Cell cycle entry in *C. elegans* development

Jerôme P. Korzelius

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Cover design: Evert Krooswijk/ Jerôme Korzelius - "When the music starts". Collage of a stained *C. elegans* L1 larva on a turntable. The needle of the turntable, where the music starts, symbolizes the cell cycle entry point.

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Voortgang van de celcyclus in *C. elegans* ontwikkeling

(met een samenvatting in het Nederlands)

Proefschrift

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Jerôme Peter Korzelius

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Promotor: Prof.dr. S.J.L. van den Heuvel

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Chapter 1

Cell cycle entry in *C. elegans* development

'If music be the food of love, play on!
Give me excess of it'

William Shakespeare - Twelfth Night'

Abstract

During development, cell division and differentiation need to be tightly coordinated to achieve the formation of a complex organism from a single fertilized oocyte. At first, the zygote will go through many rapid rounds of cell division. Transcription factors will trigger differentiation programs that will lead to specification of different cell fates. At later stages of development and in adult life, cell division and differentiation are often found to be mutually exclusive. Genes that control cell cycle entry and exit are frequently mutated in cancer. This first chapter provides an overview of the many genes involved in cell cycle regulation, with a particular focus on the developmental regulation of the cell cycle in the nematode *C. elegans*.

Cyclins and Cdk's as the main engine that drives cell proliferation

The series of events that result in cell division is called the cell cycle. The process of duplicating a cell's genome and distributing it over two daughter cells can be divided in 4 stages (Figure 1). In S (synthesis) phase, cells duplicate their DNA and these two copies are equally divided over both daughter cells in the M phase that encompasses mitosis and subsequent cytokinesis. S and M phases are segregated by 2 gap phases: G1 and G2. The cells decision whether to divide one more time or to stop dividing, is made in the G1 phase of the cell cycle. S-phase is followed by the G2 phase, wherein the cell prepares the DNA for mitosis by restoring any errors made during the replication process. During subsequent M-phase, the DNA condenses into chromosomes, which align at the metaphase plate. When the sets chromosomes are fully aligned, they will be separated and cytokinesis ensues, in which the mother cell separates its cytoplasm, yielding 2 new cells. Transitions in the cell cycle must be tightly regulated to prevent e.g. re-replication of the genome or untimely chromosome segregation.

The basic parts of the engine: The discovery of Cyclins and Cdk's as the main regulators of the cell cycle

The critical components regulating the different stages of the eukaryotic cell cycle were discovered by work on different eukaryotic model systems such as budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe)* as well as frog and sea urchin embryos. The first genetic screen for genes involved in cell division was performed in budding yeast by Hartwell and colleagues and yielded various *C*ell *D*ivision *C*ycle mutants or *cdc* genes (Hartwell et al., 1974). Experiments with *Xenopus* and *Rana* frog oocytes had identified a cytoplasmic component, dubbed MPF for maturation promoting factor, that could regulate entry into meiosis (Hara et al., 1980; Masui and Markert, 1971; Schorderet-Slatkine and Drury, 1973; Smith and Ecker, 1971). Another seminal discovery was the identification of proteins that fluctuate during each cell division cycle in sea urchin eggs (Evans et al., 1983). Due to their cyclic expression pattern these proteins were dubbed Cyclins. For years, many investigators tried to find out how these genes and molecules governed cell cycle control. The breakthrough discovery came with the biochemical isolation of MPF: this revealed that MPF actually constitutes a complex of Cyclin B and a Cyclin Dependent Kinase (Cdk) Cdk1 (also known as *CDC28* in budding yeast or *cdc2*$^{+}$ in fission yeast) (Draetta et al., 1989; Dunphy et al., 1988; Gautier et al., 1988). The conservation of these critical cell cycle control genes was elegantly demonstrated by rescuing the fission yeast *cdc2* mutant with a human cDNA encoding for Cdk1, thereby identifying the human *cdc2*$^{+}$ homologue (Lee and Nurse, 1987). Further work in these model systems forged a model in which Cyclins function together with their partners the Cyclin-Dependent Kinases (Cdk's) at all the transitions in the eukaryotic cell cycle (Figure 1).

Tuning the cell cycle engine: regulation of Cyclin/CDK activity throughout the cell cycle

Cdk's require Cyclins for their kinase function. Budding and fission yeast only has a single Cdk, Cdc2 and Cdc28 respectively, that regulates multiple cell cycle transitions by association with different Cyclins (Andrews and Measday, 1998). In contrast, mammals use a vast array of different Cyclins and Cdk's at each cell cycle transition (Murray, 2004). The large number of Cyclin and Cdk family members in mammals is probably an evolutionary consequence of the need for more refined control of the cell cycle in more complex metazoans. In this section I

will discuss four different ways in which the Cyclin/Cdk activity can be attuned in eukaryotic organisms. First, the expression of different Cyclin/Cdk's in mammals is often temporally and spatially segregated, thus providing one level of regulation. The Cyclin/Cdk complexes in mammals often show a large degree of redundancy: animals can survive even if all members of a family have been knocked out, e.g. in the case of D-type Cyclins in mice (Kozar et al., 2004). Phenotypes of single Cyclin/Cdk knockout mice are often very local, with only few tissues affected by the changes in Cyclin/Cdk activity (Cooper et al., 2006; Ortega et al., 2003).

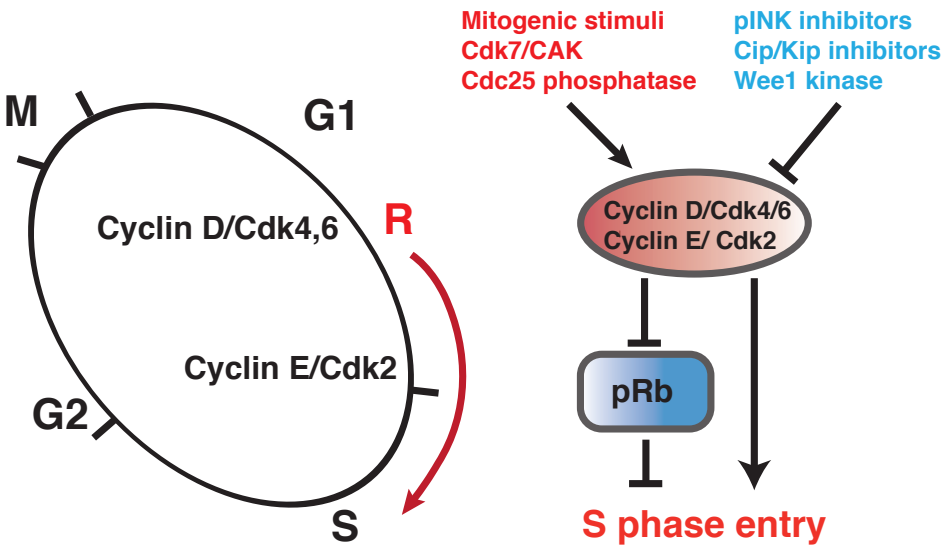


Figure 1. The cell cycle and G1/S transition regulation. Left: The cell cycle is divided into 4 phases: G1, S, G2 and M-phase. R represents the point in G1 where a cell makes the irreversible commitment to complete another round of division. Right: The Cyclin/Cdk complexes that regulate progression from G1 to S are controlled by various mechanisms. Mechanisms in blue are inhibitory, red represents factors that act positively in G1 to S progression.

The Cyclins were first identified by the steep rise and drop in their protein levels during the cell cycle (Evans et al., 1983). The abrupt rise and drop in B-type Cyclin levels at the end of mitosis suggested a protein degradation mechanism that acted at this transition in the cell cycle. In an elegant experiment, Murray et al. showed that degradation of Cyclin B is necessary for exit from mitosis, providing the first direct evidence of the involvement of proteolysis in cell cycle regulation (Murray et al., 1989). Later experiments demonstrated that degradation of mitotic Cyclins is regulated by a large Ubiquitin ligase complex called the Anaphase Promoting Complex or APC (King et al., 1995; Peters, 2002; Thornton and Toczyski, 2006). There are two different APC ubiquitin ligase complexes: APC^{CDC20} and APC^{Cdh1}. APC^{CDC20} becomes active at anaphase in mitosis and tags the mitotic Cyclins for destruction. First, APC^{CDC20} tags Cyclin A for destruction and degradation starts as early as nuclear envelope breakdown (den Elzen and Pines, 2001). Subsequently, it targets Cyclin B and Securin at the onset of anaphase when the alignment of the bivalent chromosomes at the metaphase plate is complete (Cohen-Fix et al., 1996; King et al., 1995). This will allow anaphase entry and the separation of the chromosomes. APC^{Cdh1} is important in late mitosis and early G1 to ensure that any remaining Cyclin is degraded. Altogether, these two APC complexes are essential for the completion of M phase and entry into G1.

A different ubiquitin ligase complex is crucial at another transition in the cell cycle: the G1-to-S transition. The SCF ubiquitination complex acts at this point to degrade Cyclin E as well as the Cip/Kip inhibitor p27 (reviewed in Cardozo and Pagano, 2004). The Cyclin E/Cdk2 complex promotes its own destruction at the G1/S transition by phosphorylating Cyclin E at Thr380 (Clurman et al., 1996; Won and Reed, 1996). This phosphorylation is necessary for the binding of the SCF ubiquitin ligase complex (Koepp et al., 2001). In addition, the Cyclin E/Cdk2 inhibitor p27 was discovered to be a target for ubiquitin-mediated destruction (Pagano et al., 1995) and the Cyclin E/Cdk2 complex tags p27 for destruction by the SCF by phosphorylation on the Thr187 residue (Sheaff et al., 1997). Hence, upon binding of Cyclin E, Cdk2 can phosphorylate Cyclin E and target it for destruction. Hence, protein degradation serves as the second major control mechanism that acts to control the activity of Cyclin/Cdk complexes throughout the cell cycle.

Thirdly, Cyclin/Cdk activity is regulated by 2 families of Cyclin Kinase Inhibitors (CKIs): The INK4 and Cip/Kip family (reviewed in (Kim and Sharpless, 2006) and (Besson et al., 2008). The INK4 family acts solely on the G1- specific Cdk4 and -6 kinases, whereas Cip/Kip family members can interact with all Cyclin/Cdk complexes. The latter proteins exert their function by binding the Cyclin/Cdk complex, thereby inhibiting it from phosphorylating its targets. The precise mechanism of inhibition by Cip/Kip binding is not yet fully understood. In the case of p27, the crystal structure of this protein binding to Cyclin A/Cdk-2 reveals that it might prevent ATP hydrolysis by blocking the catalytic cleft. Alternatively, it could block substrate access by occupying the substrate-binding domain of the Cyclin (Russo et al., 1996). Structural studies on the p16 INK4A protein revealed that it binds to both lobes Cdk6, preventing both ATP binding and the access to the Cyclin (Russo et al., 1998). The INK4 family members are well-characterized tumor suppressor genes, mutations in which are readily identified in a large variety of tumors (e.g. (Caldas et al., 1994; Liu et al., 1995). Curiously, the Cip/Kip family also acts in a non-inhibitory manner during G1. This is due to the requirement for Cip/Kip proteins in the formation of the Cyclin D/Cdk4 complex (Cheng et al., 1999; LaBaer et al., 1997) reviewed in (Blain, 2008). This positive requirement might explain why p27 is often haploinsufficient in tumor suppression, as a total loss of p27 might hinder the assembly of the Cyclin D/Cdk4 complex in G1 progression (Besson et al., 2008; Fero et al., 1998).

Phosphorylation of Cdk's is the fourth mechanism to fine-tune the regulation of the cell cycle. Full activity of a Cyclin/Cdk complex requires phosphorylation of Cdk's by a Cdk Activating Kinase (CAK) (Harper and Elledge, 1998). The major CAK in mammals is Cdk7, which in association with Cyclin H, phosphorylates Cdk2. Although the phosphorylation by CAK is required for full activation, this phosphorylation seems to happen by default and is not likely to be regulated at any specific point in the cell cycle. In contrast, inhibitory phosphorylation of the highly conserved Thr14 and Tyr15 residues of Cdk proteins does seem to play an important regulatory role in the cell cycle. The kinases responsible for this phosphorylation are known as Wee1 and Myt1 (Kellogg, 2003). Phosphorylation of Thr14 and Tyr15 interferes with the binding and orientation of ATP to the catalytic cleft, thereby inhibiting substrate binding. The Cdc25 family of phosphatases opposes this inhibition by de-phosphorylating these residues (Gould and Nurse, 1989; Krek and Nigg, 1991; Norbury et al., 1991). The phosphorylation and de-phosphorylation of Cdk's is a prerequisite for the switch-like behavior of Cdk activation. Furthermore, the activity of both these Wee1 and Cdc25 appears to be highly controlled throughout development. Wee1 ('wee' being colloquial for small) was originally found in fission yeast as a small mutant that

entered mitosis prematurely, creating small cells (Russell and Nurse, 1987). Regulation of Cdc25 activity in *Drosophila* as well as *C. elegans* can control the timely entry of cells into mitosis or S-phase (Clucas et al., 2002; Edgar et al., 1994; Kostic and Roy, 2002).

An engine running smoothly: Cyclins and Cdk's create a stable oscillator that drives the cell cycle

The control mechanisms described in the previous section all act on Cyclin/Cdk levels and activity and are necessary to create a stable oscillator that makes all-or-nothing decisions at cell cycle transitions. This all-or-nothing nature of the transitions is critical, as the onset of later events in the cell cycle is dependent on the completion of earlier events. Fluctuations in the levels of Cyclins, together with activators and inhibitors like Cdc25 and Wee1, result in the switch-like activation of Cdk proteins, like Cdk1 in mitosis (Pomerening et al., 2003). This kind of switch-like activation system is called bi-stable: Cyclin/Cdk complexes go from low activity to maximum activity in a switch-like manner. Good examples are the initiation of S-phase or the segregation of chromosomes at the end of M-phase. However, to create a true oscillator out of the bi-stable activation of the Cyclin/Cdk complexes, one needs to create a negative feedback mechanism. This is created by the APC and SCF complexes, which can destroy the Cyclin/Cdk complexes at the proper time. In the case of the Cyclin B/Cdk1 complex in mitosis, APC is activated by Cyclin B/Cdk1 activity and this results in the destruction of the Cyclin B/Cdk1 complex. Hence, the Cyclin/Cdk complex marks itself for death once it becomes fully active (see (Cross, 2003) for a full description of this model). In summary, the cell cycle is a regulatory module in a cell that is both remarkably dynamic as well as robust enough to create a perfect output every time: the division of a cell. Eventually, the cell can make the decision to exit the cycle and stop dividing. Reciprocally, a cell can decide to re-enter the cell cycle if conditions are favorable. The intricate signaling decisions that govern this process will be discussed in the next section.

