a *CAK1*/*csk1* ancestor, sequence analysis cannot ascertain orthology. It is interesting that the primary sequence of *C.e.* H01G02.2 and *H.s.* CCRK indicates that they may be constitutively active. However, RNAi analysis suggests that *C.e.* H01G02.2 will not function as a CAK. It will be interesting to learn whether *D.m.* AC014407 or *H.s.* CCRK have CAK activity.

The absence of clearly identifiable *CAK1*/*csk1* orthologs in metazoa lends support to a model for CAK function in metazoans originally proposed by Fisher et al., 1995

(discussed below), and suggests a model for the evolution of CAK regulation in which the *CAK1*/*csk1* gene was lost in metazoa. The model envisions an evolutionary progression from an ancestral state that was similar to the current situation in budding yeast, in which *CAK1*/*csk1* provides the only CAK activity for all CDKs (Fig. 2.4). In a later ancestor, similar to fission yeast, the transcription-CDK, Mcs6/CDK7, acquired the ability to function as a CAK and functions redundantly with Cak1/Csk1 to activate other CDKs. Although redundant for CAK activity, neither Cak1/Csk1 nor Mcs6/CDK7 are in danger of being eliminated, as Cak1/Csk1 is required to activate Mcs6/CDK7 and Mcs6/CDK7 is an essential transcription component. The final evolutionary progression is to a state similar to metazoans in which *CAK1*/*csk1* has been eliminated. To allow this elimination, an alternate CAK for Mcs6/CDK7 is necessary. It has been observed that CDK2-Cyclin A and CDC2-Cyclin B can phosphorylate CDK7 *in vitro* (Fisher et al. 1995; Martinez et al. 1997) and therefore could activate CDK7 *in vivo*. However, this leaves a theoretical quandry in that for CDK7 to become active, it must first activate its own CAK. A potential solution, proposed by Fisher et al., is that CDK7 can be activated by binding to the assembly factor MAT1 (Fisher et al. 1995). MAT1 facilitates interaction between CDK7 and Cyclin H, and the trimeric complex is active without Thr170 phosphorylation. In higher eukaryotes, MAT1 is found in complex with CDK7 both within and outside of the TFIIH transcription complex (Fisher et al. 1995). In contrast, in budding yeast, MAT1 is solely found within the transcription complex (Feaver et al. 1997), indicating that it would not be available to activate Kin28. The model suggests that the ability of MAT1 to form a complex with Mcs6/CDK7 independently of the TFIIH complex made Cak1/Csk1 activity completely redundant and

therefore allowed its loss during evolution. Much of this model remains untested, in particular it is not known if: 1) other CDKs function as CAKs for CDK7 *in vivo*; 2) the MAT1/CDK7/Cyclin H complex functions as a CAK *in vivo*; and 3) CDK7 is the CAK for all CDKs in metazoa.

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Table 2.1: Yeast, human and *C. elegans* CDK-related kinases used in this study

GENES	SPECIES	ACCESSION NUMBER
CDC28	*S. cerevisiae*	Z36029
KIN28	*S. cerevisiae*	X04423
PHO85	*S. cerevisiae*	Y00867
SGV1	*S. cerevisiae*	D90317
SRB10	*S. cerevisiae*	U20222
CTK1	*S. cerevisiae*	M69024
CAK1	*S. cerevisiae*	U60192
cdc2	*S. pombe*	AB004534
mcs6	*S. pombe*	L47353
csk1	*S. pombe*	S59896
SPSPCC16C4.11	*S. pombe*	AL031535
SPSPBC18H10.15	*S. pombe*	AL022304
SPSPAC2F3.15	*S. pombe*	Z99165
SPSPAC23H4.17C	*S. pombe*	Z98977

PI014	*S. pombe*	AB004534
STK9	*H. sapiens*	NM_003159
CDK1	*H. sapiens*	NM_001786
CDK2	*H. sapiens*	NM_001798
CDK3	*H. sapiens*	NM_001258
CDK4	*H. sapiens*	U37022
CDK5	*H. sapiens*	NM_004935
CDK6	*H. sapiens*	NM_001259
CDK7	*H. sapiens*	NM_001799
CDK8	*H. sapiens*	NM_001260
PISSLRE	*H. sapiens*	NM_003674
PITSLRE b 2-1	*H. sapiens*	U07704
KIAA0904	*H. sapiens*	AB020711
PFTK1/KIAA0834	*H. sapiens*	NM_012395
CHED	*H. sapiens*	NM_003718
CDK9/PITALRE	*H. sapiens*	NM_001261
KKIALRE	*H. sapiens*	NM_004196
KKIAMRE	*H. sapiens*	NP_003948
PCTAIRE1	*H. sapiens*	X66363
PCTAIRE2	*H. sapiens*	NM_002595

PCTAIRE3	*H. sapiens*	X66362
CCRK	*H. sapiens*	AF035013
cdc2	*Drosophila*	X57496
cdc2c	*Drosophila*	X57486
Cdk4/6	*Drosophila*	X99510
Cdk5	*Drosophila*	X99511
Cdk7	*Drosophila*	U56661
Cdk8	*Drosophila*	U33015
Cdk9	*Drosophila*	AF027300
Pftaire/Eip63E	*Drosophila*	X99512
Pitslre	*Drosophila*	X99513
Dcdrk	*Drosophila*	D16402
AC017581	*Drosophila*	AC017581
AC014407	*Drosophila*	AC014407
AC018104	*Drosophila*	AC018104
H01G02.2	*C. elegans*	Z92847
cdk-8/F39H11.3	*C. elegans*	Z81079
ZC504.3	*C. elegans*	Z50029
B0495.2	*C. elegans*	U21317
B0285.1	*C. elegans*	Z34533
cdk-9/H25P06.2A	*C. elegans*	Z92797
cdk-7/Y39G10AL	*C. elegans*	AF154004
K03E5.3	*C. elegans*	AF067619
cdk-4/F18H3.5A	*C. elegans*	Z50110

pct-1/C07G1.3	*C. elegans*	U58751
ZC123.4	*C. elegans*	AF043706
cdk-5/T27E9.3	*C. elegans*	Z82059
ncc-1/cdk-1/T05G5.3	*C. elegans*	S75262
Y42A5A.4	*C. elegans*	AL032618
Outgroup Taxa[a]		
KSS1	*S. cerevisiae*	Z72825
YAK1	*S. cerevisiae*	Z49417
MDS1	*S. cerevisiae*	U03280
HOG1	*S. cerevisiae*	L06279
MCK1	*S. cerevisiae*	M55984
FUS3	*S. cerevisiae*	Z35777
SLT2	*S. cerevisiae*	X59262

[a] Outgroup taxa belong to the ERK family (*KSS1*, *FUS3*, *SLT2*, *HOG1*), GSK3 family (*MCK1*, *MDS1*), and CLK family (*YAK1*) of kinases, which are the kinase families closest to CDKs (Hanks and Hunter 1995).

Table 2.2: JTT Distance Matrix of *C. elegans* and budding yeast CDK-family members.

	Cdc28	**Pho85**	Kin28	**Srb10**	**Ctk1**	**BC18H1**	**Sgv1**	**Cak1**	**Csk1**
NCC-1	**0.593**	0.758	1.167	1.287	1.231	0.874	1.155	**2.021**	2.023
K03E5.3	**0.801**	0.861	1.336	1.215	1.208	1.011	1.250	**2.086**	2.251
CDK-4	**1.188**	1.198	1.509	1.440	1.309	1.248	1.348	**2.243**	2.183
Y42a5a.4	**1.320**	1.344	1.503	1.600	1.584	1.467	1.428	**2.707**	2.464
CDK-5	0.665	**0.651**	1.256	1.235	1.233	0.996	1.266	**1.955**	2.136
PCT-1	0.819	**0.716**	1.396	1.416	1.169	1.139	1.444	**2.195**	2.045
ZC123.4	0.872	**0.751**	1.239	1.542	1.230	1.164	1.249	**2.331**	2.155
CDK-7	0.971	1.023	**0.907**	1.278	1.128	1.024	1.225	**2.235**	1.895
CDK-8	1.214	1.335	1.266	**1.019**	1.330	1.095	1.326	**2.391**	2.041
B0285.1	0.960	1.002	1.331	1.347	**0.863**	0.957	1.049	**2.068**	2.078
ZC504.3	1.030	1.117	1.244	1.237	0.991	**0.833**	1.091	**2.284**	2.079
B0495.2	0.978	0.985	1.162	1.271	0.940	**0.848**	1.132	**2.409**	1.984
CDK-9	1.145	1.185	1.329	1.423	1.049	1.151	**0.969**	**2.411**	2.285
H01G02.2	1.379	1.3502	1.415	1.552	1.396	1.463	**1.279**	**2.162**	2.038
AC014407	**1.236**	1.428	1.518	1.700	1.396	1.452	1.435	**1.982**	1.919
CCRK	**1.109**	1.157	1.206	1.315	1.301	1.263	1.382	**2.253**	2.177
Cak1	**1.955**	2.021	2.414	2.137	2.118	**2.446**	2.244	0.000	2.082
Csk1	2.064	2.137	2.147	2.079	**1.732**	2.070	**2.342**	2.082	0.000

The scores represent the number of amino acid substitutions per site; calculated with the ProtML program of MOLPHY using the JTT model of amino acid substitution. The scores of budding yeast taxa (plus fission yeast BC18H10.15) that are most similar to *D. melanogaster* AC014407, human CCRK, fission yeast Csk1, budding yeast Cak1, and *C. elegans* taxa are bold; those most dissimilar are bold and underlined. A column comparing fission yeast Csk1 is also given, but scores are not highlighted.

Table 3. JTT distance matrix for potential *C. elegans* G1 and S phase CDKs.

	CDK1	CDK2	CDK3	CDK4	CDK6
NCC-1/CDK-1	**0.411**	0.472	0.450	**1.046**	0.976
K03E5.3	0.744	0.720	**0.718**	**1.076**	0.952
CDK-4	**1.142**	1.087	1.135	0.978	**0.958**
cdc2	**0.301**	0.454	0.429	**1.037**	0.938
cdc2c	0.554	**0.399**	0.427	**0.925**	0.902
Cdk4/6	**1.040**	0.881	0.904	0.820	**0.814**

The scores represent the number of amino acid substitutions per site; calculated with the ProtML program of MOLPHY using the JTT model. The scores of the human G1 and S phase CDKs (CDK1, CDK2, CDK3, CDK4, and CDK6) that are most similar to *C. elegans* CDKs (NCC-1, K03E5.3, and CDK-4) and *D. melanogaster* CDKs (cdc2, cdc2c, and Cdk4/6) are bold, those most dissimilar are bold and underlined.

FIGURES

Figure 2.1 Alignment of the CDK kinase domains. Alpha helixes and beta sheets, determined from the crystal structure of human CDK2 (De Bondt et al. 1993), are presented above the sequence. Brackets over the sequence denote residues included in the phylogenetic analyses of Fig. 2. Plus symbols underneath the sequence indicate residues in the slow-evolving character groups S3 through S7 that were used in Fig. 3. Zeros underneath the sequence indicate residues in the slowest evolving character groups S1 and S2. The thick bar between alpha helixes αL12 and α4 denotes extent of the T-loop. The star denotes the position of the threonine or serine residue corresponding to Thr 160 of CDK2 that is phosphorylated by CAK. Outgroup taxa are presented separately below the CDKs.