START your engines: how a cell moves from G1 to S-phase

The G1 to S-phase transition (henceforth called G1/S transition), also known as the restriction point in mammalian cells or Start in yeast, is a crucial point in the cell cycle. In G1, a cell gets input from different mitogenic stimuli like nutrients and growth factors. When conditions are favorable, i.e. when nutrients and growth factors are abundant, a cell may decide to go through another round of cell division. In contrast, when resources are sparse, a cell can withdraw from the cell cycle and go into G0, a state also known as quiescence. Hence, a cell's decision to commit to another round of division is based on extracellular cues feeding information into the cell cycle. How the cell cycle reacts to mitogenic stimuli and how this results in the start of DNA replication will be covered in the next section.

Mitogenic stimuli can activate the G1 Cyclins and Cdk's

While the coupling between nutrient availability and cell division is more direct in single cell eukaryotes (Rupes, 2002), cells in multicellular organisms rely on more complex signaling pathways to translate nutritional status to cell division. More complex metazoans have developed numerous pathways that transduce information from the extracellular environment and feed this into the intrinsic cell cycle machinery. In G1, Cyclin D-Cdk4/6 activity rises and

eventually reaches a critical level, which triggers S-phase entry. Numerous mitogenic pathways influence the Cyclin D-Cdk4/6 complex during the G1 phase. The first discovery of a mitogen that controls the expression of Cyclin D was in macrophages, where the interleukin CSF1 stimulates G1 progression by upregulating Cyclin D levels (Matsushime et al., 1991). The well-known Epidermal Growth Factor (EGF) signaling activity stimulates Cyclin D expression as well: cells stimulated with EGF will activate the Ras signaling cascade, which results in the activation of the AP-1 transcription factor. AP-1 can then drive transcription of Cyclin D (Albanese et al., 1995; Bakiri et al., 2000; Shaulian and Karin, 2001).

Other growth factors like insulin activate the PI-3 Kinase pathway. One of the functions of PI3K is the activation of the Akt1/PKB kinase. PKB is a versatile protein kinase that can influence cell division and growth in a variety of ways. First, activation of PKB leads to nuclear export and inactivation of FoxO transcription factor family members, which downregulate Cyclin D and upregulate the Cip/Kip inhibitor p27 (Medema et al., 2000; Schmidt et al., 2002). Second, it inhibits Glycogen Synthase Kinase 3-beta (GSK3ß), a kinase that phosphorylates Cyclin D, tagging it for ubiquitin-mediated degradation (Cross et al., 1995; Diehl et al., 1998). Thirdly, it was recently discovered that Akt1 phosphorylates and activates the Skp2 subunit of the SCF ubiquitin ligase complex (Gao et al., 2009; Lin et al., 2009). SCF complex activation leads to increased degradation of p27 and thereby facilitates progression through G1.

Another important mitogenic factor is the transcription factor Myc. This bHLH-type transcription factor acts as a heterodimer together with Max to regulate a vast diversity of cellular responses downstream of major growth factor pathways e.g. the Ras and PI3-Kinase-dependent pathways (Adhikary and Eilers, 2005). Myc can influence both cell proliferation and cell growth. The different ways in which Myc regulates growth and proliferation are unclear, since Myc regulates the transcription of a very large number of genes in both flies and humans (Fernandez et al., 2003; Orian et al., 2003). However, there is convincing evidence that Myc can directly regulate the transcription of several cell cycle regulators that act on the level of Cyclin D/Cdk4. For instance, $Myc^{-/-}$ cells were found to have a reduced activity of Cyclin D-Cdk-4/6 complexes (Mateyak et al., 1999). Subsequently, Myc was found as part of a transcriptional complex at the Cyclin D2 promoter, providing direct biochemical evidence of Cyclin D2 regulation by Myc (Bouchard et al., 2001). In addition, cells that lack all D-type Cyclins fail to be properly transformed by Myc or Ras oncogenes, underlining the importance of Cyclin D control in oncogenic transformation (Kozar et al., 2004). Thus, a variety of growth factor inputs stimulate the activity of G1 Cyclin/Cdk complexes, paving the way for cell cycle progression.

Letting go of the brakes: RB and CKI inhibition leads to entry into S-phase

The G1 Cyclin D/Cdk4 complex becomes highly active in response to growth factor stimulation. To promote progression through G1 and entry into S-phase, this complex will have to inhibit two classes of negative regulators of G1 progression. The first one is the pRb family of pocket proteins. Pocket proteins function in many different cellular processes but play a key role in blocking G1 progression through inhibition of S-phase target gene transcription. They exert this function by binding to members of the E2F transcription factor family (reviewed in (van den Heuvel and Dyson, 2008). E2F transcription factors act together with DP as heterodimeric transcription factors. E2F proteins come in two flavors: activator E2F's and repressor E2F's. Activator E2F's positively regulate the transcription of genes required for S-phase progression,

like ribonuclease reductase and other proteins required for DNA synthesis. Inhibitor E2Fs have the opposite function: they can repress transcription from S-phase-responsive promoters. Pocket proteins have a dual role in their interaction with E2F's during G1/G0: the pRb pocket protein can inhibit activator E2F's (E2F 1-3), thereby inhibiting their function and hence inhibiting S-phase entry (Rubin et al., 2005). On the other hand, the p107/p130 pocket proteins act together with the repressor E2F's (E2F4 and -5) to recruit the chromatin remodeling complexes necessary for the repression of E2F 4 and -5 target promoters (Rayman et al., 2002; Takahashi et al., 2000).

The pRb family of pocket proteins was first identified as targets of the viral E7 (Human Papilloma Virus or HPV), E1A (Adenovirus) and large T antigen (Simian Virus 40 or SV40) oncogenic proteins (Dyson et al., 1989; Munger et al., 1989; Whyte et al., 1988). By inhibiting pRb proteins, these viral oncogenes trigger S-phase entry and transformation to a tumor-like growth phenotype, thereby hijacking the cell's metabolism to create more viral DNA. Thus, removal of pocket protein repression is a critical step to initiate S-phase gene transcription. This removal is accomplished by the phosphorylation of pRb family proteins by the Cyclin D-Cdk4/6 that accumulate during G1. pRb can be directly phosphorylated by Cyclin D-Cdk4 complexes (Kato et al., 1993) and this results in the inactivation of the pRb protein (reviewed in (Adams, 2001). This initial phosphorylation and inactivation of pRb by Cyclin D-Cdk4/6 triggers a positive feedback loop. Inactivation of pRb allows the activator E2Fs to express G1 to S-phase promoting genes, among them Cyclin E and Cdk2. Cyclin E was found to be crucial for the robust progression to S-phase in both mammalian cells and *Drosophila* (Duronio and O'Farrell, 1995; Ohtsubo and Roberts, 1993). In addition, the Cyclin E/Cdk2 complex phosphorylates the pRb protein, leading to an even more hyperphosphorylated, inactive pRb protein. This results in robust and irreversible entry into S-phase.

Next to Rb inactivation, cells still has to overcome the high levels of Cdk inhibitors that are generally present in G1 cells. Especially the Cip/Kip family inhibitor p27 is present in high levels in quiescent and differentiated cells to prevent cell cycle entry. The p27 protein is positively required for the formation of the Cyclin D-Cdk4/6 complex (LaBaer et al., 1997). Consequently, the increase in Cyclin D-Cdk4/6 complex levels during G1 is thought to titrate p27 away from Cyclin E-Cdk2 and Cyclin A-Cdk, which are inhibited by interaction with p27 (Polyak et al., 1994; Toyoshima and Hunter, 1994). However, the importance of this sequestration for G1 to S phase progression *in vivo* is still debated (see Chapter 5). Other mechanisms, such as relocation and destruction of p27, are essential for lowering its activity in G1 as well. Early in G1, oncogenic kinases such as Akt1/PKB, Src and Abl all contribute to the inactivation of p27 by phosphorylating p27, thereby promoting its nuclear export and subsequent degradation (Chu et al., 2007; Grimmler et al., 2007; Liang et al., 2002). Later, during G1/S transition, p27 promotes its own destruction by binding to the Cyclin E-Cdk2 complex: this complex phosphorylates p27 on Thr187, tagging it for destruction by the SCF ubiquitin ligase complex (Montagnoli et al., 1999). The importance of p27 in the regulation of cell division is illustrated by the knockout phenotypes. Mice null for p27 display widespread hyperplasia and increased tumor susceptibility (Fero et al., 1998; Fero et al., 1996; Malumbres et al., 2000). The p27 homologues Dacapo (*Drosophila*) and *cki-1* (*C. elegans*) exert similar functions and knockdown results in hyperplasia in various tissues as well as untimely cell cycle exit (Boxem and van den Heuvel, 2001; de Nooij et al., 1996; Hong et al., 1998).

Fire up your engines: entry into S-phase and origin firing

When a cell enters S-phase, the cell cycle needs to co-operate with the DNA replication machinery to accomplish replication of the genome. DNA replication needs to be precise and the DNA needs to replicate once, and only once to accomplish faithful replication of the DNA. To accomplish this, a cell separates the preparation of the DNA for replication ("licensing") from the actual start of DNA replication ("firing"). This simple but elegant licensing model, originally formulated by Blow and Laskey in 1988, has been modified and expanded tremendously over the last 2 decades. In short, a cell starts to "license" origins of DNA replication during late mitosis through formation of pre-replication complexes (preRC's) onto the DNA. Events at the G1/S transition subsequently trigger the simultaneous activation or 'firing' of these pre-RC's. This can be achieved in numerous ways and higher metazoans have developed elaborate mechanisms to prevent re-replication. In the next section, I will start discussing how the pre-RC is composed and formed, then describe how Cyclin/Cdk's can trigger the firing of origins and finally how re-replication of the DNA is prevented.

The ORC and pre-RC formation: getting ready for the green light

Starting at mitotic anaphase in mammalian cells, members of the Origin Replication Complex (ORC) will be recruited to the future origins of DNA replication. Knowledge about how an origin is defined at the DNA level, how many there are in the genome and how they are spaced is still scant, and origin use and their definition in the literature vary greatly between different eukaryotes. The ORC complex comprises of six proteins that form a scaffold to recruit other proteins in the pre-RC (Figure 2). After ORC binding, the next step is the recruitment of Cdc6 and Cdt1. Cdc6 was identified in a screen by Lee Hartwell for mutants that fail to replicate DNA (Hartwell, 1973). Cdc6, together with Cdt1 and the ORC complex recruit the six-subunit MCM complex onto the pre-RC (Bowers et al., 2004; Maiorano et al., 2000; Randell et al., 2006; Whittaker et al., 2000). The MCM or Mini Chromosome Maintenance genes were identified in a screen for genes involved in minichromosome inheritance in *S. cerevisiae* (Maine et al., 1984). These minichromosomes were yeast plasmids containing Autonomously Replicating Sequences (ARS) and therefore could be replicated and distributed over two daughter cells similar to normal yeast chromosomes. MCM mutants were found to be defective in the proper replication of these minichromosomes. Further work demonstrated that these MCMs acted in a hexameric complex that was important for DNA replication initiation (Dutta and Bell, 1997). The exact biochemical function of the MCM complex in DNA replication remained a mystery for a long time. However, work in the last 10 years has revealed that the MCM complex is the replicative helicase that unwinds the DNA upon DNA replication. It was shown that the MCM complex was not only necessary for the initiation phase but for progression of the replication fork as well (Aparicio et al., 1997; Labib et al., 2000). In addition, all six subunits encode AAA^{+} ATP-ase family members and a subcomplex of MCM4,-6 and -7 can exhibit helicase activity upon ATP hydrolysis (Ishimi, 1997; You et al., 1999). Crystallography studies reveal that the MCM complex hexamer forms a ring-like structure, which allows it to slide down the DNA during replication (Sato et al., 2000). Curiously, DNA replication only requires a fraction of the huge excess of MCM complexes in the cell (Crevel et al., 2007; Lei et al., 1996). The function of these excess MCM complexes for DNA replication is to license extra origins in case of DNA damage during replication (Ibarra et al., 2008; Woodward et al., 2006). This backup mechanism ensures that DNA replication can continue from another origin after stalling of a replication fork. When

origins are eventually loaded correctly onto the DNA, the system is poised for replication start. The different steps in the assembly of the pre-RC are summarized in a simplified scheme in figure 2.

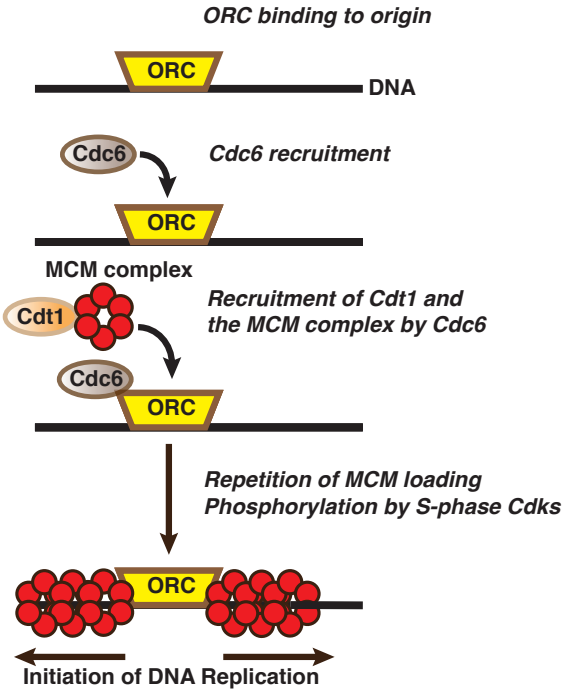


Figure 2. A simplified model of eukaryotic replication origin assembly and firing (based on *S. cerevisiae* data). Replication licensing begins in late M/early G1 phase with the binding of the ORC complex to origins of DNA replication. This is followed by recruitment of Cdt1 and Cdc6, which in turn recruit the 6-subunit MCM-2-7 helicase to the pre-replication complex. At the onset of S-phase the action of Cyclin/Cdk complexes will trigger the initiation of DNA replication.