Fig 2.1 top

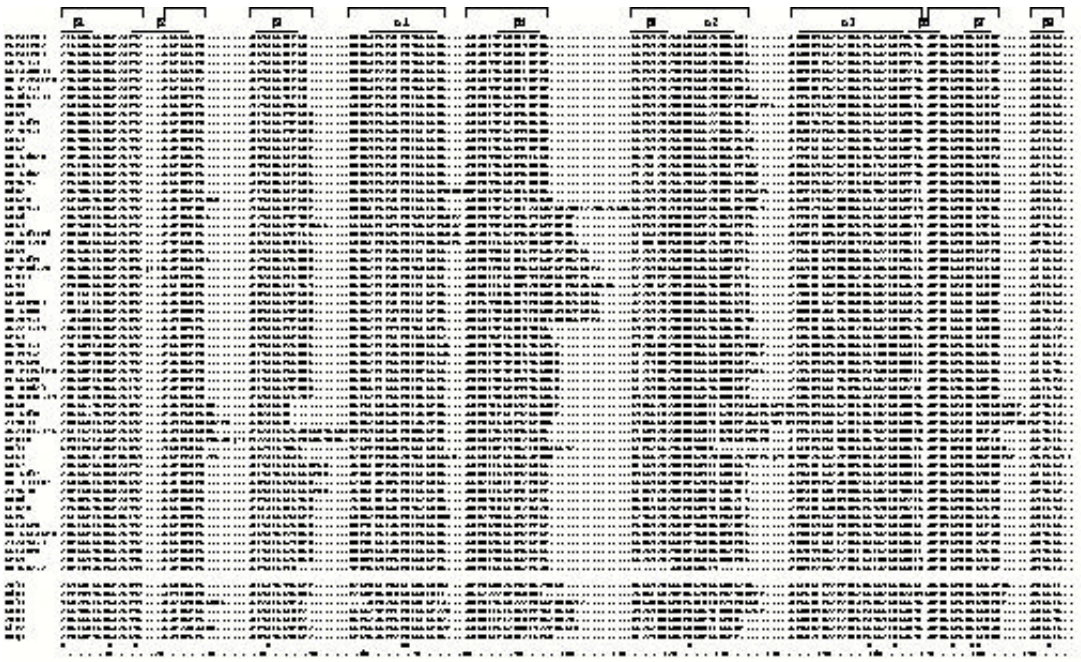


Fig 2.1.bottom

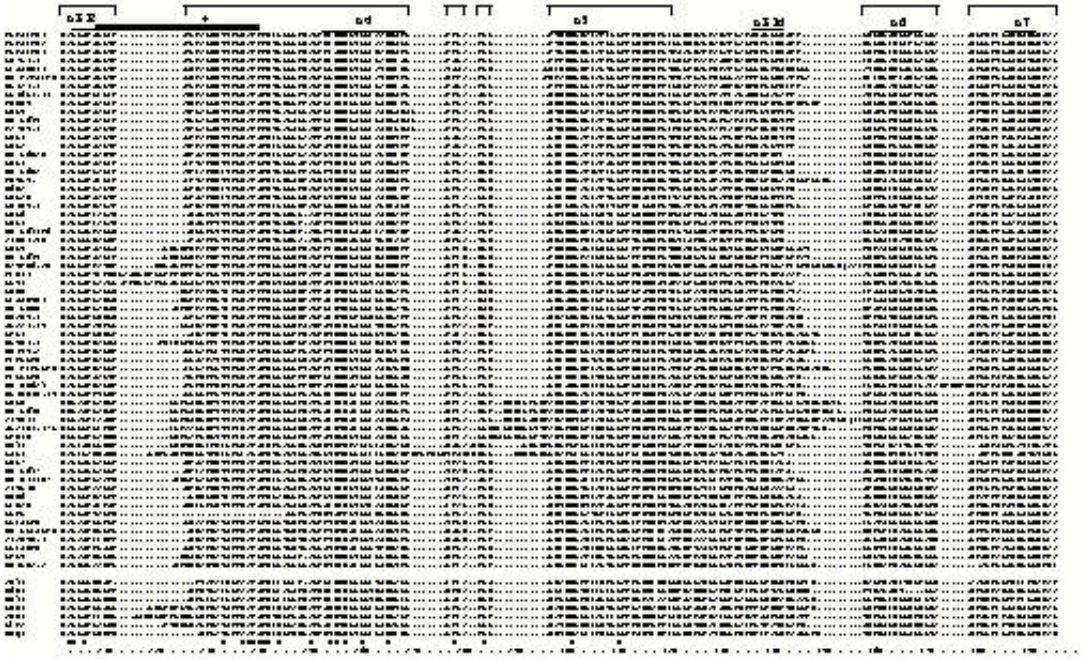
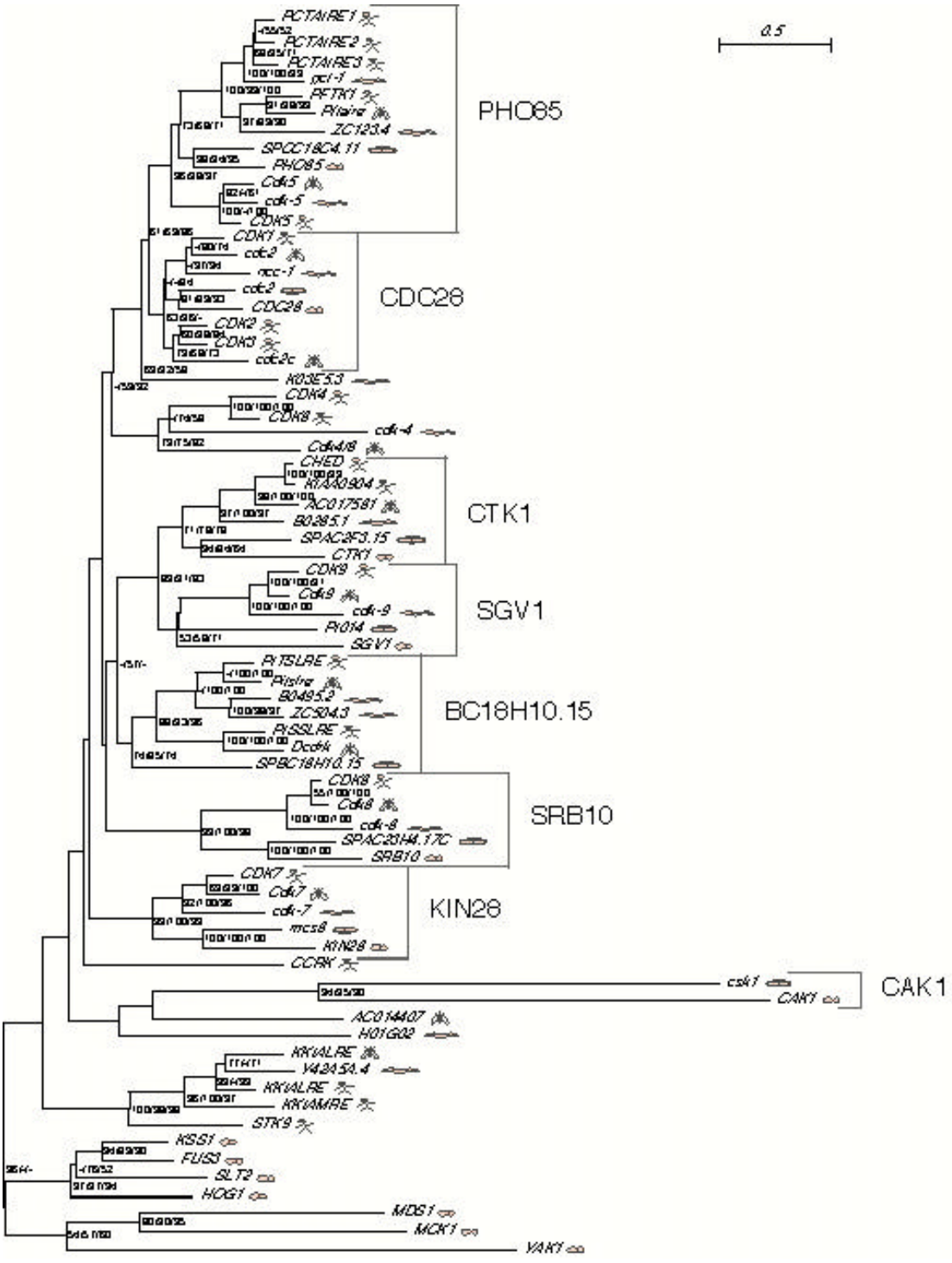


Figure 2.2. Gamma-rate corrected neighbor-joining phylogeny of *S. cerevisiae*, *S. pombe*, *D. melanogaster*, *C. elegans*, and human CDKs using the complete alignment (Fig. 2.1). The amino acid data set was modeled onto one invariable and eight gamma-rates to produce the pair-wise distances that were used to create the NJ tree. Branch lengths are proportional to the estimated number of amino acid substitutions; scale bar indicates amino acid substitutions per site. Bootstrap support values above 50% are given at branch nodes and are derived from ML, NJ of uncorrected data set, and gamma-rate corrected NJ analyses (separated by slash marks). Species are denoted by cartoon. Ancestral clades are denoted by brackets on the right.

Fig 2.2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PCTAIRE3
pct-1
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Pfsire
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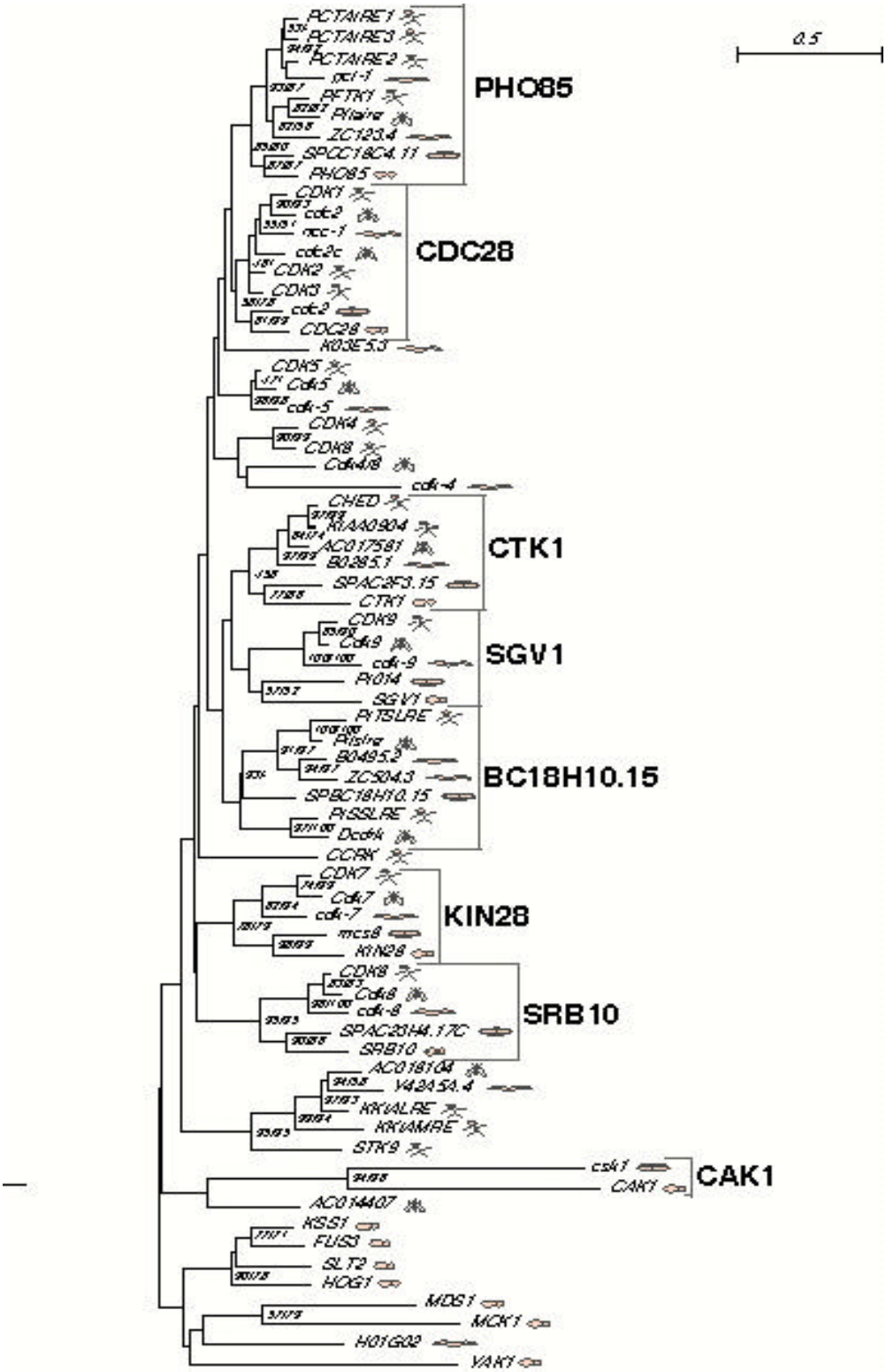
Cdk5
cdk-5
CDK5
CDK7
cdc2
ncc-1
cdc2
CDC28
CDK2
CDK3
cdc2c
K03E5.3
CDK4
CDK6
cdk-4
Cdk4/6
CHED
KIAA0904
AC017581
B0285.1
SPAC2F3.15
CTK1
CDK9
Cdk9
cdk-9
Pi014
SGV1
PITSLRE
Pfsire
B0495.2
ZC504.3
PISSLRE
Dcdk
SPBC18H10.15
CDK8
Cdk8
cdk-8
SPAC20H4.17C
SRB10
CDK7
Cdk7
cdk-7
mcs6
KIN28
CCRK
csk1
CAK1
AC014407
H01G02
KKIALRE
V43A5A.4
KKIALRE
KKIAMRE
STK9
KSS1
FUS3
SLT2
HOG1
MDS1
MCK1
YAK1

Figure 2.3. Neighbor-joining phylogeny of *S. cerevisiae*, *S. pombe, D. melanogaster, C. elegans,* and human CDKs using the S3-7 data set . The S3-7 data set (containing 95 characters) does not have significant Ftv values, suggesting that there are no taxa manifesting long-branch attraction problems. Bootstrap support values above 50% are given at branch nodes and are derived from ML and NJ analyses (separated by slash marks). Branch lengths are proportional to the estimated number of amino acid substitutions; scale bar indicates amino acid substitutions per site. Bootstrap support values above 50% are given at branch nodes and are derived from ML and NJ analyses of the S3-7 data set (separated by slash marks). Species are denoted by cartoon. Ancestral clades are denoted by brackets on the right.

Fig 2.3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PFTK1
Pfalre
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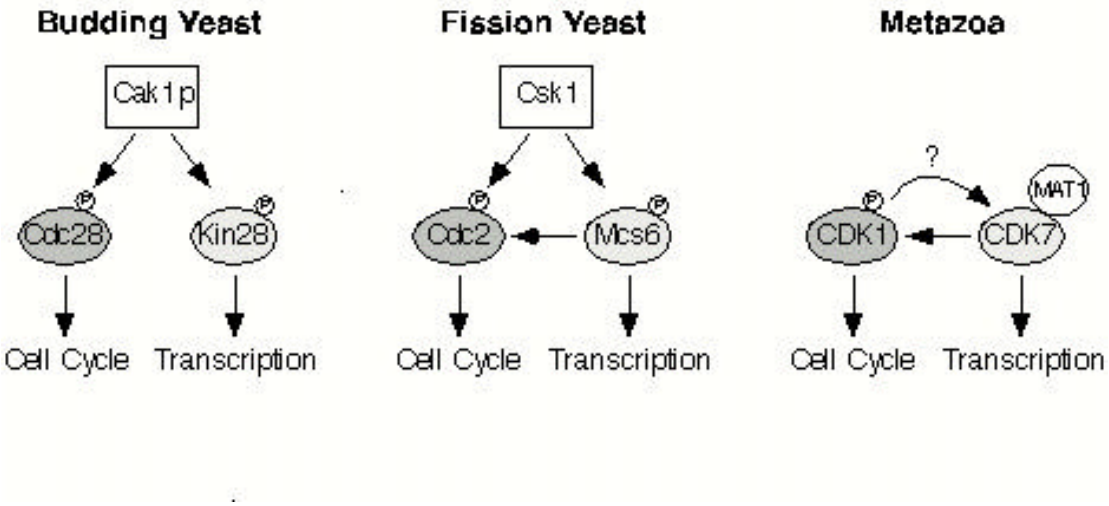
PHO85
PHO85
CDK1
cdc2
ncc-1
cdc2c
CDK2
CDK3
cdc2
CDC28
K03E5.3
CDC28
CDK5
Cdk5
cdk-5
CDK4
CDK6
Cdk4/6
cdk-4
CHED
KIAA0904
AC017581
B0285.1
SPAC2F3.15
CTK1
CTK1
CDK9
Cdk9
cdk-9
Pf014
SGV1
SGV1
PITSLRE
Pfalre
B0495.2
ZC504.3
SPBC18H10.15
PITSLRE
Dcdk4
CCRK
CCRK
CDK7
Cdk7
cdk-7
mcs6
KIN28
KIN28
CDK8
Cdk8
cdk-8
SPAC20H4.17C
SRB10
SRB10
AC018104
V4.2A5A.4
KKVALRE
KKVAMRE
STK9
csk1
CAK1
CAK1
AC014407
KSS1
FUS3
SLT2
HOG1
MDS1
MCK1
H01G02
YAK1

Figure 2.4. Model of CAK evolution in yeast and metazoa. Evolutionary progression starting with the CAK regulation now found in budding yeast (left), in which Cak1 phosphorylates and activates the cell cycle CDK (Cdc28) and the transcription CDK (Kin28). The next progression is to the CAK regulation now found in fission yeast (center), in which the Cak1 ortholog, Csk1, activates the cell cycle CDK (Cdc2) and the transcription CDK (Mcs6). Mcs6 also has CAK activity and redundantly activates Cdc2. The final progression is to the CAK regulation now found in metazoa (right), in which there is no Cak1/Csk1 ortholog. The transcription CDK, CDK7, is activated by phosphorylation by cell cycle CDKs. CDK7 in combination with MAT1 and Cyclin H is capable of being active without Thr 160 phosphorylation and this combination is capable of acting as a CAK to phosphorylate other CDKs.

Fig 2.4


Budding Yeast
Cak1p
Cdc28
Kin28
Cell Cycle
Transcription
Fission Yeast
Csk1
Cdc2
Mcs6
Cell Cycle
Transcription
Metazoa
?
MAT1
CDK1
CDK7
Cell Cycle
Transcription

CHAPTER 3

The Evolution of CDK-Activating Kinases[1]

[1]Liu, Ji and Edward T. Kipreos. 2002. From The CDK-Activating Kinases (CAK). Edited by Philipp Kaldis and published by Landes Bioscience/ Eurekah.com. Reprinted here with permission of Landes Bioscience/ Eurekah.com.

Introduction

Cyclin-dependent Kinases (CDKs) are essential regulators of the cell cycle and transcription. In the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Saccharomyces pombe*, a single CDK (Cdc28 or its ortholog Cdc2, respectively) catalyzes all major cell cycle transitions.[1,2] In higher eukaryotes, there has been an expansion in the number of CDKs that regulate the cell cycle. For example, in mammals, CDK4, CDK6, and CDK3 regulate the G1–S phase transition; CDK2 controls entry into S phase and DNA replication; and CDK1 (CDC2) is essential for mitosis.[3-7]

CDKs also regulate transcription. In budding yeast, Kin28, Srb10, and Ctk1 regulate mRNA synthesis by phosphorylating the carboxyl-terminal domain (CTD) of RNA Polymerase II.[8-11] The budding yeast CDK Sgv1 also functions to regulate transcription, but its substrates are unknown.[12] In metazoa, the ortholog of Sgv1, Cdk9, is a component of the positive transcription elongation factor b (pTEFb) that promotes productive RNA Pol II transcription elongation by phosphorylating the CTD.[13,14]

CDK activity is highly regulated in cells. There are four major regulatory mechanisms: 1) most CDKs require binding to cyclin proteins to become active; 2) CDK activity is inhibited by the binding of cyclin-dependent kinase inhibitors (CKIs); 3) phosphorylation of conserved residues in the ATP-binding pocket of the CDK inhibits its activity; and 4) phosphorylation of a conserved residue in the T-loop of CDKs is required for the activation of most CDKs.

The activating phosphorylation of CDKs is catalyzed by a CDK–activating kinase (CAK). CAK phosphorylates a conserved serine (Ser) or threonine (Thr) site in the T-loop of the CDKs. We'll refer to this site as Thr 160 (T160) based on its location in human CDK2. When not phosphorylated, the T–loop blocks the entrance of the CDK active site cleft to prevent the binding of protein substrates.[15] Phosphorylation on T160 induces a conformational change in the CDK, resulting in enhanced CDK–cyclin interaction and substrate binding.[16] Both cell cycle CDKs such as CDK1, CDK2, and CDK4, and transcription CDKs such as CDK7 and Kin28 have been shown to require CAK phosphorylation to be active.[17-23] A loss of CAK activity leads to cell cycle arrest as well as transcription defects.[22-24]

Two categories of CAKs

Two categories of CAKs have been found in eukaryotes. The first category includes two orthologous genes, budding yeast *CAK1* and its fission yeast ortholog *csk1*. The second category contains orthologous genes from metazoa, *CDK7*, and fission yeast, *mcs6*. Members of both categories of CAKs belong to the extended CDK family.[25]

Cak1 and Csk1 are diverged CDK family members.[17-19] When comparing the conserved kinase domain,[26] Cak1 and Csk1 only share 20% sequence identity with Cdc28, the prototype CDK, while most yeast CDKs are more than 35% identical to Cdc28. Both Cak1 and Csk1 are monomeric CAKs, that is, they are constitutively active without the need for a cyclin partner.[17-19] Further, they don't require phosphorylation of the T160 site to be active.[17-19] In fact, neither Cak1 nor Csk1 has a Ser or Thr at the T160 position.

CDK7 and Mcs6 are more conventional CDKs. They share 40% sequence identity with CDC28, they have Thr or Ser at their T160 sites, and they both need cyclin partners to be active.[27-30] Monomeric unphosphorylated CDK7 cannot efficiently bind to its cyclin partner, Cyclin H; it can only bind Cyclin H after having been phosphorylated on its T160 site, or alternatively, after binding of the assembly factor MAT1.[31] CDK7/Mcs6 family members function not only as CAKs but also as essential transcription factors. They are components of the general transcription factor TFIIH and function as activating CTD kinases.[32] Kin28, CDK7's budding yeast ortholog, is required for the transcription of 87% of all budding yeast genes.[33]

Different organisms use CAKs in different ways. In budding yeast, Cak1 is the sole known CAK and is required for the phosphorylation of both the cell cycle CDK Cdc28 and the transcription CDK Kin28.[17,18,22,23] Kin28, which is the ortholog of CDK7 and Mcs6, does not possess CAK activity.,[8,34,35] In fission yeast, the monomeric CAK Csk1 phosphorylates and activates Mcs6. Csk1 and Mcs6 work redundantly to phosphorylate the cell cycle CDK Cdc2.[19,36] In metazoa, *Drosophila* Cdk7 was shown to function as a CAK for the M phase cell cycle CDK Cdc2, but not for the S phase CDK Cdk2/Cdc2c.[20] No *CAK1/csk1* ortholog has been found in metazoa.

Yeast CDK and CAK orthologs

Budding yeast Cak1 and fission yeast Csk1 were initially thought to be unrelated kinases, since they share little sequence homology.[19,36] However, by phylogenetic analysis, we observed that Cak1 and Csk1 group together with significant bootstrap support (Fig. 3.1).[25] Both Cak1 and Csk1 have diverged considerably not only from other CDKs but also from other kinases, with both genes missing a number of key residues

conserved in the eukaryotic protein kinase superfamily.[17] These observations suggest that *CAK1* and *csk1* are rapidly evolving genes. In phylogenetic analyses, fast-evolving genes can group together solely because of their shared dissimilarity relative to slow-evolving taxa. This misleading convergence is called long–branch attraction.[37] To study the true phylogenetic relationship of Cak1 and Csk1, we used four methods to circumvent long branch attraction: First, we included a variety of the closest related outgroup taxa in our analysis, as the inclusion of such taxa helps to minimize long branch attraction.[38,39] Second, we used both Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses, which can better resist the effects of long branch attraction.[37,39,40,41] Third, we employed gamma-corrected distances, which facilitates a more accurate phylogeny when taxa evolve at different rates.[42] Fourth, based on the theoretical framework of the S-F method of Brinkmann and Philippe,[43] we excluded the fastest-evolving characters from each taxa and used the RASA program[38,44] to confirm that the resultant new dataset was not likely to have long-branch attraction. All four strategies indicated that the Cak1-Csk1 grouping was real and not an artifact of long-branch attraction, suggesting that *CAK1* and *csk1* are orthologous genes.

Are there *CAK1/csk1* orthologs in other fungi? Using Cak1 and Csk1 as queries for BLAST searches,[45] we identified a *Candida albicans* gene, *CAC05182.1*. Just like Cak1 and Csk1, CAC05182.1 does not have a Ser or Thr at its T160 position. Our ML and NJ analysis places CAC05182.1 within the CAK1 clade with 99% and 100% bootstrap supports, respectively (Fig. 3.2). We also generated a JTT amino acid distance matrix for CAC05182.1 and budding yeast CDK family members (Table 3.2). A JTT distance measurement is an estimate of the average number of amino acid substitutions per site

between two taxa. The shorter the distance value, the higher the similarity between the two proteins. The distance matrix shows that CAC05182.1 is more closely related to Cak1 and Csk1 than to any other yeast CDKs. In fact, it is closer to Cak1 and Csk1 than Cak1 and Csk1 are to each other. Therefore, the evidence suggests that there are at least three CAK1 orthologs in yeast*: CAK1, csk1* and *CAC05182.1,* which we now refer to as *CaCAK1*.

Metazoan CDK and CAK orthologs

Cell cycle regulators are generally conserved among eukaryotes. However, no Cak1/Csk1 type of monomeric CAK has been found in metazoa. On the other hand, three experimental observations suggest that there are missing CAKs in metazoa. First, mammalian CDK7's T160-equivalent site is phosphorylated *in vivo,*[31] but the *in vivo* CAK for mammalian CDK7 has not been identified. Since the fission yeast ortholog of CDK7, Mcs6, is phosphorylated by Csk1,[19] it is reasonable to suspect that an unidentified Cak1/Csk1 ortholog may be phosphorylating CDK7 in metazoa. Second, when *Drosophila* Cdk7 activity was reduced, Cdc2 activity was impaired due to its reduced level of T160 phosphorylation. However, neither the activity nor the phosphorylation level of Cdk2 was affected, suggesting that there exists another CAK for Cdk2.[20] Finally, Nagahara et al. showed that in human cells there is a CAK activity for CDK2 and this CAK activity is distinct from the CDK7/Cyclin H activity.[24]

Could there be *CAK1/csk1* orthologs in metazoa? The completely sequenced *C. elegans* and *D. melanogaster* genomes provided an opportunity for us to approach this question.[46,47] If there are *CAK1/csk1* orthologs in metazoa and if they still share

identifiable sequence homology with the yeast *CAK1/csk1* orthologs, we should be able to recognize them by phylogenetic analysis in the completed genomes. We identified all CDK family members in these organisms by BLAST and PROFILE[48] searches and analyzed their phylogeny by Maximum Likelihood and Neighbor-Joining analyses.[25] Most metazoan CDK family members belong to other yeast CDK clades with significant bootstrap supports, indicating that they are paralogs instead of orthologs of *CAK1/csk1*. We observed that no metazoan CDK-family members group with the CAK1 clade with significant bootstrap supports (Fig. 3.1).

A true metazoan CAK1 ortholog may be too divergent to group with the yeast *CAK1/csk1* orthologs with high bootstrap support. There are several orphan metazoan CDK family members that don't group with any of the clades with significant bootstrap scores, making them potential *CAK1/csk1* orthologs. For example, the *Drosophila* protein AC017707 and the *C. elegans* protein H01G02.2 branched at the base of the CAK1 clade, and human CCRK branched nearby (Fig. 3.1).

Sequence analyses of these proteins indicated that H01G02.2 and CCRK share one characteristic with *CAK1/csk1* orthologs: they do not have Ser or Thr at their T160 sites. H01G02.2 has an aspartic acid (D) at the T160 position, which could mimic the structural effect of a phosphorylated Ser or Thr to produce a constitutively active kinase.[49,50] CCRK has a T-loop deletion and therefore presumably does not have the physical blockage towards substrate binding that the T-loop imposes. Thus, H01G02.2 and CCRK may not need to be phosphorylated by a CAK to become active.

However, a recently identified mouse CCRK ortholog, PNQLARE (GenBank acc. no. AAF89089), has an intact T-loop with a Thr at the T160 position. This finding

suggests that the vertebrate ancestor of CCRK had a T-loop and may therefore have required activating phosphorylation.

Research in our lab suggests that *C. elegans* H01G02.2 is not likely to function as a CAK. First, a deletion allele of *H01G02.2* that is a molecular null (obtained from the *C. elegans* Gene Knockout Consortium) is phenotypically wild type and fertile without obvious cell cycle defects. Second, H01G02.2 does not seem to be working redundantly with C. elegans *cdk-7*, as inactivating both *H01G02.2* and *cdk-7* by RNAi did not cause a more severe defect than inactivating *cdk-7* alone. Furthermore, while *csk1* can complement a *cak1* mutant,[19] ectopic expression of *H01G02.2* failed to complement budding yeast *cak1* mutants.[25]

Arabidopsis CDK and CAK orthologs

Our analysis suggested that there is no identifiable *CAK1/csk1* ortholog in metazoa. If the CAK1 clade is ancient, that is, if it arose very early in eukaryotic evolution, it may have been subsequently lost in certain modern eukaryotic lineages such as metazoa. If this were the case then we would expect to see it in other eukaryotic kingdoms. Vascular plants are predicted to have diverged from the major eukaryotic lineage shortly before fungi diverged.[51] We therefore performed a phylogenetic analysis on the 98% finished *Arabidopsis thaliana* genome to search for a *CAK1/csk1* ortholog.

We identified 24 *Arabidopsis* CDK family members by BLAST searches. Phylogenetic analysis groups them into five major clades, four of which contain yeast CDK family members (Fig. 3.3).

Arabidopsis cdc2a and *cdc2b* genes belong to the CDC28 clade. *cdc2a* is involved in cell cycle regulation. When a dominant cdc2a mutant was expressed in *Arabidopsis*, cell division was reduced.[52,53] *cdc2b* has been shown to be involved in cell elongation rather than cell division in hypocotyl growth in *Arabidopsis*.[54]

Arabidopsis MBK5.8 belongs to the SRB10 clade. Although nothing is known of MBK5.8's function, its yeast ortholog Srb10 and human ortholog CDK8 both function as CTD kinases that negatively regulate transcription.[55]

Arabidopsis F12B7.13 and *K9H21.7* both belong to the BC18H10.15 clade, which contains one fission yeast CDK family member *BC18H10.15*, as well as two *C. elegans*, two *Drosophila*, and two human CDK family members. Interestingly, this clade does not contain a budding yeast ortholog, suggesting it was lost (Fig. 3.3). The functions of the clade members are largely unknown, with the exceptions that the human orthologs *PISSLRE* and *PITSLRE* have been implicated in apoptosis and the G2/M cell cycle transition, respectively.[56-58]

One cluster of *Arabidopsis* CDK family members has no obvious ortholog in yeast. It branches near the SGV1 and CTK1 clades and contains 14 CDK family members: *T22H22.5, T12H1.1, K16E14.2, F21B7.1, AT4G22940, F8L10.9, F26A9.10, AT4G10010, F14J9.26, F6A14.22, F1M20.1, AAF21469.1, T4P13.23*, and *MXK3.19*. The biological functions of these genes have not yet been defined.

The KIN28 clade contains *Arabidopsis F25P22.11* and *T10F20.5* genes. F25P22.11 and T10F20.5 share more than 85% DNA sequence identity with each other, suggesting that they are derived from a recent gene duplication within plants.

Another *Arabidopsis* gene *cak1At* is located at the base of the KIN28 clade, although with insignificant bootstrap support (43% and 84% for NJ and ML analyses, respectively). A JTT distance matrix comparing Cak1At to the budding yeast CDK-family members indicates that Cak1At is most similar to Kin28 and is least similar to Cak1 (Table 3.2). Umeda et al. showed that *cak1At* can complement both a budding yeast *cak1* mutant and a fission yeast *mcs6* mutant, indicating that it has *in vivo* CAK activity.[59] They also showed that Cak1At has *in vitro* CAK activity towards Cdk2.[59] The authors classified Cak1At as a novel type of CAK, different from both Cak1 and CDK7, as Cak1At has a divergent sequence.[59] However, based on the JTT distance data and the 84% bootstrap support in our ML tree, we think that *cak1At* is more likely to be a divergent *KIN28/mcs6/CDK7* ortholog.

There are no *Arabidopsis* CDK-family members that group with the CAK1 clade. Also, the only *Arabidopsis* orphan CDK family members, *cak1At* and members of the "*Arabidopsis* CDK cluster", are unlikely to be *CAK1/csk1* orthologs, because both BLAST searches and the distance matrix showed that they are very dissimilar to CAK1 clade members. Therefore, with 98% of the genome sequenced, we have not observed a *CAK1/csk1* ortholog in *Arabidopsis*.

Giardia lamblia CAK orthologs

Giardia lamblia represents one of the most ancient eukaryotic lineages.[60] We searched the 4x sequenced *Giardia lamblia* genome by BLAST, PROFILE, and HMM searches[61] for a *CAK1/csk1* ortholog and failed to find one. In contrast, a *KIN28* ortholog in *Giardia lamblia* (*AC052571*) was readily identifiable by BLAST search. The

affiliation of AC052571 with the KIN28 clade is supported by both ML and NJ analyses with 95% and 81% bootstrap supports, respectively (data not shown) and is reflected in a JTT distance matrix (Table 3.2).

Three hypothesis on CAK1 evolution

Our analyses have failed to identify a recognizable CAK1 clade member in the plant *Arabidopsis*, or in the metazoa *C. elegans*, *Drosophila,* and humans. Assuming that a *CAK1/csk1* ortholog is not in the remaining unsequenced 2% of the *Arabidopsis* genome, three possibilities for *CAK1/csk1* evolution remain:

1) The CAK1 clade is ancient with members present in the major eukaryotic kingdoms, however, the clade members in distantly related phyla have diverged to the extent that identification by phylogeny is not possible.

2) The CAK1 clade is ancient and could be identified by phylogeny, however, its orthologs have been lost independently in both plants and animals.

3) The CAK1 clade originated in fungi and does not exist in any other eukaryotic kingdoms.

The first possibility suggests that *CAK1/csk1* orthologs exist in metazoa and plants but they are too divergent to be identified. One way to adress this possibility is to assign all of the CDK-family members in a plant or metazoan species to known ancestral CDK clades and demonstrate either that *CAK1/csk1* orthologs are present or are missing. We have been able to assign ancestry to the majority of metazoan CDK-family members with the exceptions of the orphan genes.[25] The metazoan orphan genes are quite divergent from each other and do not have counterparts in *Arabidopsis*, suggesting that they are

undergoing rapid evolution that is obscuring their relationships to ancestral clades. The sequencing of "missing-link" species may allow assignment of the ancestry of the metazoan orphan CDK family members, the members of the "Arabidopsis CDK cluster", as well as provide a definitive assignment for *cak1At.*

The second hypothesis predicts that the CAK1 clade is ancient but was lost independently in metazoa and plants. We have not found a *CAK1/csk1* ortholog in the diplomonad *Giardia,* indicating that the CAK1 clade may not have existed in early eukaryotic lineages. However, it is still possible that the CAK1 clade is ancient but arose after the divergence of diplomonads from the main eukaryotic lineage. The test of this hypothesis will be determining whether there are *CAK1/csk1* orthologs in taxa that diverged shortly before or after fungi did, such as the choanozoa.[62]

The third hypothesis, that the CAK1 clade originated in fungi, is consistent with our inability to detect *CAK1/csk1* orthologs in other species. This hypothesis generates a prediction that can be tested. If the CAK1 clade arose in fungi, then it would have come from the duplication of a fungal CDK. Finding this gene duplication event would strongly indicate that the CAK1 clade arose in fungi. Ideally, if we identified a fungal *CAK1/csk1* ortholog that shares significant sequence homology with one of the other fungal CDK genes, then this would indicate that the CAK1 clade derived from the ancestor of this CDK gene. A successful example of this type of analysis was the study of *bicoid*'s evolution.[63] *bicoid* is an essential homeobox gene and had been identified only in the closest relatives of the schizophoran fly *Drosophila*. Stauber et al. cloned *bicoid* from a basal cyclorrhaohan fly, *Megaselia abdita*, and showed by phylogenetic analysis that the gene originated from a duplication of the *Megaselia* gene *zerknullt,*

which is also conserved in vertebrates. Such an approach may be possible for the study of CAK1 evolution as well.

Summary: CAK evolution

There are two categories of CAKs in eukaryotes encompassing the orthologous genes *CAK1*/*csk1*/*CaCAK1* and *mcs6*/*CDK7*. *mcs6/CDK7* orthologs are highly conserved from *Giardia* to metazoa, indicating that this is an extremely ancient eukaryotic CDK clade, whose members are under functional constraints that limit their evolution. Members of this clade, including the budding yeast KIN28, function as central regulators of transcription.[32] The conservation of this clade may therefore be linked to its essential role in transcription rather than to its CAK activity. CAK activity for Mcs6/CDK7 orthologs has only been observed in fission yeast and in metazoa. It is an open question whether CAK activity for Mcs6/CDK7 orthologs is evolutionarily conserved beyond certain fungi and metazoa.

CAK1/csk1 orthologs have not been identified in species other than yeast. The members of the clade appear to be evolving under relaxed evolutionary constraints as they are very divergent even within yeast. Currently it is not known whether the *CAK1/csk1* genes are specific for fungi or whether their metazoan and plant orthologs have diverged to an extent that precludes their current identification.