S-phase Cyclin/Cdk's put the pedal to the metal

When the pre-RC is assembled, the cell is essentially ready to start DNA replication. The initiation of DNA replication or origin firing is linked to Cyclin/Cdk's activity during S-phase. However, the exact way in which the cell cycle machinery achieves the start of DNA replication varies widely among eukaryotes and the details are still under debate. In budding yeast, the S-phase Cyclins Clb5 and Clb6, together with Cdc28 (Cdk1) are responsible for the initiation of DNA replication. However, in *clb5 clb6* double mutants DNA replication can still initiate but takes a prolonged time to complete, suggesting the involvement of parallel pathways (Donaldson et al., 1998). In mammalian cells, the Cyclin E/Cdk2 complex is thought to be the critical regulator of S-phase initiation, promoting further expression of S-phase target genes through phosphorylation of the G1/S regulator pRb. Indeed, Inactivation of Cdk2 by a dominant-negative form of this Cdk triggered a G1 arrest in mammalian cells (van den Heuvel and Harlow, 1993). Knockout mouse models of Cdk2 and the E-type Cyclins revealed that these proteins are not required for embryonic cell proliferation. Cdk2 knockout mice can develop into adult mice, although mouse embryonic fibroblasts (MEFs) from these mice do show delayed S-phase entry (Ortega et al., 2003, Berthet et al., 2003). Cells in Cyclin E1/E2/E3 triple KO mouse embryos proliferate normally but fail to undergo endoreplication (Geng et al., 2003). Redundancy between the different G1/S Cyclin/Cdk complexes is a likely explanation for the lack of phenotype in these mouse models. However, from various other model systems there is ample evidence that both Cyclin E/Cdk2 and Cyclin A/Cdk2 complexes have essential roles in the activation of DNA

replication (Boxem et al., 1999; Coverley et al., 2002; Girard et al., 1991; Jackson et al., 1995; Knoblich et al., 1994; van den Heuvel and Harlow, 1993). However, the details on the role of Cyclin E/Cdk2 in this process remain unclear. For instance, Cyclin E1/E2/E3 triple KO cells could not re-enter the cycle from Go and E-type Cyclins were found to play an essential role in the loading of MCM2 and possibly other MCMs onto the chromatin. However, E-type Cyclins were only essential for MCM loading when cells were arrested in Go and not in actively cycling cells (Geng et al., 2003). Later on, the same authors provided proof for a role for kinase binding-deficient Cyclin E (which can no longer associate with Cdk2) in Cdt1 binding and loading of MCMs onto the chromatin (Geng et al., 2007). The connection between Cdc6 and the Cyclin E/Cdk2 complex is also still under debate: Geng et al. demonstrated that the absence of E-type Cyclins does not affect Cdc6 loading or stability. In contrast, other work on Cdk2 and Cyclin E siRNA-depleted cells has demonstrated that Cdk2-dependent phosphorylation of Cdc6 plays a critical role by preventing degradation through the APC^{Cdh1} (Mailand and Diffley, 2005). The two different methods of inactivation (siRNA versus genetic knockdown) might explain these results. Another explanation is that Cyclin E/Cdk2 can act at different times during progression from G1 to S: at the earliest stages when the pre-RC is formed up to the moment of origin firing and replication initiation. Recently it was shown that Cyclin E/Cdk2 indirectly promotes MCM loading onto chromatin by activating the transcription of Cdc6 and Cdc7 (Chuang et al., 2009). Next to Cyclin/Cdk complexes, the highly conserved Cdc7 kinase, in conjunction with its partner Dbf4/Drf1 plays a critical role in origin firing in many species as well (Bousset and Diffley, 1998; Takahashi and Walter, 2005). It does so by directly phosphorylating MCM4, thereby promoting its association with Cdc45, a co-factor necessary for the unwinding of the DNA in higher eukaryotes (Masai et al., 2006; Sheu and Stillman, 2006). How these two kinase complexes exactly regulate the various aspects of S-phase entry: pre-RC formation, loading of multiple MCMs to origins and the activation of early and late origins, is still an actively investigated topic. Altogether, the S-phase Cyclin/Cdk complexes, in concert with the Cdc7/Dbf4 kinase, are recognized as the key cell cycle activators of DNA replication.

Letting go of the gas. The essential part of re-replication prevention

A very important feature of origin firing is that it happens once, and only once per origin. Re-replication of DNA can lead to missegregation of chromosomes in mitosis, which results in genome instability and cancer. Eukaryotic organisms have evolved many checks and double-checks to keep re-replication from occurring (reviewed in (Arias and Walter, 2007). The general theme is to stop the formation and activation of a new pre-RC on an origin once that has been fired. An important point in re-replication prevention is that origin re-firing should only be blocked at origins that have already fired, but not at late origins, which still need to fire. We can roughly determine 4 biochemical ways in which components for the pre-RC are inhibited: 1) phosphorylation 2) degradation 3) relocation and 4) association.

The Cyclin/Cdk complexes that act during S-phase play a remarkable dual role in origin firing: not only are they necessary to initiate DNA replication, they are crucial for re-replication prevention in multiple ways. Phosphorylation by Cyclin/Cdk's of Cdc6 and the MCM member Mcm4 results in degradation of Cdc and the inability of MCM complexes to re-attach to the DNA (Drury et al., 1997; Labib et al., 1999). Two other important members that are inhibited by Cyclin/Cdk activity are the ORC-members ORC2 and ORC6. In budding yeast, association of the S-phase Cyclin Clb5 with ORC6 blocks its function through association (Wilmes et al., 2004). In addition, Cdk phosphorylation blocks ORC2 and ORC6 function in the same organism

(Nguyen et al., 2001). Several papers show that the Cyclin/Cdk-dependent mechanisms are highly redundant, albeit that some are more critical than others (Green et al., 2006; Nguyen et al., 2001; Wilmes et al., 2004).

In various higher metazoans extra ways of controlling the critical licensing factor Cdt1 have evolved. The Geminin protein acts in parallel to the Cyclin/Cdk's to prevent re-replication. Geminin acts on Cdt1 by association, thereby hindering its function in MCM complex recruitment (Tada et al., 2001; Wohlschlegel et al., 2000). Furthermore, 2 different degradation pathways control levels of Cdt1 and Cdc6: the Cul4 and the SCF ubiquitin ligase complex. These 2 ubiquitin ligases can both destabilize these crucial licensing factors in S-phase to prevent rereplication. Removal of Cdc6 through degradation by APC^{CDH1} is one of the ways to prevent re-replication in mammalian cells (Petersen et al., 2000). Similarly, the degradation of Cdt1 in *Xenopus* egg extracts and mammalian cells is dependent on the Cul4-$Ddb1^{Cdt2}$ complex and the replication fork protein PCNA (Arias and Walter, 2006; Senga et al., 2006). In *C. elegans*, *cdc-6* is exported out of the nucleus in a *cul-4* dependent manner (Kim et al., 2007). Deregulation of the Cul4-dependent degradation pathway leads to accumulation of Cdt1, and can even cause substantial re-replication in *C. elegans* and *Xenopus* (Arias and Walter, 2006; Sansam et al., 2006; Zhong et al., 2003). Altogether, various parallel pathways act in concert to prevent re-replication of the DNA and ensure the faithful duplication of the genome.

Coming to a full stop: exit from the cell cycle upon differentiation

In the previous part I have discussed the mechanisms that govern the cell cycle and how entry into the cell cycle is regulated. Here, I will focus on how cells withdraw from the cell cycle. Proliferation and terminal differentiation are very often found to be mutually exclusive. Many differentiated cell types assume a shape and architecture that would not allow cytokinesis without compromising a cell's function. For instance, a neuron that would have to go though a normal division cycle after differentiation might lose its dendritic connections with the surrounding cells. Cell cycle exit upon terminal differentiation is different from transient arrest in the Go/G1 phase of the cell cycle. The type of transient arrest in G1 when cells are deprived of growth factor stimuli is commonly referred to as quiescence: a Go arrest that is reversible upon mitogenic stimulation (Coller et al., 2006). Another type of G1 arrest is commonly referred to as senescence. Cellular senescence is a mechanism by which a cell responds to DNA damage, induced by oncogenes or telomere shortening, by withdrawing from the cell cycle (Campisi, 2005). However, this section will focus primarily on cell cycle exit upon terminal differentiation and the various mechanisms by which differentiating cells accomplish cell cycle exit upon differentiation.

The usual suspects: pocket proteins and Cdk inhibitors conspire in cell cycle exit and differentiation

Cells differentiate in response to signals that lead to activation of transcription factor cascades. These transcription factors will eventually shape the cells fate by activating certain cell-type specific genes, while inactivating others such as genes required for cell cycle progression. Two critical pathways that mediate exit from the cell cycle into a post-mitotic state are the pRb pocket proteins and the Cip/Kip inhibitors (reviewed (Buttitta and Edgar, 2007). The upregulation of Cip/Kip inhibitors upon differentiation is used in many organisms as the primary mechanism

to trigger cell cycle arrest upon differentiation. The MyoD bHLH transcription factor, a key determinant of muscle fate specification, is a good example of a transcription factor triggering both differentiation genes and genes necessary for cell cycle exit. MyoD activates p21 transcription upon differentiation, promoting cell cycle exit (Guo et al., 1995; Halevy et al., 1995; Zhang et al., 1999). In *Drosophila*, the p27 ortholog Dacapo plays a similar role in neuronal differentiation in the developing eye. The bHLH transcription factors Atonal and Daughterless directly regulate Dacapo expression in developing eye neurons, together with the EGF-regulated transcription factor Pointed (Sukhanova et al., 2007). Hence, p27 expression is regulated by various transcription factors in order to coordinate differentiation with cell cycle exit. Cip/Kip inhibitors are not only a means to trigger cell cycle arrest upon differentiation, without them proper differentiation cannot even occur. The other 'usual suspect': pRb and its family members p107 and p130 have a three-way function in differentiation: First: exit from the cell cycle. In addition, pockets proteins play an important role in the promotion of the differentiated fate and thirdly, the maintenance of the differentiated fate. Numerous examples exist of each of these functions and in many cases it is very difficult to dissect the various functions from each other.

The first major insights into the function of pocket proteins in cell cycle exit and differentiation came from studies in tissue culture systems. In the G0/G1 phase of the cell cycle, pRb is found in its active, hypophosphorylated form (Buchkovich et al., 1989; DeCaprio et al., 1989). This hypophosphorylated form becomes more abundant upon lymphocyte differentiation and this form is preferably targeted by viral oncogenes such as the SV40 large T protein (Chen et al., 1989; Ludlow et al., 1989). Studies of the pRb family function in mice are hindered by lethality and the extensive redundancy between the pocket proteins Rb, p107 and p130 (Clarke et al., 1992; Lee et al., 1992; Sage et al., 2000). Triple knockout (TKO) MEFs for all pocket proteins are immortalized and can no longer undergo G1 arrest or cellular senescence (Sage et al., 2000). Unfortunately, the TKO mice die during late embryogenesis and therefore the role of pocket protein in cell cycle exit *in vivo* was hard to address. It was found that this embryonic lethality was caused by trophoblast cell hyperproliferation and the consequent malformation of the placenta. Still, placental rescue of the Rb null phenotype leads to mice that are carried to term but die soon after birth (Wu et al., 2003). Studies with Cre-inducible Rb knockout cells found that pRb was necessary to prevent cell cycle re-entry in quiescent and senescent cells (Sage et al., 2003). Next to the role of pocket proteins in cell cycle exit, there are several cases known in which pRb interacts directly with a transcription factor to establish differentiation. Similar to the Cip/Kip inhibitors, pRb was found to be essential for muscle differentiation through binding of MyoD. pRb association with MyoD is essential for cell cycle withdrawal as well as activation of late differentiation genes by the transcription factor MEF2 (Gu et al., 1993; Novitch et al., 1999). In addition, pocket protein members have a distinct role in the differentiation of adipocytes. Remarkably, pRb and p107 seem to have antagonistic roles in this process, with pRb promoting adipocyte differentiation and p107 antagonizing this (Classon et al., 2000). Next to these examples, pocket proteins have been found to play a role in differentiation of blood cells, bone, neuronal cells and lens cells of the eye (Bergh et al., 1997; de Bruin et al., 2003; Kranenburg et al., 1995; Pan and Griep, 1994; Thomas et al., 2001). In several cases, pocket protein function in differentiation is indirect: for instance, in neuronal cells during CNS development pRb prevents apoptosis of neuronal cells by inhibiting the pro-apoptotic activity of activator E2F transcription factors. In muscle cells however, pRb is directly necessary for the activation of the transcriptional program for muscle differentiation. Hence, great care should be taken when interpreting data linking pocket proteins to differentiation.

The same is true for the third role of pocket proteins in the maintenance of the differentiated state. In many cases pocket proteins do not act alone to accomplish their various roles in differentiation and proliferation control. pRb interacts with well over a hundred different proteins, mostly consisting of transcription factors and chromatin remodeling enzymes (Morris and Dyson, 2001). Biochemical studies in mammalian cell culture, *Drosophila* and *C. elegans* have found pocket proteins as integral parts of several chromatin-bound complexes. Many of the associated complexes act to repress transcription. The dREAM/MMB complex was isolated from *Drosophila* embryo extracts and contains *Drosophila* Rb/E2F family members associated with dMyb and Mip40/Mip130 proteins (Korenjak et al., 2004; Lewis et al., 2004). These complexes do not repress cell cycle genes but can repress genes that are necessary for both oogenesis and spermatogenesis. An attractive explanation would be that this complex acts in somatic cell differentiation to inhibit germline-specific gene transcription and hence aberrant differentiation. However, data from dREAM/MMB complex function in mammalian cells demonstrate that this complex can in fact repress E2F-regulated cell cycle genes in quiescent cells (Litovchick et al., 2007). Thus, there is evidence for pRb proteins and the dREAM complex regulating both cell cycle withdrawal and differentiation. Furthermore, the dREAM/MMB complex in *Drosophila* is largely homologous to the *C. elegans* SynMuvB gene class. The SynMuv (for Synthetic Multivulva) genes were found to act redundantly in *C. elegans* vulval fate specification (Bender et al., 2007). In vulval development, the SynMuvB genes *lin-35* Rb and the different dREAM orthologs cooperate to repress the expression of *lin-3* EGF in the hypodermis (Myers and Greenwald, 2005). Hence, this case further substantiates a role for Rb and the dREAM/MMB complex in repressing inappropriate gene expression in different organisms.