Acknowledgements

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Table 3.1: Extended CDK family members used in this study

GENES	SPECIES	ACCESSION NUMBER
CDC28	*S. cerevisiae*	Z36029
KIN28	*S. cerevisiae*	X04423
PHO85	*S. cerevisiae*	Y00867
SGV1	*S. cerevisiae*	D90317
SRB10	*S. cerevisiae*	U20222
CTK1	*S. cerevisiae*	M69024
CAK1	*S. cerevisiae*	U60192
cdc2	*S. pombe*	AB004534
mcs6	*S. pombe*	L47353
csk1	*S. pombe*	S59896
SPSPCC16C4.11	*S. pombe*	AL031535

SPSPBC18H10.15	*S. pombe*	AL022304
SPSPAC2F3.15	*S. pombe*	Z99165
SPSPAC23H4.17C	*S. pombe*	Z98977
PI014	*S. pombe*	AB004534
T22H22.5	*A. thaliana*	AC005388
T12H1.1	*A. thaliana*	AC009177
K16E14.2	*A. thaliana*	AB026637
F21B7.1	*A. thaliana*	AC002560
AT4g22940	*A. thaliana*	AL161558
F8L10.9	*A. thaliana*	AC022520
F26A9.10	*A. thaliana*	AC016163
AT4g10010	*A. thaliana*	AL161516
F14J9.26	*A. thaliana*	AC003970

F6A14.22	*A. thaliana*	AC011809
F1M20.1	*A. thaliana*	AC011765
AAF21469.1	*A. thaliana*	U83118
T4P13.23	*A. thaliana*	AC008261
MXK3.19	*A. thaliana*	AB019236
K9H21.7	*A. thaliana*	AB023035
F12B7.13	*A. thaliana*	AC011020
F25P22.11	*A. thaliana*	AC012679
T10F20.5	*A. thaliana*	AC034107
CAK1At/AT4g28980	*A. thaliana*	AL161574
MBK5.8	*A. thaliana*	AB005234
CDC2a	*A. thaliana*	X57839
CDC2b	*A. thaliana*	D10851
At2g38620	*A. thaliana*	AC005499
F9H16.8	*A. thaliana*	AC007369

CaCAK1/CAC05182.1	*C. albicans*	AX005954
AC052571	*G. lamblia*	AC052571

Outgroup Taxa[a]

KSS1	*S. cerevisiae*	Z72825
HOG1	S. cerevisiae	L06279
FUS3	*S. cerevisiae*	Z35777
SLT2	*S. cerevisiae*	X59262

[a] Outgroup taxa belong to the *S. cerevisiae* ERK family (*KSS1*, *FUS3*, *SLT2*, *HOG1*).[26]

Table 3.2: Distance Matrix of *Candida*, *Arabidopsis*, and *Giardia* CAK orthologs vs. yeast CDKs.

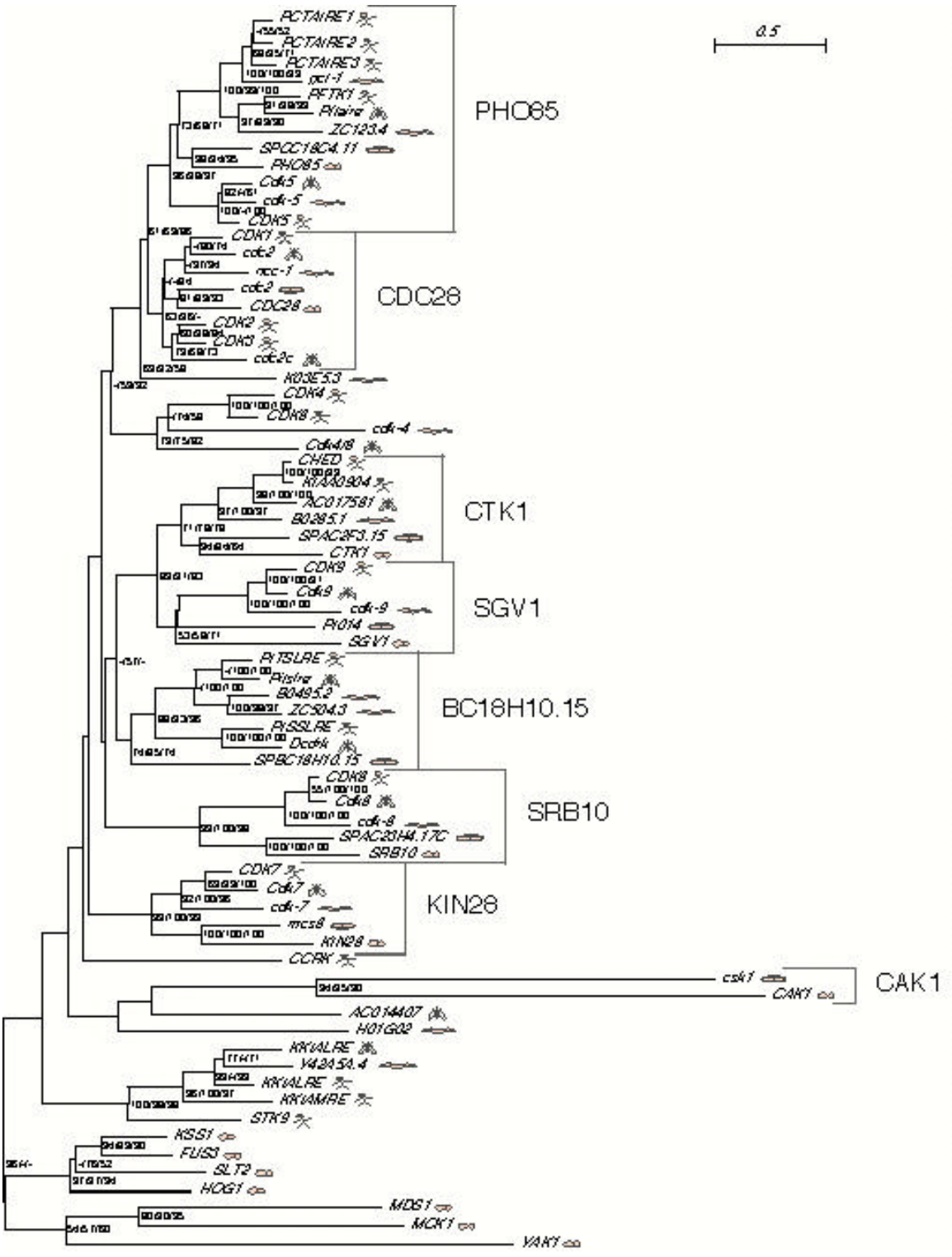
	Cdc28	**Pho85**	Kin28	**Srb10**	**Ctk1**	**Sgv1**	**Cak1**	**Csk1**
CaCAK1	2.17	2.10	2.16	**2.25**	2.16	2.12	***1.77***	1.73
Cak1At	1.23	1.39	***1.15***	1.40	1.42	1.41	**2.39**	2.18
T10F20 At	0.90	1.08	***0.80***	1.24	1.19	1.07	**1.94**	2.03
F25P22 At	0.88	1.05	***0.83***	1.22	1.18	1.05	**1.85**	2.02
AC05257	1.75	1.75	***1.37***	1.77	1.73	1.58	**2.40**	2.41

The scores represent the number of amino acid substitutions per site. Lower scores indicate taxa that are more similar, while higher scores indicate taxa that are more dissimilar. The scores of budding yeast taxa that are most similar to *C. albicans* CaCAK1, *A. thaliana* Cak1At, T10F20, and F25P22, and *G. lamblia* AC052571 are bold and b ; those most dissimilar are bold and underlined. A column comparing fission yeast Csk1 is also given, but scores are not highlighted. The scores are JTT distance scores which were created with the protml program of the MOLPHY version 2.3.[64]

Figures:

Fig. 3.1. Gamma-rate corrected neighbor-joining phylogeny of *S. cerevisiae, S. pombe, D. melanogaster, C. elegans,* and human extended CDK family members.[25] The amino acid data set was modeled onto one invariable and eight gamma-rates with the program PUZZLE to produce the pair-wise distances that were used to create the NJ tree. Branch lengths are proportional to the estimated number of amino acid substitutions; scale bar indicates amino acid substitutions per site. Bootstrap support values above 50% are given at branch nodes and are derived from ML (left), NJ of uncorrected data set (center), and gamma-rate corrected NJ (right) analyses, separated by slash marks. Species are denoted by cartoon. Ancestral clades are denoted by brackets on the right. Reprinted with permission from: Liu J and Kipreos E, *Mol Biol Evol.* 2000; 17:1061-1074. © Society for Molecular Biology and Evolution.

Fig 3.1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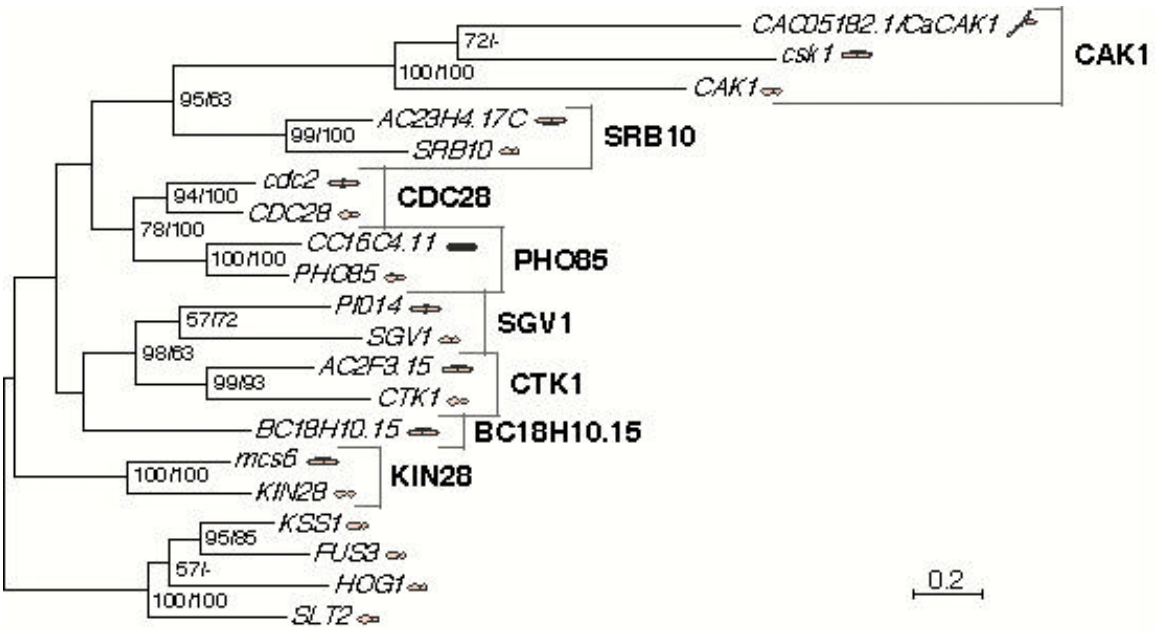
PCTAIRE2
PCTAIRE3
pct-1
PFTK1
Pfalre
ZC123.4
SPCC18C4.11
PHO85
Cak5
cak-5
CDK5
CDK1
cdc2
ncc-1
cdc2
CDC28
CDK2
CDK3
cdc2c
K03E5.3
CDK4
CDK6
cak-4
Cak4/6
CHED
KIAA0904
AC017581
B0285.1
SPAC3F3.15
CTK1
CDK9
Cak9
cak-9
Pi014
SGV1
PITSLRE
Pitslre
B0495.2
ZC504.3
PISSLRE
Dcdk
SPBC18H10.15
CDK8
Cak8
cak-8
SPAC20H4.17C
SRB10
CDK7
Cak7
cak-7
mcs8
KIN28
CCRK
csk1
CAK1
AC014407
H01G02
KKIALRE
Y43A5A.4
KKIALRE
KKIAMRE
STK9
KSS1
FUS3
SLT2
HOG1
MDS1
MCK1
YAK1
CAK1

Fig. 3.2. Neighbor-Joining and Maximum Likelihood phylogenies of *S. cerevisiae*, *S. pombe,* and *Candida albicans* CDK family members (methods see ref. 25). Branch lengths are proportional to the estimated number of amino acid substitutions; scale bar indicates amino acid substitutions per site. Bootstrap support values above 50% are given at branch nodes and are derived from ML (left) and NJ (right) analyses and are separated by slash marks. Species are denoted by cartoon. Ancestral clades are denoted by brackets on the right. The sequence alignment used for this analysis can be obtained upon request from the authors.

Fig 3.2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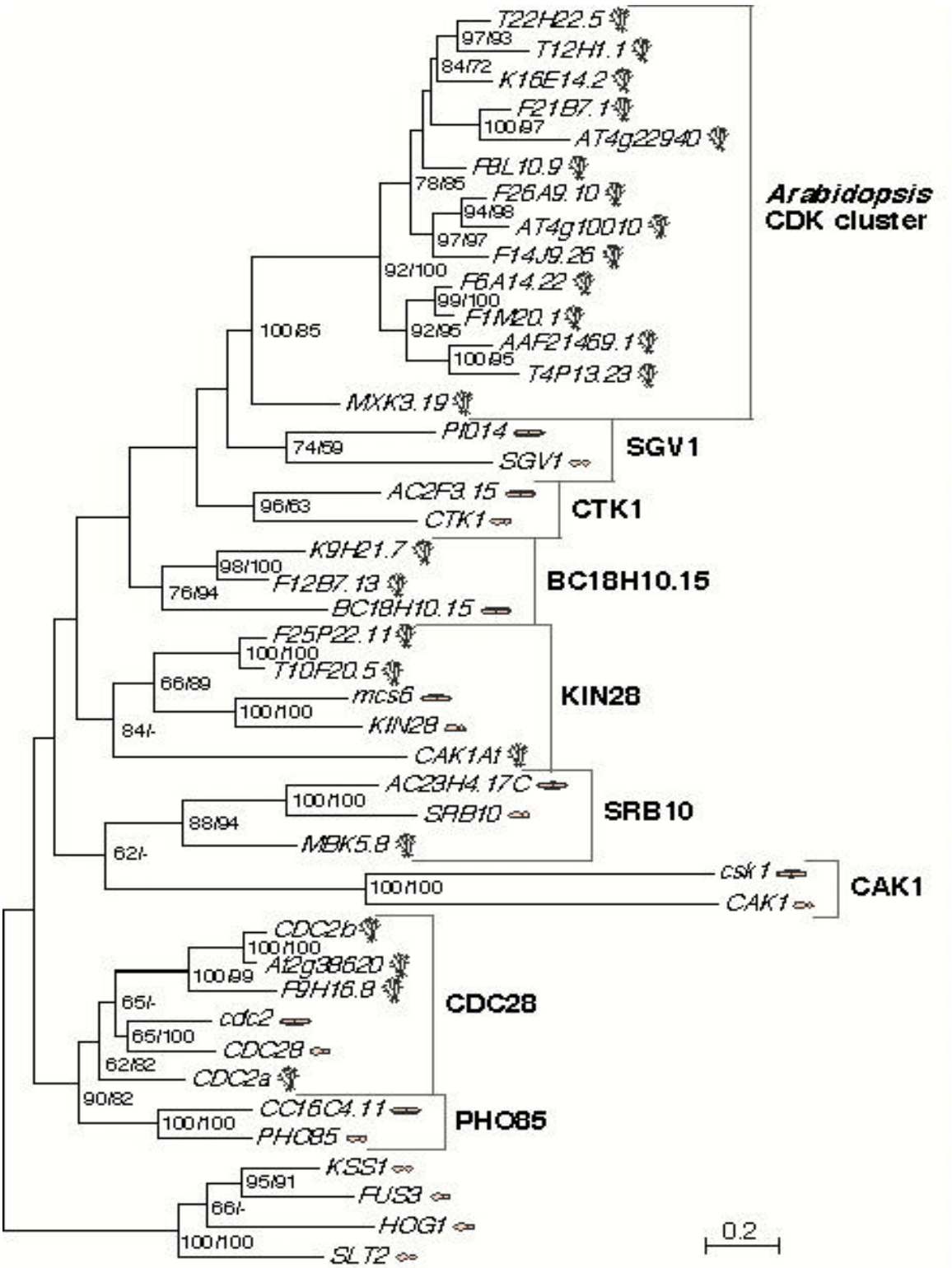
CAK1
CAK1
72/-
100/100
95/63
AC23H4.17C
SRB10
99/100
SRB10
cdc2
CDC28
94/100
CDC28
78/100
CC16C4.11
PHO85
100/100
PHO85
Pl014
SGV1
57/72
SGV1
98/63
AC2F3.15
CTK1
99/93
CTK1
BC18H10.15
BC18H10.15
mcs6
KIN28
100/100
KIN28
KSS1
95/85
FUS3
57/-
HOG1
100/100
SLT2
0.2

Fig. 3.3. Neighbor-Joining and Maximum Likelihood phylogenies of *S. cerevisiae*, *S. pombe,* and *Arabidopsis thaliana* CDK family members (methods see ref. 25). Branch lengths are proportional to the estimated number of amino acid substitutions; scale bar indicates amino acid substitutions per site. Bootstrap support values above 50% are given at branch nodes and are derived from ML (left) and NJ (right) analyses and are separated by slash marks. Species are denoted by cartoon. Ancestral clades are denoted by brackets on the right. The sequence alignment used for this analysis is available upon request from the authors.

Fig 3.3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97/93
T12H1.1
84/72
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78/85
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94/98
AT4g10010
97/97
F14J9.26
92/100
F6A14.22
99/100
F1M20.1
92/96
AAF21469.1
100/95
T4P13.23
100/85
MXK3.19
Arabidopsis
CDK cluster
PI014
74/59
SGV1
SGV1
AC2F3.15
96/63
CTK1
CTK1
K9H21.7
98/100
F12B7.13
76/94
BC18H10.15
BC18H10.15
F25P22.11
100/100
T10F20.5
66/89
mcs6
100/100
KIN28
KIN28
84/-
CAK1At
AC23H4.17C
100/100
SRB10
SRB10
88/94
MBK5.8
62/-
SRB10
csk1
100/100
CAK1
CAK1
CDC2b
100/100
At2g38620
100/99
P9H16.8
65/-
cdc2
65/100
CDC28
CDC28
62/82
CDC2a
90/82
CC16C4.11
100/100
PHO85
PHO85
KSS1
95/91
FUS3
66/-
HOG1
100/100
SLT2
0.2

CHAPTER 4

THE UBIQUITIN LIGASE CUL-2 COMPLEX IS REQUIRED FOR MEIOTIC ANAPHASE II CHROMOSOME MOVEMENT AND FOR ESTABLISHING POLARITY IN C. ELEGANS[1]

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[1]Liu, Ji; Srividya Vasudevan, and Edward T. Kipreos. Manuscript in preparation. Will submit to *CURRENT BIOLOGY*.

BACKGROUND

Meiosis is a specialized cell cycle that is adopted for gamete formation in which two rounds of cell divisions follow one round of DNA duplication. In meiosis I, chromosome homologs segregate. In meiosis II, sister chromatids segregate. The faithful segregation of chromosomes is vital for organism reproduction as mis-segregation leads to aneuploidy (chromosome loss or duplication), a condition that is usually fatal. In humans, aneuploidy caused by meiotic chromosome mis-segregation occurs in approximately 20% of pregnancies, most of which spontaneously abort (Hassold and Hunt, 2001). The underlying cause(s) of the high incidence of meiotic chromosome mis-segregation in humans are not well understood.

Chromosome segregation involves two processes. First, loss of cohesion between chromosome homologs or sister chromatids is essential for chromosome separation (Nasmyth, 2001). Second, spindle depolymerization as well as microtubule (MT) motors are required to generate a poleward force to move separated chromosomes towards the two spindle poles (Wittmann et al., 2001). In recent years, many insights have been made into the molecular mechanisms that regulate meiotic chromosome cohesion. A REC-8 Cohesin complex is required to hold chromosomes together in metaphase (Molnar et al., 1995; Klein et al., 1999; Pasierbek et al., 2001). In anaphase I, REC-8 is partially degraded from sister chromatid arms to allow the resolution of chiasmata and the separation of chromosome homologs. However, REC-8 that is located in the vicinity of the centromeres is not degraded and functions to maintain sister chromatid cohesion. In anaphase II, the remaining REC-8 is completely degraded to allow sister chromatid separation (Maguire, 1974; Klein et al., 1999; Buonomo et al., 2000). Degradation of

REC-8 in meiosis is regulated by the same Anaphase Promoting Complex/Cyclosome (APC/C) – Saperase pathway that functions in mitosis to degrade Cohesins (Nasmyth, 2001; Siomos et al., 2001). In mitosis, after chromosomes have aligned properly on the metaphase plate, the APC/C ubiquitin ligase is activated. It targets the ubiquitin-mediated degradation of Securin, an inhibitory binding protein for Saperase. The freed Saperase is then able to cleave and remove Cohesin from the chromosomes, leading to chromosome separation. During meiosis, the aurora-B kinase AIR-2 is required for REC-8 degradation, and the selective phosphorylation of REC-8 by AIR-2 has been proposed to allow the differential degradation of REC-8 on chromosome arms and at centromeric regions (Rogers et al., 2002).

In anaphase, mitotic or meiotic separated chromosomes need to move towards the spindle poles. In mitosis, three mechanisms have been shown to generate poleward chromosome movement: MT depolymerization at the spindle poles (poleward flux); MT depolymerization at the kinetochore to draw the chromosomes to the poles; and force generated by cytoplasmic dynein, a minus-end-directed MT motor protein that localizes to kinetochores (Maney et al., 2000, Banks and Heald, 2001, Wittmann et al., 2001). In contrast to the detailed information available on mitotic anaphase chromosome movement, there is little known about the molecular mechanisms responsible for anaphase chromosome movement during meiosis.

In this work, we demonstrate that the *C. elegans* ubiquitin-ligase component CUL-2 is required for anaphase chromosome movement in meiosis II. In mammalian cells, the cullin CUL2 forms a cullin/RING finger complex with the RING-H2 finger protein RBX1/ROC1, elongin C, elongin B, and the VHL tumor suppressor protein

(Kamura et al., 1999; Pause et al., 1997; Lonergan et al., 1998). VHL functions as a substrate recognition factor for the degradation of hypoxia-inducible factor-1α and -2α (HIF-α), atypical protein kinase C, and the VDU1 and VDU2 deubiquitinating enzymes (Maxwell et al., 1999; Okuda and Kimura, 1988; Li et al., 2002). It is likely that the core CUL-2 complex will interact with other substrate recognition factors as human SOCS-1 can replace VHL to target the degradation of the chromosomal translocation fusion protein TEL-JAK2 (Kamizono et al., 2001). The CUL-2/RING finger complex functions to bring substrates into close proximity to the ubiquitin-conjugating enzyme UBC5, which transfers ubiquitin to the substrate to form poly-ubiquitin chains that target the substrate for degradation by the 26S proteasome (Furukawa et al., 2002).

In *C. elegans*, CUL-2 is required for multiple cellular pathways (Feng et al., 1999). CUL-2 negatively regulates the protein level of a CDK inhibitor, CKI-1, to allow the G1-to-S phase cell cycle transition. CUL-2 is also required to promote proper cytoskeletal movements, mitotic progression, and mitotic chromosome condensation (Feng et al., 1999 and ETK unpublished data). Here we show that inactivation of CUL-2 results in a severe delay in meiotic anaphase II chromosome movement. In contrast, anaphase I chromosome segregation is normal. The Cohesin REC-8 is degraded normally in *cul-2* mutant zygotes, suggesting that CUL-2 is not required for resolving chromosome cohesion but is required for the physical movement of sister chromatids to the spindle poles. CUL-2 is also required for the establishment of anterior-posterior polarity in the embryo. In CUL-2 depleted embryos, the polarized mitotic division pattern is abnormal and polarity marker proteins are misplaced.

RESULTS

CUL-2 is required for chromosome segregation at meiosis II

We examined the chromosome dynamics in *cul-2* mutants and *cul-2* (RNAi) zygotes using live time-lapse movies of histone H2B::GFP (Fig. 1A, B). In wild-type zygotes, following fertilization, the six chromosome bivalents align in a pentagonal array to enter metaphase I. Each bivalent consists of duplicated paternal and maternal homologs (univalents) contacting each other end-to-end (Albertson and Thomson, 1993). At this stage, each dot in the histone::GFP movies represents one univalent which has two sister chromatids. Initially, the long axis of the spindle is parallel to the surface of the zygote. The spindle, along with the bivalents, rotates and becomes perpendicular to the zygote surface before the homologs segregate at anaphase I (Fig. 1A, Albertson and Thomson, 1993; Rogers et al., 2002). After a 2N polar body is extruded from the zygote, the remaining six sister chromatid pairs enter metaphase II and again adopt a similar pentagonal array configuration. At this time, individual sister chromatids are seen contacting each other end-to-end (Fig. 1A, Albertson and Thomson, 1993). Again, the chromosomes rotate and segregate at anaphase II. After the second polar body is extruded from the zygote, the remaining six chromatids decondense, marking the completion of meiosis and the entry into interphase of the first mitotic cell cycle. In wild-type, zygotes complete meiosis at approximately 30 minutes post-fertilization.

Depleting CUL-2 either by RNAi (Fire et al., 1998) or by mutational inactivation using the *cul-2(ek1)* deletion allele (Feng et al., 1999) resulted in essentially the same phenotype (Fig. 1B). In both *cul-2* (RNAi) and *cul-2(ek1)* zygotes, the chromosome dynamics are normal up to the onset of metaphase II. As in the wild-type, it takes about

15 minutes post-fertilization for *cul-2* (RNAi) and mutant zygotes to go through meiosis I (Fig1B, C). However, in *cul-2* zygotes, metaphase II sister chromatid pairs remain in a pentagonal array for about three times as long as in the wild-type. Afterwards, the chromosomes either start to decondense (in 2 out of 5 cases) or they congregate into a tight knot (in 3 out of 5 cases) and individual chromosomes can no longer be discerned (data not shown). Eventually in all cases the maternal chromosomes decondense and exit meiosis. In the 18 zygotes we observed, ten never executed anaphase II (Fig. 1B), while the other eight split their DNA unequally either before or after they started to decondense (data not shown). As a result, while meiosis II is completed in wild-type in about 15 minutes, in *cul-2* (RNAi) zygotes it takes about 50 minutes and in *cul-2(ek1)* it takes more than 60 minutes (Fig. 1B, C).

CUL-2 is not required for REC-8 degradation

A delay or failure to segregate chromosomes during the metaphase-to-anaphase transition could result from defects in either chromosome separation or chromosome movement. Chromosome separation is triggered by the degradation of the Cohesin from sister chromatids. In wild-type anaphase II, the Cohesin REC-8 is completely degraded from sister chromatids (Klein et al., 1999, Buonomo et al., 2000, Watanabe and Nurse, 1999). If REC-8 failed to be degraded, we would expect that sister chromatids would not be able to separate. Chromosome segregation could still fail if REC-8 has been degraded but the mechanical force to move the separated chromosome towards opposite poles is missing.

To determine if REC-8 is degraded normally in *cul-2* depleted zygotes, we studied REC-8 localization using immunofluorescence microscopy with an anti-REC-8

antibody (Pasierbek et al., 2001). We found that the REC-8 staining pattern in *cul-2* (RNAi) zygotes was the same as that in wild-type zygotes. In meiosis I, REC-8 is located along the axes of sister chromatids (Fig. 2E, F, M, N; Pasierbek et al., 2001), and in metaphase II, REC-8 is located at the junction of the sister chromatids (Fig. 2G, O, Pasierbek et al., 2001,). In anaphase II, REC-8 is completely lost from the separating wild-type chromatids (Fig. 2H; Pasierbek et al., 2001). In *cul-2* (RNAi) zygotes, chromosomes remain in a metaphase II pentagonal array for an extended period of time, but the vast majority of them do not have REC-8 staining (Fig. 2P). In our experiments, we observed that 100% of wild-type zygotes have metaphase II REC-8 staining (n = 6). In contrast, only 30% of *cul-2* metaphase II zygotes have REC-8 staining (n = 26). Further, when *cul-2* (RNAi) zygotes are in the later stages of the extended meiosis II, the maternal DNA often wanders away from the anterior pole of the egg. None of these late stage meiosis II zygotes have REC8 staining (data not shown). This suggests that REC-8 degradation occurs in *cul-2* inactivated zygotes with a timing similar to that of wild-type even though they remain in metaphase II without separating the sister chromatids.

Loss of cohesion by *rec-8* (RNAi) **cannot rescue the *cul-2* mutant phenotype**

The loss of REC-8 staining during metaphase II in *cul-2* (RNAi) zygotes suggests that the defect in chromosome segregation is not due to a failure to degrade REC-8. As Cohesin degradation has been found to be sufficient for anaphase chromosome separation (Uhlmann et al., 2000), this suggests that the chromosomes in the extended metaphase II arrest of *cul-2* depletion zygotes are not strictly bound together. Further, we observed that occasionally sister chromatids separate during the extended metaphase II of *cul-2*

(RNAi) and *cul-2(ek1)* zygotes (Fig. 1D). In 1/11 *cul-2* (RNAi) and 1/ 7 *cul-2(ek1)* mutants, 12 individual chromatids were observed at the metaphase II plate rather than the normal 6 joined sister chromatids, indicating that cohesion has been lost from the sister chromatids. However, in these zygotes, anaphase II chromosome movement was still lacking.

To further confirm that the failure of sister chromatid separation was not due to residual cohesion factors, we artificially eliminated sister chromatid cohesion by subjecting zygotes to *rec-8* (RNAi). With REC-8 depleted, sister chromatids lose cohesion throughout meiosis. As a result, in metaphase I, 24 chromosomes instead of six bivalents are present (Fig. 3, metaphase I). In both *rec-8* (RNAi) and *cul-2* (RNAi), *rec-8* (RNAi) zygotes, chromosome segregation during anaphase I is normal and one polar body is extruded (Fig. 3, anaphase I). In metaphase II, 12 chromosomes instead of six chromatid pairs are present after *rec-8* (RNAi) (Fig. 3, metaphase II). In the majority of both *rec-8* (RNAi) and *cul-2* (RNAi), *rec-8* (RNAi) zygotes, metaphase II chromosomes fail to segregate. However, a careful examination of the histone::GFP movies revealed that the dynamics of the chromosome movements are very different between *rec-8* (RNAi) and *cul-2* (RNAi), *rec-8* (RNAi) zygotes. In *rec-8* (RNAi) zygotes, although the chromosomes did not segregate into two DNA masses, all of the chromosomes compressed tightly together (as is seen in wild-type anaphase) and moved to the edge of the embryo at the time corresponding to anaphase II in wild-type (n = 10), as if all the chromosomes were being actively moved toward the cell cortex. After several minutes the chromosomes moved back to the cytoplasm and immediately started to decondense. As a result, *rec-8* (RNAi) zygotes finished meiosis with the normal timing of

approximately 30 minutes. This observation suggests that in *rec-8* (RNAi) zygotes anaphase II movement still occurs, albeit mono-directionally. Interestingly, the same kind of mono-poleward chromosome movement was also observed for *C. elegans* zygotes that have been depleted of the aurora B kinase AIR-2, which are incapable of degrading REC-8 (Rogers et al., 2002). The mechanism for this type of monopolar-like movement in *C. elegans* is not understood. In contrast to *rec-8* (RNAi) zygotes, in *cul-2* (RNAi), *rec-8* (RNAi) zygotes, all chromosomes stayed in the metaphase II plate without any anaphase-like compression or movement. In these double RNAi zygotes, metaphase II lasted for an extended period of time similar to that observed for *cul-2* (RNAi) zygotes. Afterwards, as for *cul-2* (RNAi) zygotes, chromosomes finally decondense either with or without chromosome splitting (Fig. 3). We conclude that removing cohesion between sister chromatids does not rescue the failure of sister chromosomes to initiate anaphase poleward movement in *cul-2* zygotes, and that the anaphase II defect in *cul-2* zygotes is due to a lack of chromosome movement instead of a failure to lose cohesion.

The morphology of the meiotic spindle is normal in *cul-2* zygotes

We studied the morphology and dynamics of the meiotic spindle in *cul-2* zygotes by both anti-α-tublin antibody staining and live β-tublin::GFP movies (Fig. 4; and data not shown). With both approaches *cul-2* meiosis II spindle morphology appeared normal. The only defect we detected was that the meiosis II spindle in *cul-2* zygotes lasted three times as long as in the wild-type: the wild-type meiotic II spindle lasted 12.2 ± 0.68 minutes while the *cul-2* meiosis II spindle lasted 36.2 ± 2.45 minutes (n = 6 for each

case). Significantly, the meiosis II spindle duration in *cul-2* zygotes generally corresponds to the time the chromosomes spend in the pentagonal array (data not shown).