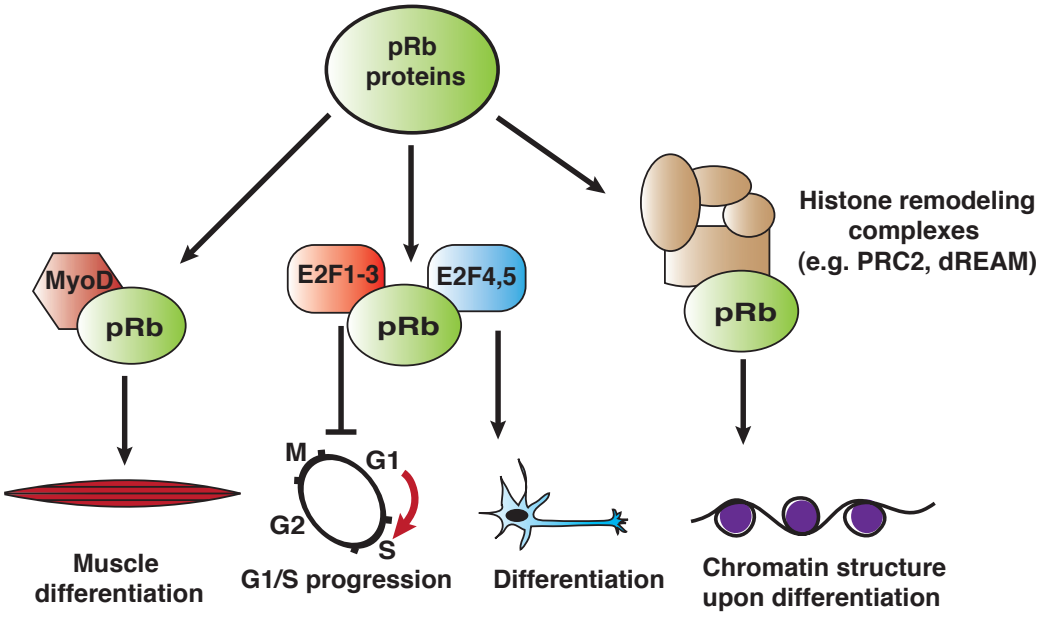


Figure 3. The diverse roles of pRb pocket proteins in cell cycle exit and differentiation. Pocket proteins such as pRb can influence cellular differentiation in various ways. Left: In certain tissues, pRb can interact directly with transcription factors such as MyoD to regulate expression of muscle-specific genes. Middle: Through interaction with activator (red) and repressor (blue) E2F transcription factors, pRb controls G1 to S progression and expression of differentiation genes, respectively. Right: pRb and other pocket proteins are often found as part of histone remodeling complexes such as the Polycomb complex (PRC2) that control either the stable repression or activation of genes in differentiated cells.

Another remarkable manner in which pRb promotes gene silencing is through the sequential interaction with HDAC1 and the histone methyltransferase Suv39H1 and heterochromatin protein HP1. pRb can recruit Suv39H1 and consequently HP1 to target gene promoters such as the Cyclin E promoter to provide stable repression of transcription (Nielsen et al., 2001). The tentative model that is postulated by the authors is that pRb first recruits HDAC1 to deacetylate the Lysine 9 (K9) residue on histone H3 and subsequently recruits Suv39H1 and HP1 to methylate the H3K9 residue, thus providing stable repression. pRb can indeed interact with the histone deacetylase HDAC1 to repress transcription and this functional interaction seems to be conserved in other organisms like *Drosophila* and *C. elegans* (Brehm et al., 1998; Korenjak et al., 2004; Lu and Horvitz, 1998; Luo et al., 1998). This simple 2-step model illustrated how pRb can function as a hub for chromatin remodeling activity in controlling gene expression. In recent years, it was shown that pRb interacts with a number of histone demethylase enzymes. The Rbp2 protein was found as an interactor of pRb that was important for the function of pRb in cellular differentiation (Benevolenskaya et al., 2005). The Helin and Kaelin labs independently found that demethylation of the activating chromatin mark histone H3 lysine 4 (H3K4) is the biochemical function of Rbp2 (Christensen et al., 2007; Klose et al., 2007). Rbp2 functions to repress cell cycle gene promoters late in differentiation (Lopez-Bigas et al., 2008). The pRb/Rbp2 interaction provides an exemplary case wherein pRb indirectly enforces cell cycle exit upon differentiation by preventing cell cycle genes from becoming active after terminal differentiation. In summary, the pRb family of pocket proteins regulate both cell division and cell fate through a large variety of interactions. The various ways in which the G1/S regulator pRb acts at the nexus of cell proliferation and differentiation are summarized in figure 3.

Proliferation of differentiated cells: driving with the handbrake on?

The previous section has shown that proper and irreversible exit from the cell cycle is essential for normal cell differentiation. However, little is known about the molecular mechanisms that prevent a cell from re-entering the cell cycle once it is terminally differentiated. Although the coincidence of cell division and terminal differentiation is rare, there are several examples that will be covered in this section.

Work on terminally differentiated muscle myotubes has provided several interesting insights into the possibility of cell cycle re-entry in differentiated cells. Muscle satellite cells were allowed to differentiate and Rb was removed from the differentiated myotubes by Cre-induced recombination. Acute Rb deletion was sufficient to trigger E2F/S-phase marker gene expression. However, it could not trigger DNA replication and mitosis (Camarda et al., 2004). Curiously, the same study shows that co-expression of CyclinD-Cdk4 could trigger DNA-replication, suggesting that there is an Rb-independent role of the CyclinD-Cdk4 complex in G1 progression. Another study shows, using siRNA to interfere with all three pocket proteins in myotubes, that acute pocket protein removal does lead to BrdU incorporation and S-phase (Blais et al., 2007). However, mitosis still seems to be defective in these cells, suggesting that there are other mechanisms acting in parallel to Rb to prevent full cell cycle re-entry. A more recent study showed that the reduction of various CKIs triggers cell cycle re-entry in myotubes as well as senescent and quiescent cells (Pajalunga et al., 2007). In addition, *in vivo* examples of cell cycle re-entry feature Rb as an important means in the establishment and maintenance of cell cycle arrest. Studies in a mouse Rb knockout model provided direct evidence of cell cycle re-entry of terminally differentiated hair cells in the inner ear (Sage et al., 2005). Floxing out the Rb gene in postmitotic hair cells caused cell cycle re-entry, whereas the cells retained their normal function.

Another instance in which cell cycle re-entry and terminal differentiation co-occur is found in *Drosophila*. In *Rbf* and *Dacapo* (pRb and p27) mutant clones, differentiated neurons in the *Drosophila* eye continue to divide after the last mitotic wave. This proliferation of differentiated cells coincides with the expression of neuronal differentiation markers like ELAV and Senseless (Baonza and Freeman, 2005; Firth and Baker, 2005). Another study of cell cycle re-entry in the *Drosophila* eye and wing discs provided a more detailed analysis of the controls on cell cycle exit upon differentiation. Using conditional clonal overexpression, Buttitta et al. found that simultaneous expression of an activator E2F, E2F1 and a G1/S Cyclin/Cdk combination is sufficient to reverse cell cycle exit. In cells that have not undergone terminal differentiation, expression of either E2F1 or Cyclin E together with Cdc25 is enough to stimulate cell cycle entry. This suggests the existence of a double assurance mechanism in terminally differentiated cells, which requires both Cyclin/Cdk and E2F activity to be deregulated in order to re-enter the cell cycle. Furthermore, the frequency in which cells re-enter the cell cycle seems to be tissue and timing dependent, with consistently more re-entry seen in the eye than in the wing tissue (Buttitta et al., 2007). This suggests that there are differences between terminally differentiated tissues in their ability to re-enter the cell cycle. Perhaps the most striking example of this comes from a conditional pRb family knockout mouse model. Mice homozygous mutant for p130, heterozygous for p107 and carrying a pRb Lox allele that was knocked out during retinal development, are born with normal retinas. However, these mice develop eye tumors after several weeks, which arise from terminally differentiated horizontal interneurons (Ajioka et al., 2007). Importantly, the authors show that these proliferating interneurons are fully functional differentiated neurons. Moreover, these tumors can metastasize but the cells lose their neuronal fate upon metastasis. It remains a mystery why pocket-protein knockout alone is sufficient to drive tumorigenesis in the eye, while other differentiated tissues are unaffected by the loss of pocket proteins alone. Recently, it was shown that retinoblastoma tumors in humans could arise from a cone cell precursor that has lost one allele of pRb only (Xu et al., 2009), highlighting the difference between men and mice. Furthermore, this paper provides us with some clues about the difference between various differentiated cells in becoming tumorigenic. The cone cell precursors have high levels of the proto-oncogenes MDM2 and N-Myc. Therefore, these cells are more likely to become tumorigenic when one of the negative regulators of cell cycle progression is removed. These results are in contrast with the increasingly common notion in the cancer biology field that tumors can only arise from undifferentiated progenitor cell populations in adult tissue.

Many of the studies on cell cycle re-entry have been performed in a tissue culture differentiation system. As an obvious drawback, these systems lack the ability to faithfully mimic the complex interactions of cells in their organismal context. Furthermore, there are limited possibilities to perform genetic screening in a tissue culture system. Therefore we set out to use the *C. elegans* bodywall muscle as a model system for cell cycle re-entry of differentiated cells. In the next chapter I will expand on *C. elegans* and the various aspects of developmental regulation of cell division in this nematode.

The nematode *C. elegans* as a model organism for developmental control of cell division

The nematode *Caenorhabditis elegans* is a soil-dwelling nematode that has a short life cycle of about 3 days at 20 ºC. It is hermaphroditic, with a small (0.1%) natural incidence of males. Furthermore, the animals can be easily grown under laboratory conditions. Sydney Brenner first saw the potential of using this nematode as a model system for development and neurobiology. He isolated the standard wild-type *C. elegans* strain (N2) and performed the first genetic screen (Brenner, 1974). The transparency of these animals, together with their reproducible pattern of development, allowed researches to track all cell divisions in this nematode: from the fertilized 1-cell embryo all the way to adulthood (Sulston and Horvitz, 1977). The *C. elegans* larva hatches with 550 cells and subsequently goes through 4 larval stages, L1 to L4 (Figure 4). Specific cells called blast cells continue to divide during larval development to provide in the growth of these animals. The tractability of the division in these different blast cells resulted in screens for mutants with aberrant cell division patterns (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981). Studies in *C. elegans* have been instrumental for our understanding of a wide variety of processes (Jorgensen and Mango, 2002). Fundamental biological processes such as apoptosis, RNA interference (RNAi) and microRNA regulation were first characterized in *C. elegans* and the field was rewarded with no less than 3 Nobel prizes in the last 6 years. Study of the cell cycle in *C. elegans* allows us to discover how cell division is regulated in a developmental context and which factors control division of cells during the life of a nematode. In the next section I will cover work that was aimed to understand the post-embryonic regulation of the cell cycle, focusing in particular on G1/S transition.

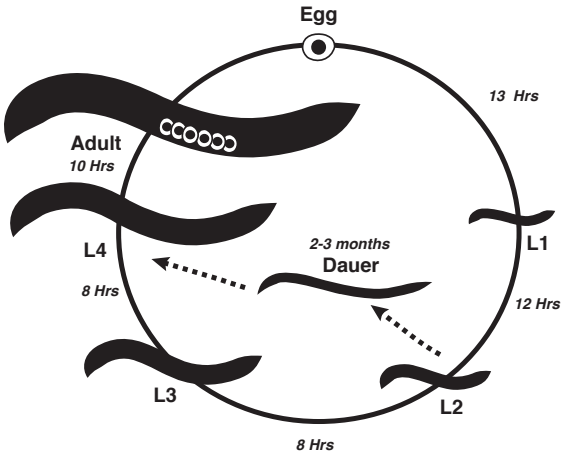
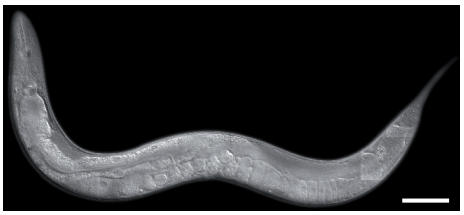


Figure 4. The nematode *Caenorhabditis elegans* and its life cycle. Top: An adult *C. elegans* animal. Scalebar is 100 µm. Bottom: The life cycle of *C. elegans* from fertilized egg to adult. In the absence of food, L2 larvae can enter an alternative L3 stage called the Dauer stage (dotted lines) which is long lived and extremely resistant to environmental stress.



Control of G1 progression in C. elegans: a conserved pathway with fewer parts

Although studies of mammalian cells in tissue culture identified many of the components that are important for G1 progression, it cannot answer the question how these regulators function *in vivo* to control cell division during multicellular development and homeostasis. More importantly, genetic approaches in model organisms can help to identify the various signals that act upstream of the intrinsic cell cycle machinery to control the division program during development.

A search for Cdk orthologs in *C. elegans* identified multiple Cdk members in this nematode, with single genes representing the different Cdk families (Boxem et al., 1999). Two of the genes that were found to be critical for G1 progression encode the only Cyclin D and Cdk4/6 family members in *C. elegans cyd-1* and *cdk-4* respectively (Boxem and van den Heuvel, 2001; Park and Krause, 1999). *cyd-1* and *cdk-4* mutants show similar defects: absence of post-embryonic blast cell division and DNA replication. In addition, a few cells that divide late in embryogenesis, such as the coelomocytes, fail to divide in *cyd-1* mutants, suggesting a need for G1 control in this tissue (Yanowitz and Fire, 2005). However, most of the embryonic cell divisions can occur without the need for G1 control. In mammalian cells, the main function of the CyclinD/Cdk4 complex is phosphorylation and inactivation of Rb. The *C. elegans* Rb homologue *lin-35* was defined by a SynMuv class B mutation that affects vulva development (Ferguson and Horvitz, 1989; Lu and Horvitz, 1998). In addition, *lin-35*/Rb plays its canonical role in the regulation of G1 progression. The *lin-35* gene acts downstream of both *cyd-1* and *cdk-4* and the lack of cell division and DNA replication in these mutants can be partially rescued by *lin-35* inactivation and *lin-35* mutants extra nuclear divisions in the intestinal lineage (Ouellet and Roy, 2007). In mammalian systems, the CyclinE/Cdk2 complex acts later during G1 progression at the G1/S transition to ensure entry into S-phase. The *C. elegans cye-1* gene is essential for cell cycle progression at an earlier stage than *cyd-1*, since RNAi of *cye-1* leads to embryonic arrest at approx. the 100-cell stage. The *cye-1* putative null mutant is sterile, lacks late post-embryonic cell divisions and has various fate specification defects in the vulva (Fay and Han, 2000). Analysis of the *cye-1* promoter reveals that it has several E2F binding sites, suggesting that its expression is induced upon activation of E2F transcription factors in G1/S transition (Brodigan et al., 2003). However, the study of E2F transcription factor family members in *C.elegans* has yielded conflicting results. There are 2 different E2F-like transcription factors in *C. elegans*, *efl-1* and *efl-2*. The *efl-1* gene is a SynMuv B gene and acts, like *lin-35* Rb, to repress cell division and therefore *efl-1* is likely to be the *C. elegans* repressor E2F (Boxem and van den Heuvel, 2002). Inconsistent with this is the role of *efl-1* in the germline. In the germline, *efl-1* and *efl-2* act together with *dpl-1*, the *C. elegans* DP homologue, to positively regulate gene transcription. Microarray analysis revealed that *efl-1* and *dpl-1* are likely to function as a heterodimer since their removal affects the expression of a similar set of targets. These targets seem to be genes that are not involved in cell cycle but in oogenesis and early embryonic development (Chi and Reinke, 2006). In conclusion, a recognizable activator E2F complex that acts to promote progression through the cell cycle has not been identified yet in *C. elegans*. Strangely enough, S-phase target genes like the *rnr-1* ribonucleotide reductase and *cye-1* do have multiple canonical E2F binding sites in their 5' UTRs, which does suggest E2F involvement in S-phase gene expression (Brodigan et al., 2003; Kirienko and Fay, 2007).

While *C. elegans* does not seem to have pINK family kinase inhibitors, it does have two members of the CipKip inhibitor family named *cki-1* and *cki-2* (Hong et al., 1998). Knockdown of *cki-1* results in extra divisions in many different blast cell types, including intestine, V-cells and P-cells (Boxem and van den Heuvel, 2001; Hong et al., 1998). Furthermore, combined inactivation of *lin-35* and *cki-1* leads to a number of cells far exceeding that of the wildtype in these blast cell lineages (Boxem and van den Heuvel, 2001). In addition to *cki-1* and *cki-2*, several other conserved regulatory molecules exert their control on Cyclin/Cdk complex activity. Like mammals, *C. elegans* has various protein complexes involved in Cyclin ubiquitination and degradation. Mutants in the SCF ubiquitin ligase complex components *cul-1* Cullin and its F-box factor *lin-23* have extensive hyperplasia in multiple post-embryonic cell lineages, leading

amongst others to defects in vulval and gonadal development (Kipreos et al., 2000; Kipreos et al., 1996). As in mammals, the SCF complex in *C. elegans* might control *cye-1* levels. Consistent with this idea, loss of *cul-1* can partially suppress some of the phenotypes associated with loss of *cye-1* (Fay and Han, 2000). Loss of *lin-23* and another ubiquitin ligase component of the APC family *fzr-1* both act synergistically with *lin-35* in controlling cell division (Fay et al., 2002). As mentioned previously, Cyclins and Cdk's can be phosphorylated in order to attune their activity. A gain-of-function allele of the *cdc-25.1* phosphatase was picked up in two independent screens for mutations that could enhance the number of intestinal nuclei (Clucas et al., 2002; Kostic and Roy, 2002). Knockdown of *cye-1* can suppress the extra division phenotype in the intestine of *cdc-25.1(gf)* animals. Thus, the Cdc25 phosphatase has a conserved role as a negative regulator of Cyclin/Cdk complexes in *C. elegans* there are two Wee1-like kinases that seem to play a role in *C. elegans* cell cycle progression. The *wee-1.3* gene was initially found as a dominant gain-of-function allele with defects in spermatogenesis due to developing spermatocytes that fail to enter mitosis (Lamitina and L'Hernault, 2002). Later on, the *wee-1.3* mutant was found to have defects in oocyte maturation, a defect that was dependent on *cdk-1* (Burrows et al., 2006). Null mutants for *wee-1.3* cause embryonic lethality and early larval arrest phenotypes. These results support a role for *C. elegans wee-1.3* as an inhibitory kinase of *cdk-1* that prevents entry into mitosis. Together, these data point to a pivotal role for Cdk activity regulators in controlling G1/S transition in *C. elegans*.

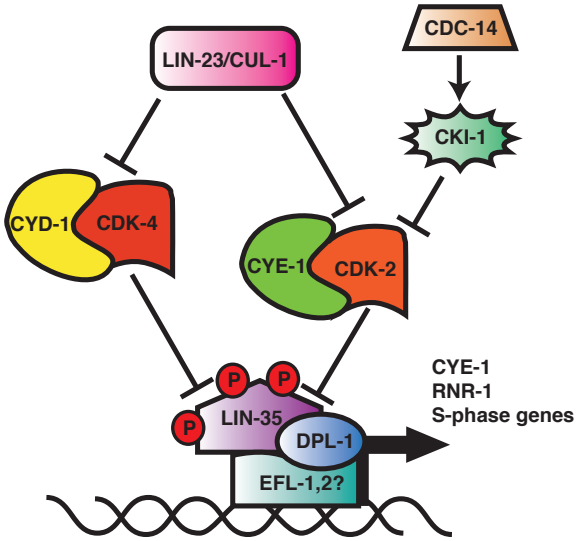


Figure 5. Regulation of G1/S transition in *C. elegans*. Two Cyclin/Cdk complexes, CYD-1/CDK-4 and CYE-1/CDK-2, control progression from G1 to S in *C. elegans*. In G1, CYD-1/CDK-4 inactivates the Rb orthologue LIN-35, presumably by phosphorylation, and activates the expression of S-phase specific genes, including CYE-1. This results in an increase in CYE-1/CDK-2 activity and leads to further inactivation of LIN-35. This positive feedback loop ensures robust and irreversible S-phase entry. Negative regulation of the G1 Cyclin/Cdk complexes by the LIN-23/CUL-1 SCF complex and the p27 orthologue CKI-1 act to prevent G1 progression during development.

We can summarize our understanding of the G1/S transition in *C. elegans* in the following scheme (Figure 5). The *C. elegans cyd-1/cdk-4* and *cye-1/cdk-2* complexes act as positive regulators of G1 progression. The negative regulators: *lin-35* Rb, *cki-1* Cip/Kip and SCF components constitute parallel pathways that control entry into S-phase. A major challenge in understanding the complexity of cell cycle regulation during metazoan development is to identify the factors that act upstream of the core machinery to regulate cell cycle progression. Furthermore, work in *C. elegans* has also uncovered some unusual roles for cell cycle components in establishing cell polarity and fate. I will discuss examples of both in the next section.

Regulation of G1 progression during post-embryonic development in C. elegans

During post-embryonic development, several blast cell lineages continue to divide to accommodate the growing animal. The P cells are a set of blast cells at the ventral side of the body that give rise to neurons, cells that fuse with the hypodermal syncytium hyp7 and vulval cells. The six P cell descendants that give rise to the vulva are called vulva precursor cells or VPCs. These precursors arise during P cell division in mid-L1 and temporarily arrest for 2 larval stages until mid-L3, when the vulva begins to develop (Sulston and Horvitz, 1977). A screen was performed to identify factors controlling this temporary G1 arrest. It was found that cell cycle arrest is maintained in the VPCs by high levels of *cki-1* and loss of *cki-1* leads to extra VPC divisions during the L2 stage (Hong et al., 1998). Transcriptional induction of *cki-1* by the *lin-1* ETS and *lin-31* Forkhead transcription factors increases *cki-1* levels in the VPCs to promote G1 arrest in late L1 larvae (Figure 6B) (Clayton et al., 2008). At the mid-L3 stage, the RAS/MAP kinase pathway is activated in the VPCs, which leads to phosphorylation and inactivation of LIN-1 and LIN-31 (Tan et al., 1998). Next to genes that function to regulate transcription of *cki-1*, this screen also identified a novel regulator of *cki-1*. The *cdc-14* gene was defined by a mutation that caused premature division of the VPCs during the L2 stage (Saito et al., 2004). As previously mentioned in this introduction, the mammalian p27 is degraded in a phosphorylation-dependent manner. The *cdc-14* phosphatase might act to stabilize the CKI-1 protein through dephosphorylation, which prevents it from being recognized by the SCF complex and degraded by the proteasome.

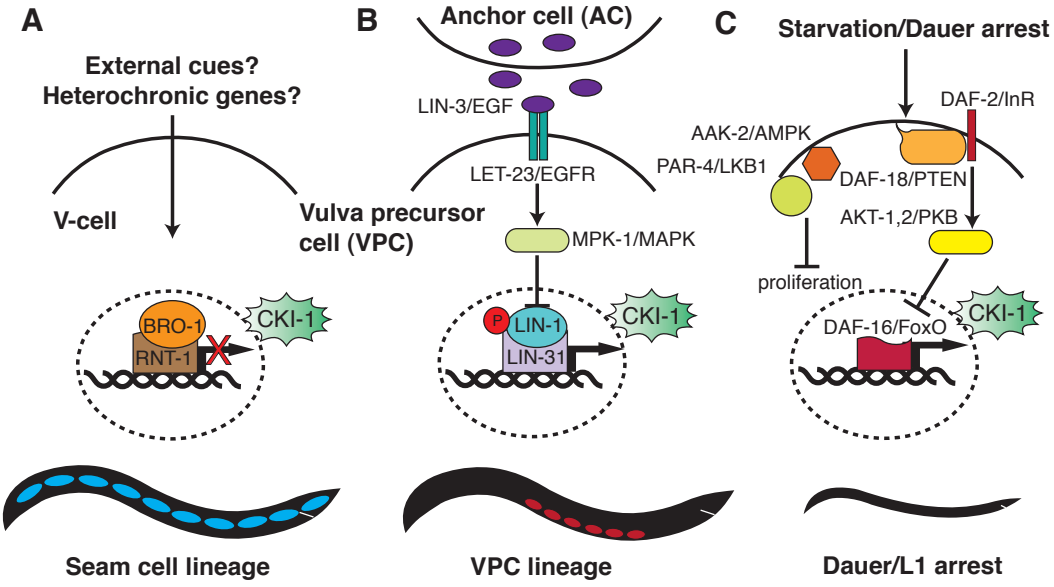


Figure 6. The *cki-1* Cip/Kip inhibitor acts in different cells and multiple developmental times to control cell division in *C. elegans*. Left: in the seam cell lineage, the transcription factor RNT-1, together with its co-factor BRO-1, controls the division frequency of the V-cells through transcription of *cki-1*. Middle: VPC cells remain quiescent from late L1 to mid-L3 due to high levels of CKI-1, induced by the transcription factors LIN-1 and LIN-31. These transcription factors get inactivated at mid L3 by a LIN-3/EGF MAP kinase pathway. Subsequently, the induced VPCs will start to divide and form the vulva. Right: in the absence of food, L1 or Dauer larvae arrest cell division through the action of two pathways: The DAF-2 insulin pathway negatively regulates the activity of the DAF-16 FoxO transcription factor. In the absence of food, DAF-16 moves to the nucleus and activates transcription of *cki-1*, thereby ensuring arrest in G1. Alternatively, a *par-4*/LKB1 and *aak-2*/AMPK-dependent pathway represses cell proliferation in the germline by an as of yet unknown mechanism.

Temporal regulation of cell division also plays an important role in the seam or V-cells, a group of blast cells that contribute to the hypodermis or skin of the animal. During each larval stage seam cells divide asymmetrically to give rise to an anterior daughter cell that fuses with the hypodermis (hyp7) and a posterior seam cell daughter that will divide again in the next larval stage. This stem-cell like division pattern is repeated during every larval stage up to adulthood, when seam cells differentiate and form alae, a hypodermal ridge-like structure that presumably aids movement of the animal. A complex network of transcription factors and microRNAs is known to regulate the timed division of the seam cells (Pasquinelli and Ruvkun, 2002). The Runx-like transcription factor *rnt-1* is one of the factors required to control seam cell number. Loss of *rnt-1* leads to fewer seam cell divisions (Kagoshima et al., 2005; Nimmo et al., 2005). In mammals, Runt can promote tumor growth together with its co-factor CBF. Similarly, overexpression of *rnt-1* together with its co-factor *bro-1*/CBF leads to widespread seam cell hyperplasia, with cells failing to adopt the fused hypodermal fate. One of the ways in which *rnt-1* might regulate seam cell division is through *cki-1* regulation (Figure 6A). Reduction of *cki-1* by RNAi could partially suppress the loss of divisions in the seam cell lineage of *rnt-1* animals and *rnt-1* could control the expression of a *cki-1::GFP* transcriptional reporter (Nimmo et al., 2005). However, the evidence for direct regulation of *cki-1* expression by *rnt-1* is limited. In fact, the role of *cki-1* in regulating seam cell fate seems to be more complex. *cki-1(RNAi)* animals have changes in seam cell division timing and number as well as changes in seam cell fate (Fujita et al., 2007; Hong et al., 1998). The role of *cki-1* and *cye-1/cdk-2* in the seam cell lineage will be addressed in more detail in chapter 4 of this thesis.

Next to its role in cell division during normal development, *cki-1* also plays a role in the developmental arrest that takes place when *C. elegans* are exposed to unfavorable conditions such as insufficient nutrients and overcrowding (Figure 6C). The larvae can arrest at two points in development: at the beginning of the L1 stage when animals have just hatched or at the end of the L2 stage. The latter arrest involves entry into a specialized L3 stage called the Dauer stage. As compared to animals raised in the presence of food, Dauer larvae are extremely long-lived and also very resistant to many forms of physiological stress like heat, alkalyzing agents and heavy metals (Fielenbach and Antebi, 2008; Hu, 2007). L1 arrested animals will not start somatic or germline development but retain the same number of cells as the hatched larva. Similar to the Dauer stage, L1 arrest is controlled by *cki-1* and the insulin signaling pathway. Loss of *cki-1* leads to inappropriate cell cycle entry in starved L1 and Dauer larvae (Hong et al., 1998). A direct link between the insulin signaling pathway, *cki-1* and L1 arrest was made by the Sternberg lab. They demonstrated that *daf-16* FoxO can control *cki-1* transcription (Baugh and Sternberg, 2006). *daf-16* mutants fail to arrest properly during L1 starvation and show a loss of *cki-1* expression in starved L1's. This is similar to the situation found in mammals, where FoxO factors can induce cell cycle arrest by direct p27 upregulation (Medema et al., 2000). However, the maintenance of cell cycle arrest in the germline seems to be independent of *daf-16* and *cki-1*. In this tissue, the PTEN homologue *daf-18* functions together with the *aak-2*/AMPK and *par-4*/LKB1 kinase to regulate germ cell arrest in starved L1 animals (Fukuyama et al., 2006; Narbonne and Roy, 2006). *daf-18* might function in a parallel pathway together with *aak-2* and *par-4* to control the response to starvation through different downstream regulators than *daf-16*/FoxO (Figure 6). More recently it was discovered that AAK-2 can be directly phosphorylated by the PAR-4 kinase. AAK-2 kinase activity is responsible for both longevity and stress resistance of starved L1's (Lee et al., 2008; Narbonne and Roy, 2009). It would be interesting to see if these kinases directly influence cell cycle regulators by phosphorylation or

whether this is done indirectly through as of yet unknown downstream targets. In summary, both transcription factors as well as extrinsic cues such as nutrient availability can influence cell cycle progression. The *cki-1* Cip/Kip homologue seems to play a prominent role in the negative regulation of cell cycle progression during development. Unfortunately, much less is known about what determines the expression of positive cell cycle regulators e.g. *cyd-1* and *cye-1* during development.