RNAi of the other CUL-2 ubiquitin ligase complex components phenocopy the *cul-2* meiosis II chromosome segregation defect

In mammalian cells, CUL-2 forms a Cullin-RING finger complex with RBX1, elongin C, elongin B, and VHL (Kamura et al., 1999, Pause et al., 1997, Lonergan et al., 1998). The complex functions as a ubiquitin ligase that interacts with the UBC5 ubiquitin conjugating enzyme to add ubiquitin onto substrate proteins (Furukawa et al., 2002).

To test the hypothesis that CUL-2 functions to promote meiotic progression in the context of a conserved ubiquitin ligase complex, we inactivated predicted CUL-2 complex components to determine if they produce the same phenotype. dsRNA for *C. elegans* orthologs of RBX1 (*rbx-1*), elongin C (*eln-1*), and UBC5 (*ubc-2*) were injected into histone H2B::GFP hermaphrodites and the progeny were analyzed. As shown in Fig. 5, both *rbx-1* (RNAi) and *eln-1* (RNAi) zygotes have meiotic phenotypes similar to *cul-2* (RNAi) zygotes: a normal meiosis I followed by an extended metaphase II delay with no anaphase II chromosome movement. This result provides support for the proposal that CUL-2, RBX-1, and ELN-1 function in the same ubiquitin ligase complex to initiate anaphase II chromosome movement. In contrast, RNAi of the elongin B ortholog, *eln-2*, or the VHL ortholog, *vhl-1*, did not produce any embryonic phenotype (H. Feng and ETK, unpublished data). This is not likely to be due to a reduced susceptibility of *cln-2* or *vhl-1* to RNAi, because a vhl-1 mutant also failed to produce any phenotype (Epstein

et al., 2001). Therefore, ELN-2 and VHL-1 are either not within the complex that acts to promote anaphase II, or they are not essential for that complex's function.

RNAi of *ubc-2* produced two kinds of phenotypes. Two zygotes were followed 12-18 hours after dsRNA injection of the adult hermaphrodites. They finished meiosis I with slightly prolonged timing (20 minutes and 30 minutes, respectively) and arrested at metaphase II. The other three zygotes were observed 24 hours after dsRNA injection, and they all arrested at metaphase I stage (data not shown). These results indicate that UBC-2 is required for progression through both meiotic divisions, probably interacting with a different ubiquitin ligase to promote meiosis I progression.

CUL-2 is required for the establishment of polarity

We found that *cul-2* zygotes have various anterior-posterior (A-P) polarity defects. In wild-type zygotes, the first mitosis produces a larger anterior cell AB and a smaller posterior cell P (Sulston and Horvitz, 1977; Sulston et al., 1983; Fig. 6, left column). At the two-cell stage, the polarity marker protein PAR-2 is exclusively on the cell cortex of the P cell, and another polarity marker protein PAR-6 is exclusively on the cell cortex of the AB cell (Fig. 6, left column; Boyd et al., 1996; Hung and Kemphues, 1999). In *cul-2* zygotes, AB can be either bigger or smaller than P, and PAR-2 and PAR-6 can be located on the cortex of either AB, P, or both, irrespective of the division pattern (Fig. 6, middle and right columns).

In *C. elegans*, the establishment of A-P polarity has been proposed to be a microtubule (MT) structure-directed process (Hird and White, 1993; Goldstein and Hird, 1996; O'Connell et al., 2000; Wallenfang and Seydoux, 2000). To study the initial

polarity pattern in *cul-2* zygotes, we co-stained zygotes with anti-α-tubulin and anti-PAR-2 antibodies. In wild-type, PAR-2 is always initiated posteriorly near the sperm asters after meiosis (Fig. 7A). In *cul-2* (RNAi) animals, however, 43% (10/23) of the zygotes had an anterior cortical or a lateral cortical PAR-2 patch before they formed obvious sperm asters, suggesting that the polarity cue in these cases was not derived from the sperm asters. Among them, 80% initiated PAR-2 at the anterior cortex, and 20% initiated PAR-2 at the lateral cortex (n = 10). Among the eight zygotes that had an anterior cortical patch, six had the meiotic spindle positioned close to the cortex where PAR-2 is localized (Fig. 7B), while in the other two zygotes, the meiotic spindles were not near the cortical PAR-2 patch (Fig. 7C). In those zygotes with lateral PAR-2 patches the meiotic spindle was not nearby (Fig. 7D). The observation of PAR-2 cortical localization distant from both the meiotic spindle and sperm asters was confirmed by following *cul-2* (RNAi) zygotes expressing PAR-2 and β-tubulin, both of which were tagged with GFP. We observed clear examples in which the PAR-2 cortical patch initiated distant from both the meiotic spindle and sperm asters (data not shown).

In summary, 60% of *cul-2* zygotes have PAR-2 cortical patches in the vicinity of the meiotic spindle, suggesting that PAR-2 localization was initiated by meiotic spindle polarity cues in those cases. This correlates with observations by Wallenfang et al. that an extended duration of the meiotic spindle can reverse polarity cues and direct PAR-2 onto the anterior cortex (Wallenfang and Seydoux, 2000). However, in 40% of *cul-2* zygotes, the PAR-2 patch does not appear to be initiated by any MT cues, as they do not originate in the vicinity of any MT structures. We conclude that CUL-2 is required for

the initiation of polarity and that in *cul-2* (RNAi) zygotes PAR-2 cortical localization can occur through mechanisms other than MT-induced localization.

DISCUSSION

CUL-2 is required for anaphase II chromosome movement but not for sister chromatid separation

Our results demonstrate that in embryos devoid of CUL-2, anaphase II chromosome movement either fails to occur or is severely delayed. There are three lines of evidence that indicate that this defect is not due to a failure of the sister chromatids to separate from one another. First, immunostaining using anti-REC-8 antibody showed that the Cohesin REC-8, which is required to hold sister chromatids together (Pasierbek et al., 2001), is degraded normally in *cul-2* mutants. Second, we observed a small percentage of *cul-2* mutant zygotes in which sister chromatids separated during meiosis II but still failed to move to the spindle poles. Finally, in *rec-8* (RNAi), *cul-2* (RNAi) zygotes, all chromatids appeared to separate completely but again failed to segregate.

The pentagonal array of sister chromatids is maintained in *cul-2* mutants during metaphase II despite the fact that REC-8 has been degraded. This suggests that once formed, the pentagonal array configuration does not depend on REC-8-mediated cohesion between sister chromatids, but instead can be maintained by other mechanisms, perhaps MT-chromosome interactions. However, sister chromatid cohesion does appear to stabilize the pentagonal array configuration, as we occasionally observe, as mentioned, a breakdown of the structure in which individual chromosomes separate from each other (Fig. 1D).

Previously, we reported that *cul-2* mutant embryos are defective in mitotic chromosome condensation (Feng et al., 1999). However, meiotic chromosomes in *cul-2* appear to be well condensed. Under the compound microscope, *cul-2* meiotic chromosomes are morphologically indistinguishable from those of the wild-type. Furthermore, it is unlikely that the observed anaphase chromosome movement defect would result from a defect in chromosome condensation, as the *C. elegans smc-4* mutant, which is defective for chromosome condensation, undergoes normal anaphase II movement, although DNA bridges form between the separating chromosome masses (Hagstrom et al., 2002).

Mitotic anaphase chromosome movement has been extensively analyzed. Both MT spindle dynamics and MT motors have been shown to be required for mitotic anaphase poleward force generation (Wittmann et al., 2001). While it is possible that similar mechanisms will be employed for meiotic chromosome movements, this has not yet been determined. Interestingly, the morphology and dynamics of the meiotic spindle during anaphase are quite different from the mitotic spindle, suggesting that meiosis and mitosis might employ different mechanisms of chromosome segregation. We have observed, in agreement with earlier observations (Albertson and Thomson, 1993), that during both anaphase I and II the meiotic spindle pole MTs appear to depolymerize and become progressively less visible, while the amount of midzone MTs between the separating chromosomes increases dramatically so that at the end of anaphase almost all MTs are located between the separating chromosomes. In contrast, using the tubulin::GFP time lapse movies, we found that during mitosis, the majority of the anaphase MTs are polar MTs with very few midzone MTs. The reorganization of MTs to

the midzone that occurs in meiotic anaphase suggests that chromosomes may be "pushed" apart by MT polymerization between the chromosomes rather than being "pulled" to the spindle poles. Such a mechanism is not dominant in *C. elegans* mitosis, as ablation of the MTs between the separating chromosomes was found to produce both increased velocity and distance of chromosome separation (Grill et al., 2001). Therefore, in C. elegans, mitotic midzone MTs are not required to generate force to "push" chromosomes apart but instead may function to limit the speed and/or extent of chromosome movement.

We observed that the meiotic II spindle maintained its normal shape and size during the extended metaphase II in *cul-2* mutant zygotes (data not shown), indicating that MT depolymerization has been delayed in the zygote. This lack of depolymerization could be a primary defect for *cul-2*'s anaphase movement failure, given that MT depolymerization at the spindles is correlated with anaphase chromosome separation. Alternatively, the lack of depolymerization could be a secondary consequence of the failure to induce chromosome movement.

Anaphase I and anaphase II chromosome movements are differentially regulated

We have shown that in *cul-2* mutant zygotes, anaphase I is normal but anaphase II is severely delayed. This is not likely due to a purdurance of maternal product in meiosis I relative to meiosis II, since the mutant zygote is derived from *cul-2*(*ek1*) homozygous hermaphrodites, which have no detectable CUL-2 protein in oocytes or early embryos (Feng et al., 1999). We conclude that CUL-2 is required for meiosis II but not meiosis I. Our results therefore suggest that anaphase I and anaphase II are differentially regulated.

The observation that CUL-2 is required specifically for anaphase II suggests that there is a CUL-2-dependent activity that is required for anaphase II but not for anaphase I. Observations of meiotic anaphase I and II spindles in wild-type did not reveal any major differences in spindle dynamics (data not shown; Albertson and Thomson, 1993). One major difference between metaphase I and II is that the chromosome-spindle pole attachments are different. In metaphase I, both sister chromatids are attached to the same spindle pole, while in metaphase II, sister chromatids are attached to opposite poles (Albertson and Thomson, 1993). FISH analysis of chromosomes suggested that there is a switch of the kinetochore-spindle attachments between meiosis I and II (Albertson and Thomson, 1993). The end where the two sister chromatids connect to each other during meiosis I is not the end for attachment in meiosis II. It is possible that CUL-2 is required for the attachment of spindle MTs to the free chromosome ends in metaphase II and that the segregation defect in *cul-2* is due to a mis-attachment of the MTs. However, it's obvious that some aspects of spindle-chromosome interactions are still present in *cul-2* zygotes, since the metaphase II chromosomes position perfectly in the pentagonal array and rotate normally in *cul-2* zygotes.

Predicted CUL-2/Ring finger complex components share the same anaphase II chromosome movement defect

In mammalian cells, CUL2 forms a cullin-ring finger complex with elongin C, elongin B, RBX1, and VHL proteins (Kamura et al., 1999; Pause et al., 1997; Lonergan et al., 1998). The complex acts as a ubiquitin ligase (E3 enzyme) and interacts with the E2 ubiquitin conjugating enzyme UBC5 to covalently link ubiquitin onto substrate proteins and

thereby mark them for degradation (Furukawa et al., 2002). Here we showed that depletion of the *C. elegans* orthologs of elongin C and RBX1, *eln-1* and *rbx-1*, respectively, resulted in the same meiotic phenotype as CUL-2 depletion. Other embryonic *cul-2* phenotypes, e.g, formation of multiple nuclei and cytoskeletal extensions, are also present in *eln-1* (RNAi) and *rbx-1* (RNAi) embryos, suggesting that CUL-2 is regulating zygotic development as part of a cullin/RING finger E3 complex (H. Feng and ETK, unpublished data). Inactivation of either elongin B or VHL orthologs produced no embryonic phenotypes (H. Feng and ETK, unpublished data). Further, a *vhl-1* deletion mutant also has no embryonic phenotypes (Epstein et al., 2001). These results suggest that neither elongin B nor VHL are required for CUL-2/RING finger complex regulation of anaphase II. Therefore, it is likely that the core CUL-2 complex employs a different substrate recognition component than VHL for its meiotic functions. RNAi depletion of the UBC5 ortholog, *let-70*/*ubc-2* (Jones et al., 2002), produced both metaphase I and metaphase II arrests, indicating that UBC-2 is required for both anaphase I and anaphase II. The requirement of UBC-2 for anaphase I suggests that UBC-2 interacts with other ubiquitin ligases to promote this aspect of meiotic progression. In total, our results provide the first indication that a cullin/Ring finger complex is required for anaphase chromosome movement.

CUL-2 is required for the establishment of polarity

We have shown that *cul-2* embryos are defective in establishing anterior-posterior (A-P) polarity. In *C. elegans*, the establishment of A-P polarity has been shown to closely correlate with MT-directed processes. In wild-type embryos, the position of the sperm pronucleus/centrosome complex (SP/CC) defines the posterior end of the embryo and the polarity marker PAR-2 is always located at the posterior cortex (Goldstein and Hird, 1996, Boyd et al., 1996). In a mutant that is defective in sperm aster formation, the A-P axis is disrupted (O'Connell et al., 2000). Further, in APC/C mutants that have prolonged meiotic spindles resulting from a metaphase I arrest, polarity is reversed and PAR-2 is initiated on the anterior cortex near the meiotic spindle (Wallenfang and Seydoux, 2000). In *cul-2* mutants, the initial PAR-2 patch was established either on the anterior cortex or the lateral cortex but not on the posterior cortex. Interestingly, contrary to the virtually complete correlation of PAR-2 localization with the meiotic spindle reported by Wallenfang and Seydoux, we found that the PAR-2 initiated cortical localization distant to any MT structure in 40% of the *cul-2* (RNAi) zygotes, indicating that in these cases PAR-2 is localized independently of MT structures. Therefore, the polarity defect in *cul-2* is not likely a mere secondary defect of meiosis delay.

METHODS

Strains and alleles

C. elegans strains were cultured as previously described (Brenner, 1974). Strains and alleles used were Bristol N2; AZ212 [unc-119(ed3) ruIs32(pAZ132: pie-1/GFP/histoneH2B)]; AZ244 [unc-119(ed3) III, ruIs57(pAZ147: pie-1/GFP/tubulin)];

cul-2(ek1)/unc-64(e246) III, JH1446 {KK866 [pMW1.03(SacII), pRF4(EcoRI), N2 genomic (Scal)]}; JH1473 {KK866 [pMW1.03(SacII), pRF4(EcoRI), N2 genomic (Scal)]}; WH204 (GFP/tubulin), and ET108 [*cul-2* (ek1)/unc-64 (e246), histone-GFP]. All GFP starins were maintained at 24°C except for ET108, which was maintained at 20°C. All other strains were maintained in 20°C.

RNA-mediated interference (RNAi)

RNAi was performed either by injection of dsRNA into hermaphrodites or by feeding with dsRNA-expressing bacteria. dsRNA injection followed the procedure of Liu and Kipreos (2000). RNAi feeding followed the protocol of Timmons et al. (2001). The *rec-8, cul-2* double RNAi experiment followed the protocol of Davis et al. (2002), except that after *rec-8* (RNAi) injection of adult hermaphrodites, F1 progeny were allowed to grow to the L4 stage and then put on *cul-2* (RNAi) bacteria feeding plates for 24 hours before observation.

Time-lapse epifluorescence microscopy

Time-lapse movies were made on embryos *in utero*. Young adult hermaphrodites were mounted on 4% agarose pads and anesthetized in 0.1% tricaine, 0.01% tetramisole in M9. A Zeiss Axioplan microscope equipped with a Hamamatsu ORCA-ER CCD camera, LUDL hardware controller, automated filter wheels and shutters, and a G4 Mac computer equipped with Openlab software were used for automatic time-lapse imaging.

Immunofluorescence microscopy

We used anti-REC-8, anti-PAR-2, and anti-α-tubulin antibodies. The immunofluorescence protocol of Pasierbek et al. (2001) was followed for anti-REC-8 and anti-α-tubulin antibodies. The immunofluorescence protocol of Boyd et al. (1996) was followed for the anti-PAR-2 antibody. The secondary antibodies used were goat anti-mouse antibody conjugated to rhodamine (Cappel) and goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Molecular Probes). Slides were incubated with 1 µg/ml DAPI prior to mounting in 90% glycerol/PBS with 1 mg/ml p-phenylenediamine.

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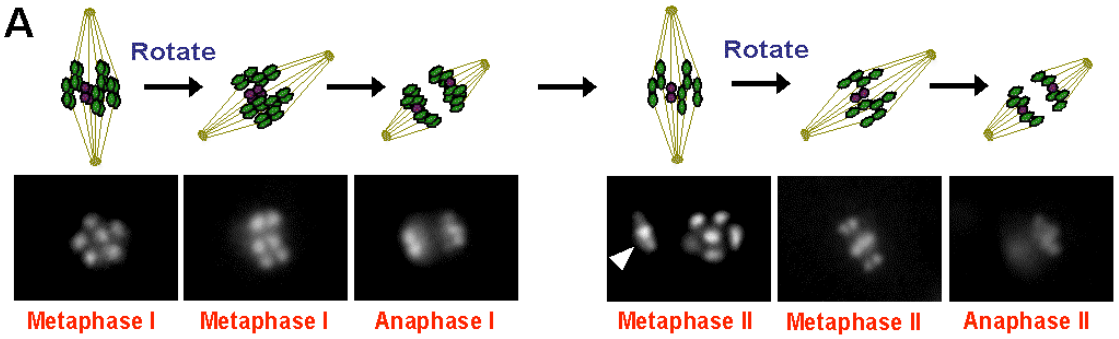
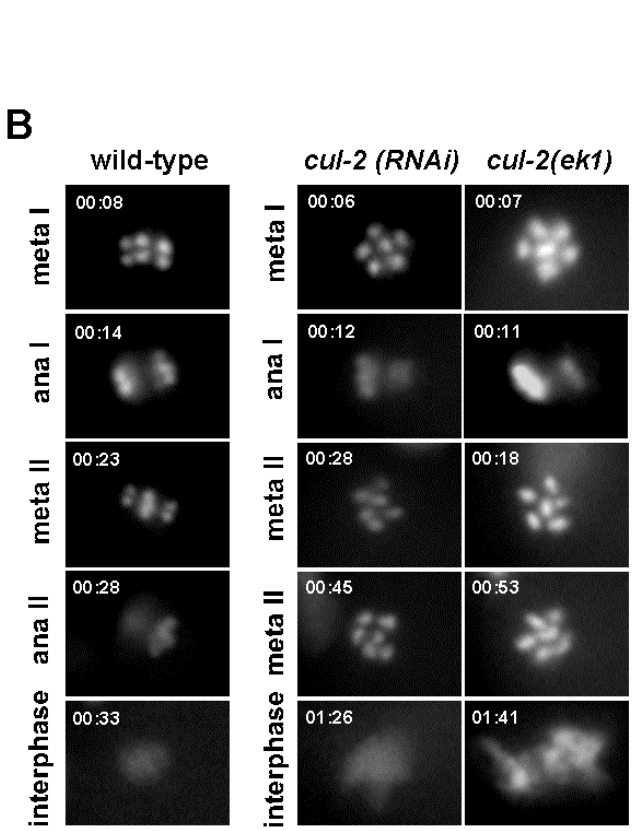
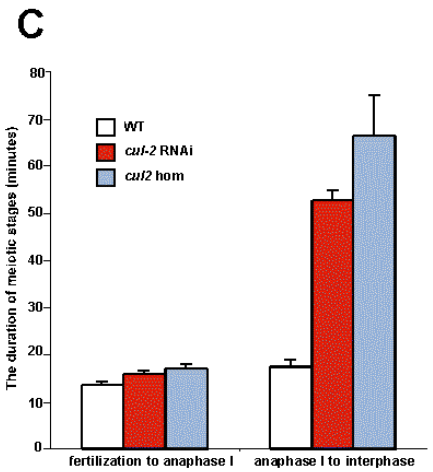
Figure 5.1. *cul-2* (RNAi) and mutant zygotes undergo a metaphase II delay. **A**: Diagrams (top) and histone H2B::GFP epifluorescence images (bottom) of meiotic chromosome orientation in wild-type embryos. The diagrams correspond to the histone H2B::GFP images when viewed from the top of the page down. The X chromosome, at the center of the pentagonal array (Albertson and Thomson, 1993), is labeled purple in the diagram. For both metaphase I and II, after the spindle has rotated, only three chromosomes can be observed in a given focal plane of the histone H2B::GFP images. The anterior of the zygote is to the left. The arrowhead denotes a polar body. **B**: Histone H2B::GFP epifluorescence movie sequences of live wild-type (left), *cul-2* (RNAi) (middle), and *cul-2*(*ek1*) mutant (right) zygotes. Time after fertilization is indicated in the upper left corner of images (hr:min). The stage of meiosis is indicated on the left of the image; interphase denotes the initial time of chromosome decondensation after meiotic exit. Note that *cul-2*(RNAi) and *cul-2*(*ek1*) mutant zygotes spend excessive time in metaphase II. **C**: The duration of meiotic stages in wild-type and *cul-2* animals as observed in histone H2B::GFP movies. **D**: Histone H2B::GFP epifluorescence movie images of 12 separated metaphase II chromosomes in *cul-2*(RNAi) and *cul-2(ek1)* embryos.


A
Rotate
Rotate
Metaphase I
Metaphase I
Anaphase I
Metaphase II
Metaphase II
Anaphase II


B
wild-type
cul-2 (RNAi)
cul-2(ek1)
meta I
ana I
meta II
ana II
interphase
00:08
00:14
00:23
00:28
00:33
00:06
00:07
00:12
00:11
00:28
00:18
00:45
00:53
01:26
01:41


C
The duration of meiotic stages (minutes)
WT
cul-2 RNAi
cul2 hom
fertilization to anaphase I
anaphase I to interphase

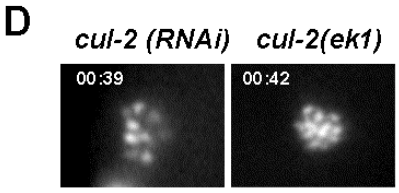

D
cul-2 (RNAi)
cul-2(ek1)
00:39
00:42

Figure 5.2. The Cohesin REC-8 is degraded normally in *cul-2*(RNAi) zygotes. Immunofluorescence images of REC-8 antibody staining and DAPI staining of DNA at the indicated meiotic stages for wild-type (top) and *cul-2* (RNAi) zygotes (bottom). Note that in late metaphase II *cul-2* (RNAi) zygotes, REC-8 has been degraded but the chromosomes fail to segregate. Arrowhead denotes polar body.

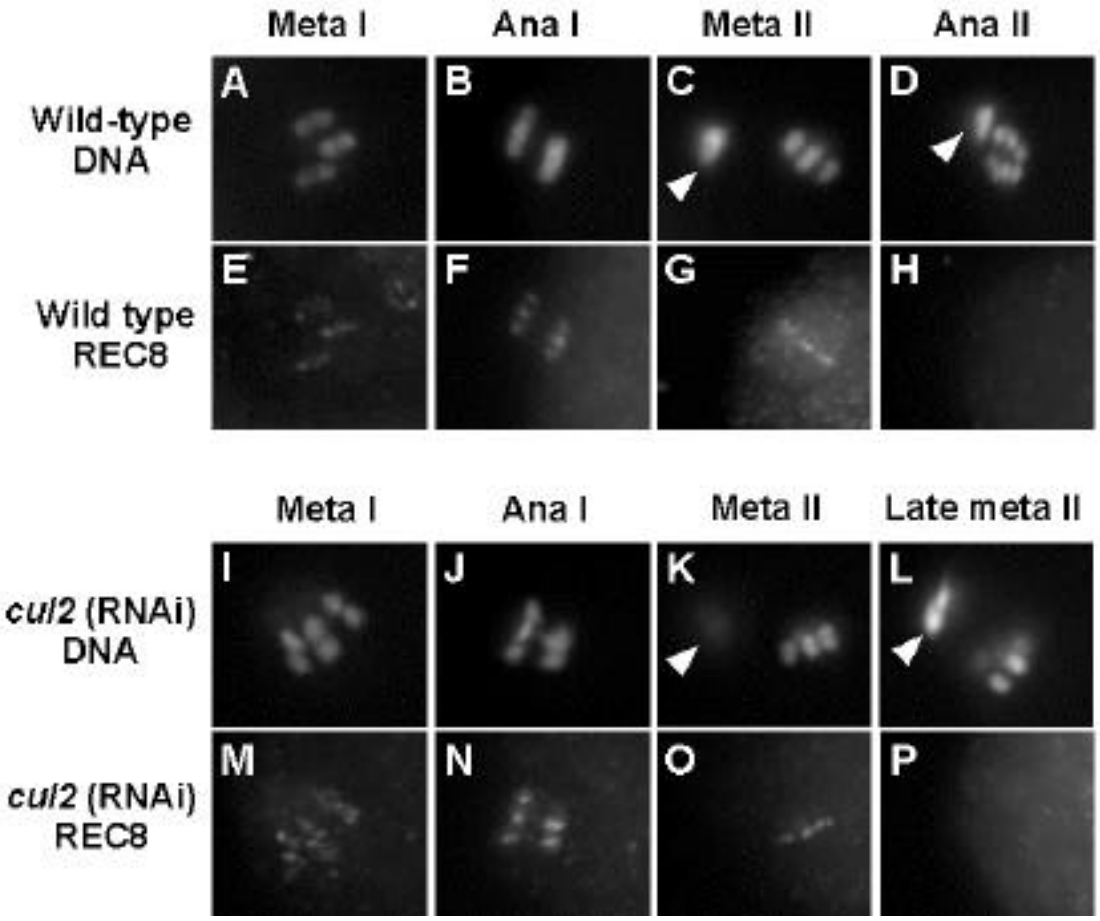

Meta I
Ana I
Meta II
Ana II
Wild-type DNA
A
B
C
D
Wild type REC8
E
F
G
H
Meta I
Ana I
Meta II
Late meta II
cul2 (RNAi) DNA
I
J
K
L
cul2 (RNAi) REC8
M
N
O
P

Figure 5.3. Depletion of *rec-8* by RNAi does not rescue the segregation defect in *cul-2* (RNAi) zygotes. Histone H2B::GFP fluorescence movie sequences in live *rec-8* (RNAi) alone or *cul-2* (RNAi), *rec-8* (RNAi) embryos. Time from fertilization (hr:min) is indicated in the upper left corner of images. Interphase denotes the time of chromosome decondensation after the completion of meiosis. Stars denote meiotic chromosomes. Arrowheads denote polar bodies.

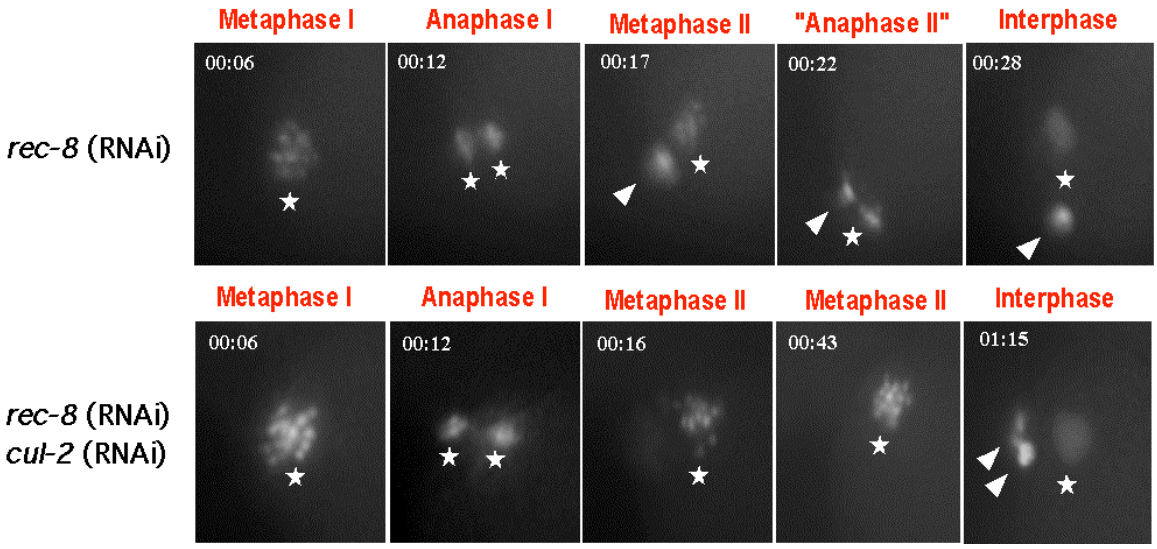

Metaphase I
Anaphase I
Metaphase II
"Anaphase II"
Interphase
rec-8 (RNAi)
00:06
00:12
00:17
00:22
00:28
Metaphase I
Anaphase I
Metaphase II
Metaphase II
Interphase
rec-8 (RNAi)
cul-2 (RNAi)
00:06
00:12
00:16
00:43
01:15

Figure 5.4. The morphology of the meiotic spindle is normal in *cul-2* animals. Epifluorescence images of anti-α-tublin antibody (left) and DNA (DAPI, right) staining in metaphase II for wild-type (top) and *cul-2* (RNAi) embryos (bottom).

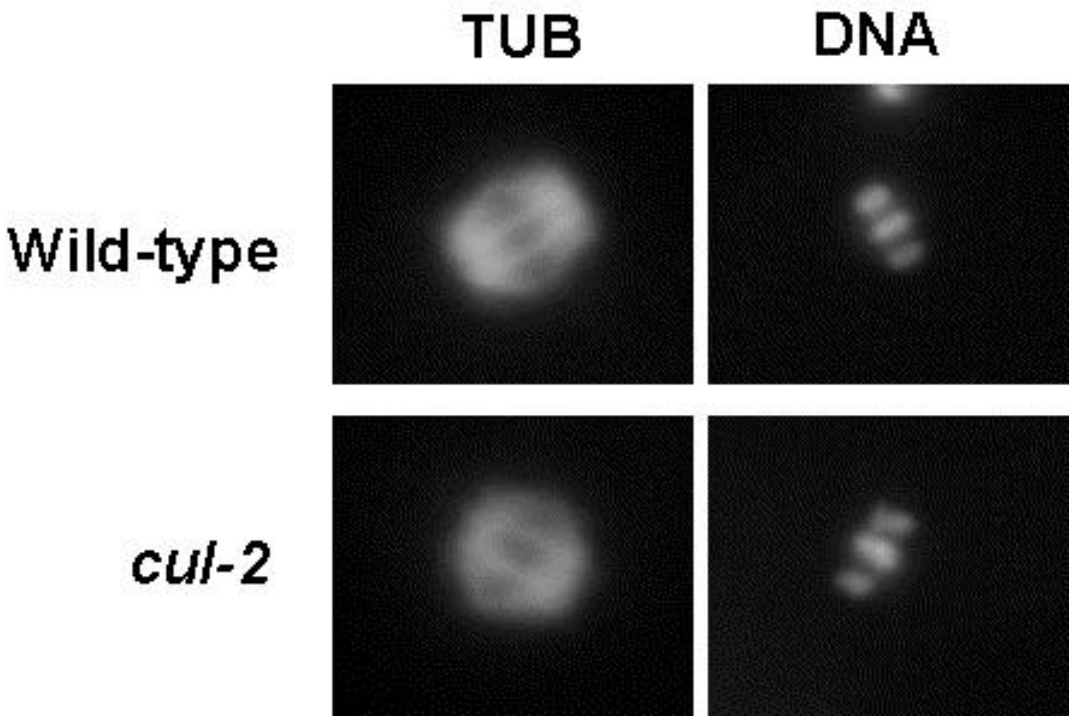

TUB
DNA
Wild-type
cul-2

Figure 5.5. *eln-1* (RNAi) and *rbx-1* (RNAi) zygotes phenocopy *cul-2* (RNAi) zygotes. Histone H2B::GFP epifluorescence movie sequences of live *cul-2* (left), *eln-1* (center), and *rbx-1* RNAi (right) zygotes. Time from fertilization is indicated in the upper left corner of images (hr:min). The stage of meiosis is indicated on the left of the image.