Basic parts performing extraordinary functions: roles of G1/S regulators in various other cellular processes

Work in *C.elegans* has uncovered interesting roles for G1/S regulators in cell division timing as well as fate determination during development. The previously mentioned fate transformation in the seam cell lineage upon G1/S regulation is one example (Fujita et al., 2007; Hong et al., 1998). Furthermore, several mutations in G1/S regulators cause defects in fate specification of the somatic gonad precursor lineage (Z1 and Z4) (Fujita et al., 2007; Kostic et al., 2003; Tilmann and Kimble, 2005). The Z1 and Z4 cell each divide to generate four cells, one of which will become the Distal Tip Cell (DTC), which migrates to guide the developing gonad (Kimble and Hirsh, 1979). It was observed that *cki-1(RNAi)* animals had extra gonadal arms that were derived from aberrant fate specification in the Z1/Z4 lineages (Kostic et al., 2003). Fujita et al. observed that CYE-1 levels were higher in the non-DTC Z1.ap and Z4.pa cells and conversely, CKI-1 levels are higher in the DTC-fated daughters Z1.aa and Z4.pp. (Fujita et al., 2007), suggesting that this asymmetric distribution of G1/S regulators contributes to fate determination in this lineage. In line with these results, the Kimble lab found that the division of Z1 and Z4 is disrupted in a temperature-sensitive *cyd-1* mutant (Tilmann and Kimble, 2005). *cdk-4* mutants have a similar phenotype and this can be partially rescued by removal of any of the downstream negative G1/S regulators like *lin-35*/Rb and *efl-1*/E2F. Although both groups demonstrate that proper fate specification in the somatic gonad precursors requires the G1/S-promoting *cye-1/cdk-2* and *cyd-1/cdk-4* complexes, the explanations for this phenotype differ. Tilmann and Kimble observe that the fate change in Z1, Z4 daughter cells is inversely correlated with the localization of the Wnt pathway transcription factor POP-1/Tcf, which is affected by loss of *cyd-1*. POP-1 is upregulated in non-DTC precursors but POP-1 asymmetry was normal in the V and T seam cells (Tilmann and Kimble, 2005). In addition, *cyd-1* might affect cell fate in the somatic gonad through inhibition of the *efl-1*/E2F-mediated repression of the *fkh-6*/Forkhead transcription factor, which is required for somatic gonad differentiation (Chang et al., 2004). Fujita et al. use a temperature-sensitive *wrm-1*/β-catenin mutant to demonstrate that Wnt signaling is responsible for the asymmetric distribution of CYE-1/ and CKI-1. Furthermore, they report that the asymmetry of another Wnt component important in asymmetric cell division, LIT-1 is still normal in *cye-1* mutants. Although these data are seemingly contradictory, G1/S regulation is likely to be important at multiple times during somatic gonad fate specification and therefore might act both up- and downstream of the Wnt//β-catenin asymmetry pathway in somatic gonad fate specification.

The question remains as to what degree this phenotype of G1/S regulators is caused by a lengthening or shortening of the G1 phase, or that *cyd-1* and *cye-1* indeed have distinct roles in cell fate that are unrelated to their role in G1/S regulation. Both studies claim that it is unlikely that the fate change caused by *cyd-1* or *cye-1* mutation is due to an increase in G1 length. Extension of cell cycle length with either Hydroxyurea and *gon-4(RNAi)* did not lead to gonadal precursor misspecification. However, HU stalls replication fork progression during

S-phase and hence does not lead to a lengthening of G1. Furthermore, the *gon-4* mutant does display a loss of proper Z1/Z4 specification due to the severe delay in division. Curiously, the particular temperature-sensitive *cyd-1* allele isolated in the Tilmann and Kimble study only affects divisions in the somatic gonad, suggesting a dose-dependent effect of *cyd-1* removal. A more detailed analysis of the role of G1/S regulators in somatic gonad differentiation will be necessary to resolve these issues.

There are examples of G1/S regulators involved in cell fate decisions in several other tissues. A *cyd-1* mutation was identified in a screen for mutants with an aberrant number of coelomocytes, scavenger cells that are thought to play a similar role as the kidney in higher organisms (Yanowitz and Fire, 2005). In the *cyd-1(cc600)* mutant the two embryonic coelomocyte precursors do not divide anymore but rather precociously differentiate into functional coelomocytes. Curiously, other cell divisions are not affected by this splicing mutation of *cyd-1*, not even in a trans-heterozygous animal carrying a null allele of *cyd-1* and the *cc600* allele. The coelomocytes are one of the last cells to divide during embryogenesis. Therefore, this late embryonic division might have a true G1-phase, in contrast to most other embryonic cell divisions. Lowering the level of *cyd-1* will cause a premature arrest in G1, resulting in the precocious differentiation of the undivided coelomocyte mother cell. However, it is not known why the coelomocyte division is especially sensitive to low levels of *cyd-1*. Similar precocious differentiation was reported for vulval precursor cells (VPCs) in *cye-1* mutants. *cye-1* mutant animals have more VPCs adopting a vulval cell fate, but these ectopically induced VPCs precociously differentiate after one round of division (Fay and Han, 2000). The authors demonstrate that the lengthening of G1, the time in which the VPC are normally induced (Ambros, 1999), is likely to be the cause of the increase in VPC fate induction in the *cye-1* mutant. Altogether, these examples show that G1 progression regulators can determine cell fate in different cell types. But although it might seem that G1/S regulators are directly involved in fate specification in some of these cell types, the effect might still be due to the canonical role of G1/S regulators in the cell cycle.

The *C.elegans* Rb homologue *lin-35* however has obvious roles outside of G1/S regulation (van den Heuvel and Dyson, 2008). Next to its previously mentioned role in vulval fate specification, *lin-35* is also involved in repression of transgene expression, maintenance of soma-germline distinction and RNAi hypersensitivity. Transgenes in *C. elegans* form extrachromosomal arrays made up of hundreds of copies of the transgene that form a large chromosome-like structure and gets inherited during cell division in both the germline and the soma (Mello et al., 1991). Such transgenic arrays are silenced in the germline and not in the soma (Kelly et al., 1997). *lin-35* and other genes in the SynMuvB class were found to be involved in somatic transgene silencing (Hsieh et al., 1999) and in addition, *lin-35* mutants were hypersensitive to RNAi (Kennedy et al., 2004; Wang et al., 2005). How these two processes are connected and the particular role that *lin-35* plays in these processes has long remained a mystery. A first clue into the mechanism came from the observation that *lin-35* and other members of the SynMuvB class have aberrant expression of germline-specific markers in the soma. Ectopic expression of the germline-specific *pgl-1* marker and P granules was observed when *lin-35* or *let-418* Mi2, a NuRD complex component, were mutated (Unhavaithaya et al., 2002; Wang et al., 2005). Removing the Polycomb-like gene *mes-4* and other *mes* genes enhanced this phenotype. Furthermore, *mes-4* and *zfp-1*, which encodes a zinc-finger protein that also has an enhanced RNAi mutant phenotype, can both suppress the SynMuv phenotype of a class A-B double mutant (Lehner et al., 2006). So it appears that *lin-35* together with SynMuvB genes

and other chromatin remodeling factors determine soma-germline distinction. The soma-germline transformation in *lin-35* mutants might explain the RNAi hypersensitivity and somatic transgene silencing defects. Enhanced RNAi and transgene silencing in the germline are likely to be side effects of the defense mechanism that protects the germline against transposons and retroviruses. For instance, the Tc1 transposon is silenced in the germline but not in the soma (Emmons and Yesner, 1984). This repression requires the RNAi mechanism: fortuitous read-through products of the transposon can trigger the production of dsRNA against the transposase (Sijen and Plasterk, 2003). This will trigger the destruction of the transposase, hence preventing the transposon from hopping around in the genome and causing DNA damage. The RNAi mechanism can also protect against viral replication in the germline (Lu et al., 2005; Wilkins et al., 2005). Since the germline DNA is crucial to faithfully pass on the DNA to the next generation, it needs to have extra control mechanisms in place to protect this particular DNA from damage. The germline-like fate of the soma in *lin-35* mutants results in misexpression of germline-specific RNAi factors in the soma, thus making RNAi all the more efficient in the soma of this mutant. Recently, the discovery was made that long-lived mutants also have a soma-to-germline transformation and that longevity required germline factors like *pie-1* and *pgl-1* (Curran et al., 2009). Hence, next to protection against molecular parasites, the germline fate also enhances protection against cellular stress caused by ageing.

In summary, the *lin-35* gene has pleiotropic roles in cell division and fate, RNAi and soma-germline distinction. Furthermore, screens for synthetic interactors with *lin-35* revealed roles in pharyngeal morphogenesis, intestinal homeostasis, ribosomal RNA metabolism and DNA damage control (Ceron et al., 2007; Fay et al., 2004; Kirienko et al., 2008). The gene regulatory program of *lin-35* reveals little overlap in genes expressed at different stages or in different tissues (Chi and Reinke, 2006; Kirienko and Fay, 2007). It appears that *lin-35* can act as a broad transcriptional regulator in various tissues and at different times during development. Since pocket proteins cannot bind DNA directly, *lin-35* must act together with different co-factors to regulate gene expression and cell fate.

The C. elegans bodywall muscle as a model for cell cycle re-entry of terminally differentiated cells

So far, we have seen important roles for both *lin-35*/Rb and *cki-1* Cip/Kip in controlling the division and fate of post-embryonic blast cell divisions. However, most tissues in *C. elegans* remain unaffected by loss of these negative cell cycle regulators and cell cycle re-entry of differentiated cells has not been observed in the single or double mutants. Similar to mammals, this is likely to reflect a critical difference between cells that are terminally differentiated and cells that temporarily arrest but retain the ability to divide. We use the *C. elegans* bodywall muscle (bm) as a terminally differentiated cell type in which to study this process in further detail.

The *C. elegans* bodywall muscle is formed during embryogenesis from 4 different blastomeres (Figure 7A). After embryogenesis has been completed, the bodywall muscle makes up 81 cells of the 550 cells. In addition to this, the mesoblast (M) blast cell gives rise to 14 additional post-embryonic muscle cells in the hermaphrodite. The mesoblast is located in the mid-posterior of the animal and starts to divide 6-7 into L1 development (Figure 7B). The first division is dorso-ventral and subsequent M-blast daughters divide along the A-P axis. 14 of the mesoblast descendants become striated bodywall muscle cells. Two of the M-blast daughters, the sex myoblasts (SM) migrate to the anterior during the L3 stage to form eight non-striated sex

muscle cells in the vulva. Two other mesoblast daughters become the coelomocytes (Sulston and Horvitz, 1977). The muscle cells lie in 4 quadrants that run the longitudinal axis of the animal. Upon formation of the myoblast cells during the late gastrulation stage, the muscle cells subsequently migrate and stretch out during the elongation stage. Eventually, muscle cells in *C. elegans* are not fused together, but linked together by muscle adhesion plaques. Structures called dense bodies and M-lines provide the connection with the surrounding hypodermis and cuticle. In addition, they provide a frame for the assembly of the sarcomere: the actin-myosin structure that forms the contractile unit of the muscle (Moerman and Williams, 2006).

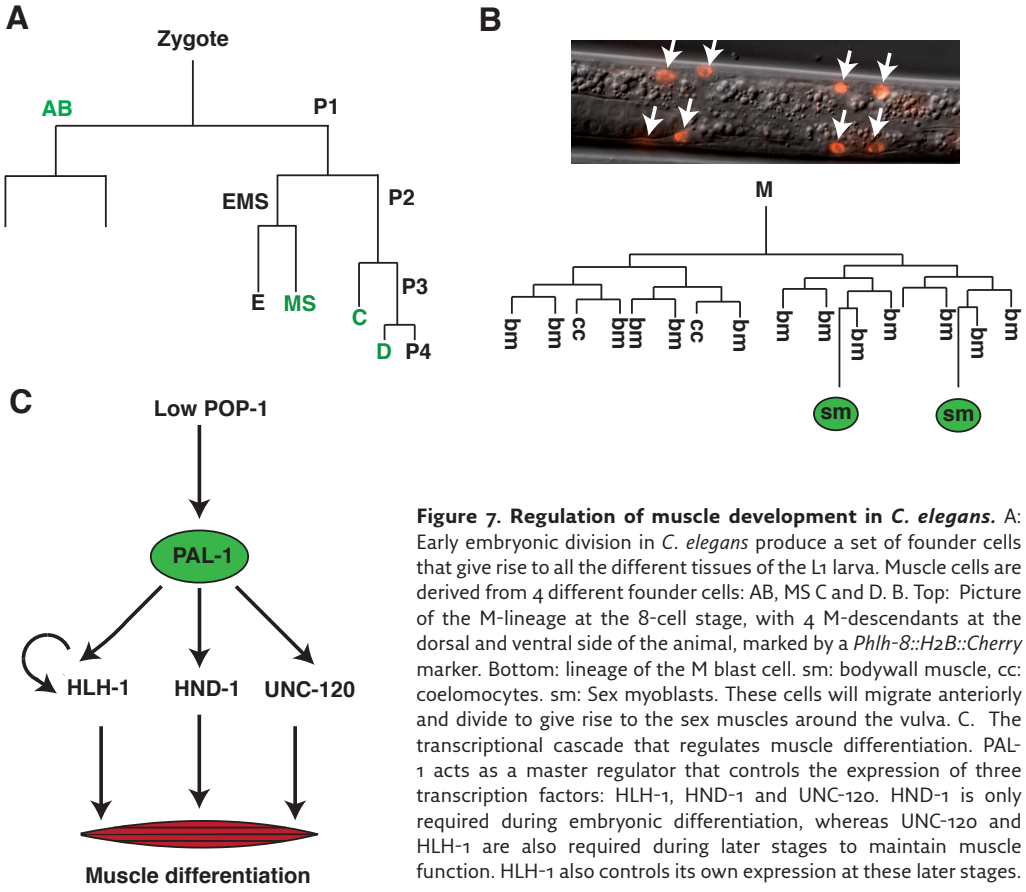


Figure 7. Regulation of muscle development in *C. elegans*. A: Early embryonic division in *C. elegans* produce a set of founder cells that give rise to all the different tissues of the L1 larva. Muscle cells are derived from 4 different founder cells: AB, MS C and D. B. Top: Picture of the M-lineage at the 8-cell stage, with 4 M-descendants at the dorsal and ventral side of the animal, marked by a *Phlh-8::H2B::Cherry* marker. Bottom: lineage of the M blast cell. sm: bodywall muscle, cc: coelomocytes. sm: Sex myoblasts. These cells will migrate anteriorly and divide to give rise to the sex muscles around the vulva. C. The transcriptional cascade that regulates muscle differentiation. PAL-1 acts as a master regulator that controls the expression of three transcription factors: HLH-1, HND-1 and UNC-120. HND-1 is only required during embryonic differentiation, whereas UNC-120 and HLH-1 are also required during later stages to maintain muscle function. HLH-1 also controls its own expression at these later stages.

Specification of bodywall muscle fate starts early in embryogenesis. Initially, the Caudal-like transcription factor PAL-1 becomes restricted to the P2 blastomere by the action of the transcription factors SKN-1, POP-1/Tcf and the translational regulator MEX-3 (Baugh et al., 2005; Hunter and Kenyon, 1996). PAL-1 directly activates transcription of the HLH-1/MyoD transcription factor in the blastomeres that give rise to the majority of the bodywall muscles (Lei et al., 2009). MyoD type transcription factors regulate muscle fate across a wide range of metazoans (Krause, 1995). HLH-1 itself can trigger widespread differentiation towards a muscle fate when expressed inappropriately in blastomeres of the *C. elegans* embryo (Fukushige and Krause, 2005). Although the ectopically formed bodywall muscle cells in these embryos had numerous features of muscle cell differentiation, they did not contract. In fact, *hlh-1* null

mutants actually develop contractile bodywall muscle, although these contractions are weak and the animals die as early larvae with abnormal morphology (Chen et al., 1992; Chen et al., 1994). This suggested that there must be parallel pathways that act in the specification of muscle fate. Indeed, it was found that *hlh-1* acts in parallel with *unc-120* and *hnd-1* to regulate proper fate specification in the muscle (Fukushige et al., 2006). The *unc-120* gene encodes for the serum response factor (SRF) homologue, which is required for the production of muscle structural proteins in response to growth signals in mammals. Similar to *hlh-1*, this gene is continuously expressed in the bodywall muscle to regulate muscle homeostasis. In contrast, the *hnd-1* HAND-like bHLH transcription factor is only expressed transiently during the early stages of myoblast specification. Both *hnd-1* and *unc-120* have weak myogenic potential in early blastomeres compared to *hlh-1*. In addition, *unc-120* myogenic activity was dependent on *hlh-1* activity, whereas *hnd-1* was not. When all three factors were eliminated, no detectable muscle differentiation took place in the embryos. Altogether this reveals that these three genes fulfill partially redundant roles but all contribute to the proper specification of bodywall muscle fate (Figure 7C). *hlh-1* also cooperates with several other factors to control fate specification in the muscle cells derived from the M blast cell. Postembryonic loss of *hlh-1* in the M lineage leads to fate transformations in this lineage: *hlh-1* mutant animals lack postembryonic bodywall muscles and have too many sex myoblasts (Harfe et al., 1998). In addition, expression of *hlh-1* is highest in the bodywall muscle descendents of M. This leads to the model that *hlh-1* controls striated muscle cell fate in the M-descendants that become bodywall muscle cells (Harfe et al., 1998). It does so in concert with *fozi-1*, a novel nuclear protein that has an expression pattern overlapping with *hlh-1* in the M-lineage. Double inactivation of *fozi-1* and *hlh-1* all but eliminated post-embryonic bodywall muscle cells derived from M (Amin et al., 2007).

Since differentiation of the bodywall muscle has been extensively studied, it makes it an ideal model for the study of the inverse relationship between differentiation and proliferation. There are several additional reasons for studying this particularly in the bodywall muscle. Firstly, we know when the cells differentiate and which factors are important in this process. Bodywall muscle differentiation is completed before the end of embryogenesis and these bodywall muscle do not divide anymore during subsequent larval stages. Furthermore, the bodywall muscle is a relatively large tissue in *C. elegans* that is easily recognizable under the microscope. In addition, the bodywall muscle is a tissue that is susceptible to manipulation by RNAi, in contrast to neurons in *C. elegans*. The bodywall muscle is also not essential for postembryonic viability in *C. elegans* and there are only very few muscle mutants that result in embryonic or early larval arrest. Study of the bodywall muscle has yielded many early and late differentiation marker genes, which could be used for tissue-specific expression of constructs. On the whole, the *C. elegans* bodywall muscle provides a suitable system for the study of cell cycle re-entry in differentiated cells. This allows us to address some fundamental questions about cell proliferation in differentiated cells. What is necessary and sufficient to get a terminally differentiated cell to re-enter the cell cycle? Does cell cycle re-entry coincide with a loss of differentiation status? To what extent can these differentiated cells proliferate? And can we identify factors that prevent re-entry of differentiated cells?

Outline of this thesis

Over the past 30 years, our understanding of the cell cycle has grown exponentially. However, knowledge about how the cell cycle gets regulated in the context of a whole developing organism has not increased at the same rate. There is not enough known about which signaling factors trigger cell cycle entry or exit at defined moments in development. In addition, how different tissues vary in their response to cell cycle disruption is a critical question in development and cancer biology that needs to be answered. Studies in model organisms like *Drosophila* and *C. elegans* contribute important insights in cell cycle regulation during development.

I have used *C. elegans* to address several questions related to the developmental control of cell division. Chapter 2 elaborates on the role of DNA replication during *C. elegans* development and describes the cloning and characterization of the *lin-6* gene, the *C. elegans* MCM-4 homologue. We study the phenotypes of *lin-6* mutants both embryonically and postembryonically and address the apparent uncoupling between different developmental processes in this mutant. Chapter 3 describes work on cell cycle re-entry in the differentiated bodywall muscle. Muscle-specific expression of positive G1/S Cyclin/Cdk regulators is used to trigger cell cycle re-entry. Description of the phenotype and the use of different cell cycle markers in combination with microarray analysis provide us with valuable insights in the extent to which differentiated cells can re-enter the cell cycle. Chapter 4 goes into detail about the function of the G1/S regulators *cki-1* and *cye-1/cdk-2* in the seam cell development. We show that *cki-1* does not only influence the number and frequency of seam cell divisions but that it also influences the fate outcome of the seam cell divisions. Chapter 5 addresses a long-standing question in the cell cycle field: the presumptive kinase-independent role of CDK-4 in the sequestration of CKI-1/p27. The use of endogenous levels of wild-type and kinase-dead CDK-4 to rescue a *cdk-4* null mutant provided conclusive results about this issue in *C. elegans*. Chapter 6 will be a summarizing discussion about the results presented in this thesis and will give directions for future research.

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Chapter 2

C. elegans lin-6/MCM-4 is a general DNA replication component with essential tissue-specific functions

Jerome Korzelius [1], H. Robert Horvitz [2] and Sander van den Heuvel [1]

[1] Developmental Biology, Utrecht University,
Padualaan 8, 3584 CH Utrecht, The Netherlands.

[2] Howard Hughes Medical Institute, Department of Biology,
Massachusetts Institute of Technology,
Cambridge, Massachusetts, United States of America.

'When he goes back to his home, that's when it's
Back to the lab again, yo'

Eminem – 'Lose Yourself'

Abstract

DNA replication and its connection to M phase restraint are studied extensively at the level of single cells, but rarely in the context of a developing animal. The *C. elegans* abnormal cell lineage mutant *lin-6* lacks DNA synthesis in the post-embryonic somatic cell lineages, while entry into mitosis continues. These mutants grow slowly and either die during larval development or develop into sterile adults. We show that *lin-6* encodes the MCM-4 component of the MCM2-7 pre-RC and replicative helicase complex. The LIN-6/MCM-4 protein is expressed in all dividing cells during embryonic and postembryonic development and associates with chromatin in late anaphase. Induction of cell cycle entry and differentiation continues in developing *lin-6* larvae, even in cells that went through abortive division. In contrast to *lin-6(RNAi)* embryos and cells in the soma of *lin-6* mutants, the gonad continues DNA replication until late larval development. Expression of LIN-6 solely in the epidermis is sufficient to rescue the growth retardation and lethality of *lin-6* mutants. Thus, *lin-6/mcm-4* has conserved functions in DNA replication and replication checkpoint control, but also shows surprising tissue specific requirements. While the somatic gonad and germline show advanced ability to cope with lack of zygotic *lin-6/mcm-4* function, replication and checkpoint control in the epidermis are critical for growth and survival of the whole organism.

Introduction

A crucial part of the cell division cycle is DNA replication, which takes place during the Synthesis (S) phase of the cell cycle (Arias and Walter, 2007). DNA replication must be highly accurate and tightly controlled to maintain genomic integrity over many rounds of division and multiple generations. The developmental context adds additional constrains on S phase regulation. For instance, in meiosis, M phases follow each other without intervening S phase, while in endoreduplication cycles, rounds of DNA replication continue in the absence of M phases. Yet, during the division of most somatic cells, DNA duplication should happen once and only once, and M phase should not initiate until S phase is complete. Stalled replication forks and DNA damage activate a checkpoint that delays cell cycle progression. Activation of this replication/damage checkpoint involves the Chk1 kinase and forms part of normal *Drosophila* and *C. elegans* development. *Drosophila* Chk1 *(grapes)* is required for decelerating embryonic cell cycles at the midblastula transition (Fogarty et al., 1997; Sibon et al., 1997) while the *C. elegans* ortholog *chk-1* contributes to different cell cycle timing of early blastomers (Brauchle et al., 2003). Thus, DNA replication and replication checkpoint control have developmental functions that go beyond the duplication of individual cells.

Studies of single cell eukaryotes, *Xenopus* egg extracts and mammalian cells in culture have generated substantial insights in the process of DNA replication. To accomplish faithful duplication of the DNA in each cell cycle, the cell treats the 'licensing' of the DNA for replication and the actual start of DNA replication as separate events (Arias and Walter, 2007). In the licensing phase of the cell cycle, pre-replication complexes (pre-RC's) are assembled at the future origins of DNA replication. The sequential action of ORC1-6 proteins, Cdc6 and Cdt1 load the MCM2-7 DNA helicase onto the origins during late mitosis and early G1 (Bell and Dutta, 2002). The MCM2-7 complex is thought to act during S-phase as the helicase that unwinds the DNA at the replication origins (Aparicio et al., 1997; Labib et al., 2000; Pacek and Walter, 2004). CDK (Cyclin-dependent kinase) and DDK (Dbf-4 dependent Cdc7 kinase) control activation of the MCM2-7 helicase while at the same time preventing new recruitment of MCM2-7 complexes. This way, DNA synthesis is limited to a single round (Nguyen et al., 2001; Petersen et al., 1999; Piatti et al., 1996; Schwob and Nasmyth, 1993).

Our understanding of the control of DNA replication in an organismal context is less advanced. The nematode *C. elegans* provides an attractive model in which this question can be addressed. Work from various researchers has demonstrated that conserved modules with less-redundant parts regulate various cell cycle transitions and checkpoint controls in *C. elegans* (Kipreos, 2005; O'Neil and Rose, 2006; van den Heuvel, 2005). Hence, it is attractive to examine the control of DNA replication and activation of the DNA damage/replication checkpoint in a developmental context and tissue-specific manner in *C. elegans*. Previous studies have shown that inactivation of basic DNA polymerase subunits leads to disruption of the normal asymmetric division pattern of the early embryo and cause lineage specific delays in cell division (Brauchle et al., 2003; Encalada et al., 2000). The cell cycle delay involves activation of a conserved ATR/Chk1 replication checkpoint pathway (Brauchle et al., 2003). In addition, RNAi knockdown of *cul-4* Cullin4 was found to result in a dramatic DNA re-replication phenotype in *C. elegans* larvae (Kim and Kipreos, 2007; Zhong et al., 2003). Further analysis revealed that the CUL-4/DDB-1 E3 ubiquitin ligase prevents re-replication through regulation of two parallel aspects of replication licensing. CUL-4/DDB-1 directly targets the licensing factor CDT-1 for destruction by the proteasome (Kim and Kipreos, 2007; Zhong et al., 2003). In addition, CUL-4 also acts to promote

the nuclear export of CDC-6 through a CDK-phosphorylation-dependent pathway (Kim et al., 2007). A mammalian CUL-4/DDB-1 E3 Ubiquitin ligase complex was also found to promote Cdt1 degradation and prevent re-replication (Arias and Walter, 2006; Senga et al., 2006), illustrating the potential of *C. elegans* in identifying novel regulators of the cell cycle.

In this study, we describe the molecular and genetic characterization of the *C. elegans lin-6* gene. The *lin-6(e1466)* mutation was identified in the first systematic search for mutants with defects in the normally invariant post-embryonic cell lineages of *C. elegans* (*lin* mutants) (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981). We show that *lin-6* mutants maintain temporal expression of S phase and differentiation genes, yet fail to replicate DNA and lack the G2/M checkpoint that senses incomplete replication. Mapping and cloning revealed that *lin-6* encodes the *C. elegans* MCM4 ortholog MCM-4, a member of the six-subunit MCM2-7 pre-RC and replicative helicase complex. *lin-6* mutants show a high degree of larval lethality and growth retardation, which reflected a specific requirement for *lin-6* in the hypodermis. Our results support a conserved function of *lin-6/mcm-4* in DNA replication and replication licensing. Moreover, our results emphasize the intrinsic independence of developmental processes such as DNA replication, mitosis, differentiation, growth and molting. Consequently, checkpoint mechanisms that make later events dependent on completion of earlier events can have effects for the whole organism.