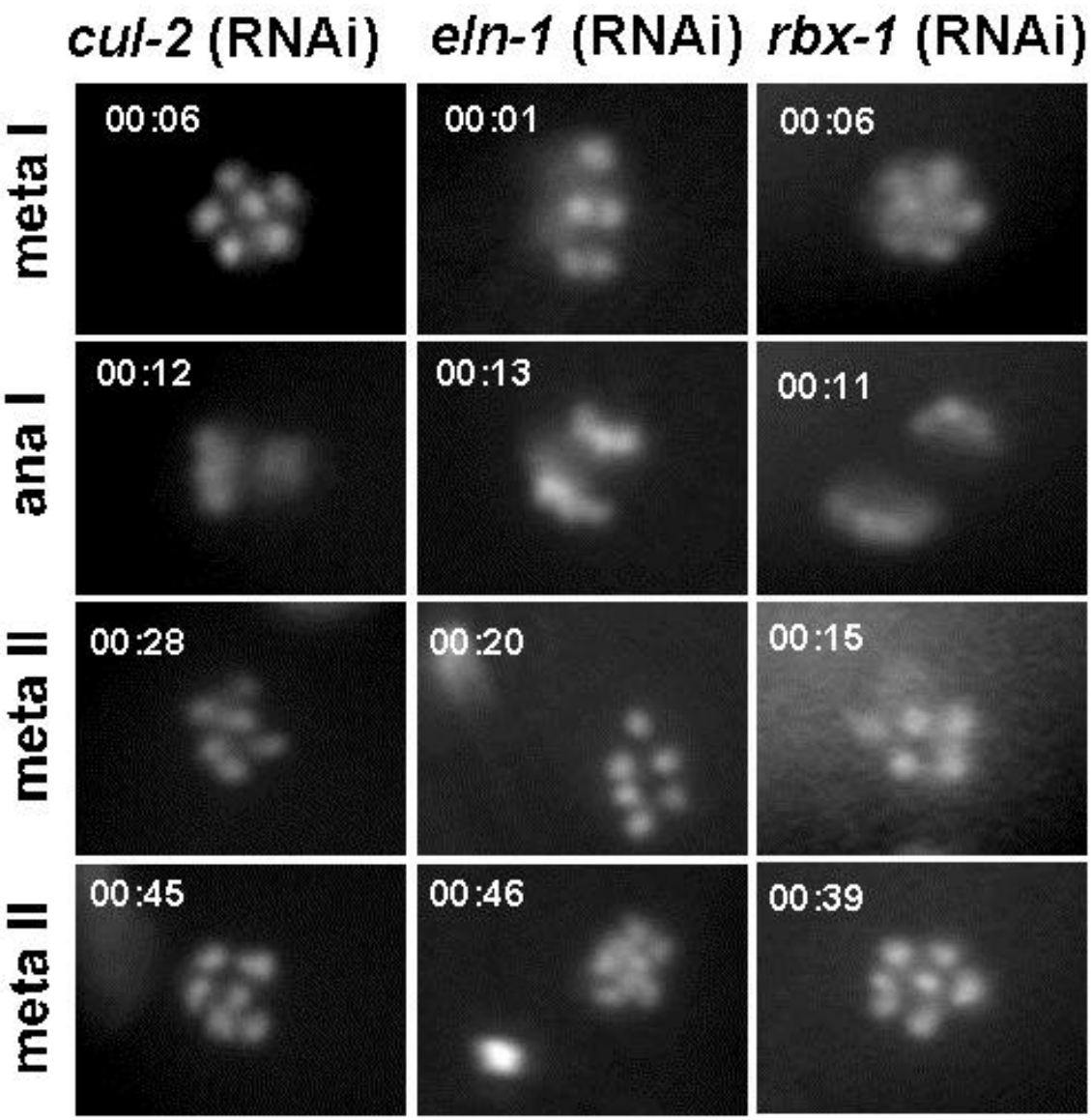

cul-2 (RNAi)
eln-1 (RNAi)
rbx-1 (RNAi)
meta I
ana I
meta II
meta II
00:06
00:01
00:06
00:12
00:13
00:11
00:28
00:20
00:15
00:45
00:46
00:39

Figure 5.6. Polarity defects in *cul-2* (RNAi) embryos. Matched DIC and PAR-2::GFP or PAR-6::GFP epifluorescence images of two cell-stage embryos. In wild-type (left column):, the anterior AB cell is bigger than the posterior P cell, PAR-2 forms a posterior cortical patch, and PAR-6 forms an anterior cortical patch. In *cul-2* (RNAi) embryos, the AB cell can be either bigger (40%, n = 31; middle column) or smaller than P (60%, n = 31; right column), and PAR-2::GFP and PAR-6::GFP proteins can be located on the cortex of AB and/or P, irrespective of the division pattern. Posterior is to the right.

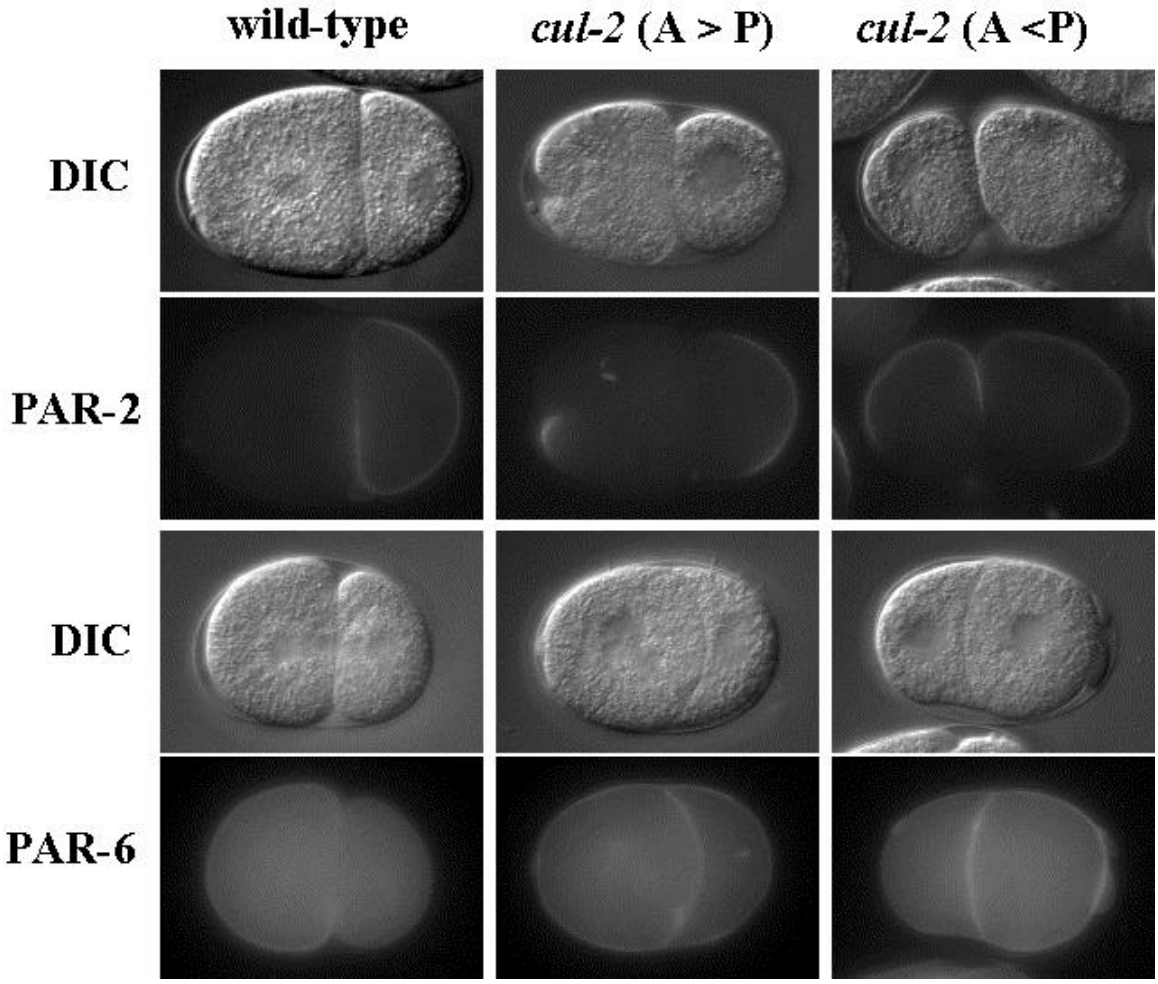
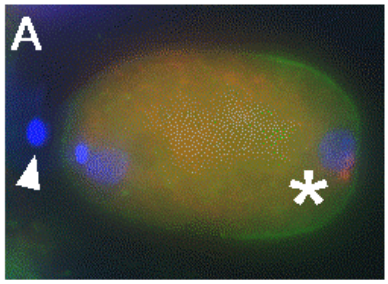
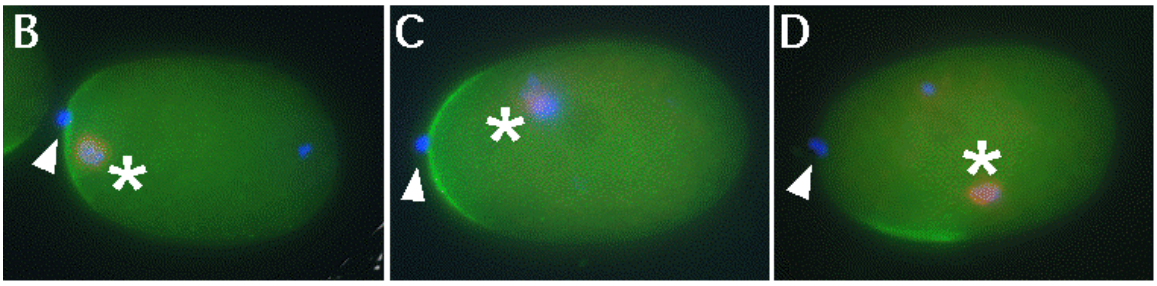

wild-type
cul-2 (A > P)
cul-2 (A <P)
DIC
PAR-2
DIC
PAR-6

Figure 5.7. Location of the initial PAR-2 cortical patch in *cul-2* (RNAi) and wild-type zygotes. Immunofluorescence images of PAR-2 antibody staining for PAR-2 (green), α-tubulin antibody staining for MT structures (red and denoted by stars), as well as DAPI staining for DNA (blue) in wild-type (top) and *cul-2* (RNAi) (bottom) zygotes. **A**: in the wild-type, PAR-2 is initiated posteriorly near the two sperm asters. In *cul-2,* PAR-2 can be initiated either **B**: anteriorly near the meiotic spindle;, or **C**: anteriorly without close association with a MT structure, or **D**: laterally without close association with a MT structure. Arrowhead denotes polar body. Posterior is to the right.

wild-type



cul-2 (RNAi)



CHAPTER 5

GENERAL DISCUSSION

In this dissertation, I have presented work that contributes to our understanding of cell cycle regulation by providing a comprehensive phylogenetic analysis of CDKs in model eukaryotic organisms and by identifying novel regulators of meiosis. I will briefly summarize the major conclusions of my work and discuss implications and possible future directions for this research.

My first dissertation project was an evolutionary analysis of CDKs in the completely or nearly-completely sequenced genomes of *S. cerevisiae*, *S. pombe*, *A. thaliana*, *C. elegans*, *D. melanogaster*, and humans. With the coming of the "genome age", large numbers of CDKs have been discovered in all eukaryotic kingdoms. However, their evolutionary relationships were not clear. Before our work, there was only one study made seven years ago on CDK phylogeny (Riley and Krieger, 1995). Since no eukaryotic genome had been sequenced at that time, the authors were only able to obtain sequence data for limited numbers of CDKs from protists, yeasts, and higher eukaryotes (Riley and Krieger, 1995). The availability of complete genome sequences enabled us to extract sequence data accurately and efficiently. More importantly, since the assignment of orthology completely depends on the relative phylogenetic similarity between entries, an accurate assignment of orthology can only be achieved when the

sequences of all family members are compared. There are many examples in the pre-genome era literature in which orthology was assigned erroneously to paralogs before the identification of the real ortholog. In our work, we comprehensively collected all CDK sequences from whole genomes. As a result, we were able to assign orthology to the majority of the CDKs with high confidence. Since orthologs tend to have conserved functions, our analysis will help in the design of future studies to test for conserved functions among CDK orthologs.

The CDK phylogenetic project also allowed us to focus on the relationships of CDK-activating kinases (CAKs). The identification of CAKs is still an open area of cell cycle research as a number of these important cell cycle regulators have not yet been identified. Before our study, the budding yeast CAK, Cak1, was thought to be a novel CDK without orthologs in other species. Through the use of sophisticated phylogenetic tools, including maximum likelihood and gamma rate distribution analyses, we succeeded in identifying two orthologs for Cak1: Csk1 in fission yeast, and CaCAK1 in *Candida albicans*. This expansion of the Cak1 clade may aid in the identification of key residues that are conserved in all Cak1 family members. Both Cak1 and Csk1 are unique among CDKs in their ability to be fully active as monomers, without the need for activating T-loop phosphorylation or cyclin binding. Residues that are conserved among the Cak1 family members may help to elucidate the unique structural features that allow the Cak1 clade members to function differently from other CDKs.

Our study suggested that the CAK1 clade originated in fungi, since no plant or metazoan CDK could be placed in the CAK1 clade with high confidence values. However, our analysis identified a number of orphan metazoan CDKs that could not be

placed into defined clades. These orphan CDKs could be functionally divergent from other more conventional CDKs and potentially could serve as candidates for unidentified metazoan CAKs.

In my second dissertation project, we identified the CUL-2 ubiquitin-ligase complex as a regulator of both meiotic anaphase and polarity. This study provides the first evidence that cullin/RING finger complex-dependent ubiquitination is needed to regulate anaphase and polarity.

In *cul-2* (RNAi) or mutant zygotes, sister chromatids stay in metaphase II for a prolonged period of time without any poleward movement. However, we found that the first step of anaphase initiation, the dissolution of sister chromatid cohesion, was executed normally. A study in budding yeast (Uhlmann et al., 2000) has suggested that the degradation of cohesin was able to initiate anaphase and allow sister chromatids to segregate to opposite poles, even when APC/C was not activated. On the other hand, another study in Xenopus egg extract showed that even after cohesin has been degraded, APC/C activity is still required to degrade the chromokinesins Xkid to allow anaphase chromosome movement (Funabiki and Murray, 2000), suggesting that cohesin degradation is not sufficient for initiating anaphase movement. Our study showed that in C. elegans, meiosis II cohesin degradation is not sufficient for initiating anaphase movement. However, in *cul-2* (RNAi) animals, mitotic anaphase was normal (data not shown). By comparing meiosis and mitosis in the same organism, our study suggests that mitotic anaphase and meiotic anaphase are regulated differently, thus laying the groundwork for future studies in understanding the differential regulation of meiotic versus mitotic anaphases. As discussed in our manuscript, we also showed that CUL-2 is

required specifically for anaphase II but not for anaphase I, indicating for the first time that anaphase chromosome segregation is regulated differently in these two meiotic phases. Given the similar spindle dynamics that we observe for anaphase I and anaphase II, we currently do not have any firm explanation for the different requirement for CUL-2 in the two phases.

The APC/C has been shown to be required for many events in mitotic anaphase: cohesin degradation to initiate anaphase, Xkid degradation to initiate anaphase movement; and cyclin B degradation to exit mitosis. It is thus reasonable to ask whether APC/C is playing a role in causing the failure of anaphase chromosome movement in *cul-2* zygotes. The degradation of REC-8 in *cul-2* mutant zygotes indicates that APC/C is likely activated in *cul-2* mutants, although no published study has clearly shown a requirement of APC/C for meiosis II cohesin degradation. We are currently in the process of studying APC/C activity in *cul-2* (RNAi) zygotes. We have successfully made GFP transgenic lines for several predicted APC/C substrates: the Xkid ortholog, cyclin A, cyclin B1, cyclin B3, and REC-8. Comparing their expression/degradation pattern in wild-type versus *cul-2* (RNAi) zygotes will not only allow us to monitor APC/C activity, but also let us test if the dynamics of the worm Xkid ortholog, cyclin A, cyclin B1, cyclin B3, and REC-8 proteins correlates with specific *cul-2* phenotypes such as anaphase movement, meiosis exit, cytoplasmic flow, and polarity.

We have also shown that the CUL-2 complex is required for establishing polarity in the *C. elegans* zygote. The most intriguing observation we have made is that PAR-2 can localize to the lateral cortex in the absence of any associated MT structures. PAR-2 location normally depends not only on MT structures but also on the location of the PAR-

3/PAR6/protein kinase C complex (Rappleye et al., 2002). Future work in the laboratory will monitor the initial PAR-6::GFP pattern in *cul-2* zygotes. If again the establishment of PAR-6::GFP is found not to correlate with the MT pattern, then we would be providing the first evidence in *C. elegans* that the earliest aspects of polarity can be initiated independent of MT structures.

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Riley, D. E., and Krieger, J. N. (1995). Molecular and phylogenetic analysis of PCR-amplified cyclin-dependent kinase (CDK) family sequences from representatives of the earliest available lineages of eukaryotes, J Mol Evol *41*, 407-13.

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