Materials and methods

Strains and culturing

Strains were cultured on NGM plates seeded with *E. coli* OP50 according to standard conditions. Feeding RNAi was performed on NGM plates supplied with 50 µm/ml Ampicillin and 2 mM IPTG. Animals were synchronized by hypochlorite treatment and hatching eggs in M9 medium with 0.05%Tween-20. L1 larvae were then transferred to NGM plates with OP50 and allowed to develop for the appropriate amount of time. Experiments were conducted at 20°C unless indicated otherwise. Strains used were: N2 Bristol wild-type, CB3475 *lin-6(e1466)/szT1[lon-2(e678)];+/szT1*, MT1442 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1*, JK2739 *lin-6(e1466) dpy-5(e61)/hT2[bli-4(e937) let-?(q782) qIs48]*, SV987 *cyd-1(he112) rol-1(e91)/mnC1; heIs30[Prnr-1::cyb-1DesBox::3XVenus]*, SV1035 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx349[Plin-6::lin-6::mCherry]*, SV1032 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1; heEx347[Prnr-1::cyb-1DesBox::3XVenus]*, SV1055 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx358[Pelt-2::lin-6::mCherry]*, SV1056 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx359[Pelt-2::lin-6::mCherry]*, SV1057 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx360[Pdpy-7::lin-6::mCherry]*, SV1058 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx361[Pdpy-7::lin-6::mCherry]*, SV1059 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx362[sur-5::GFP;myo-2::TdTomato].*

Molecular cloning of lin-6

Deficiencies *tDf3*, which includes *lin-6*, and *tDf4*, which does not include *lin-6*, were used to connect the *lin-6(e1466)* mutation to the physical map. YACs from the region were used in transgenic rescue experiments and Y74C10 was observed to rescue the *lin-6* phenotype. This ~330 kb YAC was labeled with α^{32}P-ATP and used as a probe to isolate cDNAs from a *C. elegans* library. 43 cDNAs were identified and assigned to at most 9 different genes. RNA interference of one of these genes caused cell cycle defects that closely resembled *lin-6* mutants. One of

the 8 independent cDNAs for this gene (clone 6.10) was used as a probe to isolate genomic clones from a *C. elegans* phage library. Two of the identified λ clones partly rescued the *lin-6(e1466)* allele, suggesting that the genomic DNA includes the *lin-6* gene and that the cDNAs were derived from *lin-6*. We obtained the nucleotide sequences from 4 independent cDNAs and determined the exon and intron sequences of the corresponding genomic DNA from WT and *e1466* animals (Figure 4). All cDNAs contain sequences from 7 exons, a 3' poly(A) tract and are predicted to encode a protein of 823 amino acids. This likely is the full length protein for three reasons: the first methionine is preceded by a stop, 3 codons upstream. Several cDNAs and ESTs start at about the same nucleotide and Northern analysis of wild-type mRNA revealed a single transcript consistent with the size of the cDNA.

Reporters and Transgenics

Plin-6::lin-6::mCherry::lin-6 3'UTR was generated by amplifying a 5.7 KB fragment, encoding *lin-6* and 2.4 KB of promoter sequence, from genomic N2 DNA using Phusion polymerase (Finnzymes) and cloning this fragment into the pGEMT vector (Promega). Subsequently, the mCherry coding sequence (a kind gift of R. Tsien) together with the *unc-54* 3' UTR was amplified by PCR with Phusion and was fused in frame with the *lin-6* coding sequence in the pGEMT vector. The *unc-54* 3'UTR was replaced with the 650 bp *lin-6* 3'UTR, which was amplified from genomic N2 DNA, to yield the *Plin-6::lin-6::mCherry::lin-6 3'UTR*. The *Plin-6::mCherry::lin-6 3'UTR* and *Plin-6::lin-6::mCherry::unc-54* 3'UTR constructs were injected into the MT1442 strain at a concentration of 30 ng/µl. *myo-2::GFP* and *lin-48::GFP* were used as co-injection markers, respectively. The transgenes rescued *lin-6(e1466)* larval development, resulting in adults that produced dead embryo's. Both constructs gave similar expression patterns in 4 independent transgenic lines

Pdpy-7:: LIN-6::mCherry and *Pelt-2:: LIN-6::mCherry* were created by replacing the *lin-6* promoter from the *Plin-6::mCherry::lin-6 3'UTR* construct by a 500 bp (*dpy-7*) or a 5 Kb (*elt-2*) promoter fragment. Constructs were co-injected with *sur-5::GFP* (50 ng/µl) and *myo-2::TdTomato* (10 ng/µl) into the CB3475 or MT1442 strain.

Prnr::CYB-1DesBox::3XVenus was created by cloning a tandem *C. elegans*-optimized Venus (a kind gift of T. Ishihara, Kyushu University, Kyushu, Japan), in frame with an N-terminal fragment of *C. elegans cyb-1*/Cyclin B1. This CYB-1DesBox fragment contains a putative destruction box for APC-dependent degradation. The codon usage was altered (optimized) to prevent co-suppression of the endogenous *cyb-1* gene. CYB-1DesBox was expressed as a translational fusion with tripleVenus, controlled by the *rnr-1* ribonucleotide reductase promoter in the pVT501 vector (a kind gift of V. Ambros). This construct was injected into MT1442 *lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1)* at 40ng/µl with *lin-48::TdTomato* as a co-injection marker. Transgenic lines were created by micro-injection as described (Mello et al., 1991). To examine reporter gene expression, animals were washed off the plates, anaesthetized with 10mM Sodium Azide and mounted on slides with a 2% agar pad. Pictures were taken with an Axioplan 2 microscope and Axiocam mRM camera (Zeiss Microscopy).

Immunostaining and antibodies

EdU labeling and staining was performed according to a protocol developed by S. Crittenden and J. Kimble using the Click-IT EdU Alexa Fluor 594 kit (Invitrogen). In short, MG1693 (Thymidine deficient) bacteria were grown in 100 ml of minimal medium containing 20 µM EdU. Worms were fed on NGM+ampicillin plates with these bacteria for the appropriate time. Worms were fixed by freeze-cracking on Poly-L-Lysine coated slides in liquid nitrogen and subsequent fixation in Methanol (5 min. at -20 C) and Acetone (20 minutes at -20 C). Slides were washed 1 X in PBS with 0.1 % Tween-20 and afterwards permeabilized by incubation with PBS + 0.1% Triton X-100. Slides were dried and the animals encircled with a PAP liquid blocker pen. The Click-IT reaction was subsequently performed on slides according to the manufacturer's instructions. Afterwards, slides were blocked using 10% donkey serum and 1% BSA. Monoclonal mouse anti-GFP (Sigma) was used at a 1:100 dilution. Donkey anti-mouse FITC (Jackson Immunoresearch Laboratories) was used as a secondary antibody. Slides were mounted in Prolong Anti-Fade Gold (invitrogen) supplied with 2 µg/ml DAPI. Propidium iodide and BrdU staining were performed as previously described (Boxem et al., 1999) . Immunostaining of *C. elegans* embryo's was performed as previously described (van der Voet et al., 2009). Primary antibodies used are anti-rabbit MCM-4 (Bleed/Rabbit 62-3) at 1:100 dilution and Anti-Nuclear Pore Complex mAB414 (Abcam) at 1:100 dilution.

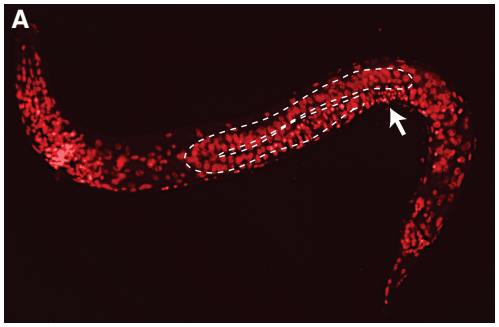


Table 1. Total progeny from four successful crosses between a single *lin-6(e1466)*/+ male and *lin-6(e1466)/dpy-5(e61)* or *tDf3/dpy-5(e61*) hermaphrodite. For both types of crosses, approximately one quarter of the progeny is Stu (Sterile, Thin and Uncoordinated). Such animals develop slowly, vary in size and either arrest at a late larval molt or live as Stu adults. Embryonic lethality (Emb) is similar to the wild-type. A: PI staining of a *lin-6(e1466)* mutant animal with one gonad marked(dotted line). Note the extensive development of the gonad and the presence of sperm in the spermatheca (A, arrow). The total number of propidium iodide stained cells in the somatic gonad and germline were counted in five adults of each genotype.

Genotype crosses (n=4)	Phenotype progeny			Cell count gonad
	Emb (n)	Stu (n)	WT (n)	
lin-6/+ X *lin-6*/+	0.9%(12)	25.8%(332)	73.3%(943)	122±19, sperm 72 ±54
lin-6/+ X *tDf3*	1.0%(14)	23.2%(313)	75.8%(1023)	131±18, sperm 83 ±57

Results

lin-6 mutants enter mitosis without DNA replication

Based on DNA staining and observations with Nomarski optics, *lin-6(e1466)* mutant animals were reported to initiate mitosis while DNA replication appeared absent outside of the germline (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981). As this phenotype is rare, we first confirmed and strengthened these observations. The *lin-6(e1466)* allele is fully recessive (Table 1). Trans-heterozygotes of *lin-6(e1466)* and the deficiency *tDf3* showed defects that were indistinguishable from those observed in animals homozygous for *lin-6(e1466)* (Table 1). Thus, *e1466* is probably a strong loss-of-function or null allele of *lin-6*.

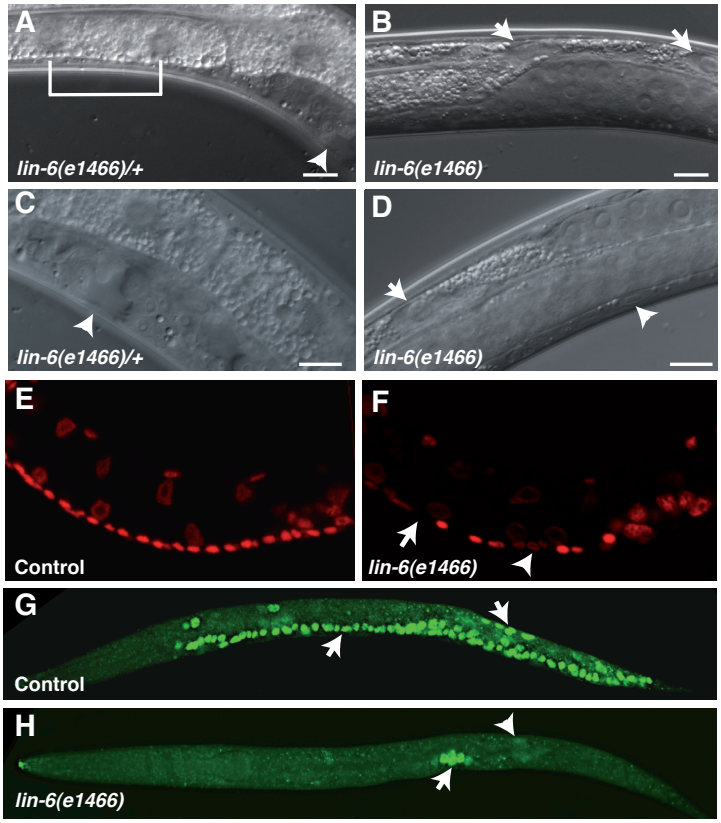


Figure 1. *lin-6* mutant animals are defective in DNA replication and cell division. A-D: DIC-images of *lin-6(e1466)* heterozygotes (A,C) and *lin-6(e1466)* homozygous mutant animals (B,D). *lin-6(e1466)* heterozygotes have normal cell divisions in the intestine and P-cell descendants that form the vulva (A,C, brackets, arrowheads). In contrast, *lin-6(e1466)* homozygous mutant animals lack post-embryonic cell division in these tissues (B,D, arrows and arrowheads). Note the significant size of the gonad in *lin-6(e1466)* homozygotes. E,F: Propidium Iodide (PI) staining of a *lin-6* mutant larva shows the empty ventral cord (F, arrow), while some P-cells have divided to form small fragmented nuclei (F, arrowhead). G,H. BrdU staining visualizes DNA replication in various postembryonic lineages in a wild-type animal, grown in the presence of BrdU from 6-14 hrs of larval development. Labeled cells include the daughter cells of the ventral cord precursors (G, left arrow) and the mesoblast (G, right arrow). The *lin-6* mutant lacks post-embryonic DNA replication, except in the gonad (H, arrow). Limited DNA replication is sometimes detected in mesoblast daughters (H, arrowhead), as well as the V_5 seam cells and Q neuroblasts (not visible).

In the presence of maternal wild-type product, *lin-6(e1466)* homozygous animals complete embryogenesis and hatch as normal first stage larva (L1). Starting in the first larval stage, cell divisions fail (see below) yet the animals continue to grow at a rate that is reduced and more variable than wild-type animals (Figure 1A-D, Figure 8). The divisions of all post-embryonic blast cells outside the gonad are defective and form nuclei that are abnormally small (Figure 1F, arrowhead). Some of these nuclei remain in close contact, probably as a result of incomplete mitosis and cytokinesis, while others fully separate (Figure 1A-F, Figure 8C,D). Germline development is severely delayed in most mutants, but somatic gonad and germline divisions often continue until late development. As a result, *lin-6* animals that reach the adult stage generally have extensive gonads, with reflexed arms and sperm cells, but without oocytes (Figure 1A-D, Table 1).

We used incorporation and immunohistochemical detection of the thymidine analog BrdU as a sensitive assay for DNA synthesis. Wild-type animals grown in the presence of BrdU from 0-14 hrs of larval development incorporated BrdU in the post-embryonic blast cell lineages (Figure 1G). In *lin-6* mutants, only cells in the somatic gonad and germline continued DNA replication (Figure 1H). Occasionally, limited DNA synthesis could be detected in the blast cells that initiate post-embryonic division soon after hatching. This includes the neuroblasts QR and QL, the mesoblast M and the hypodermal nuclei V5R and V5L (Figure 1H, arrowhead and data not shown). We did not detect any BrdU incorporation in the precursor cells of the ventral nerve cord (P cells) (Compare figure 1G-H). We used DIC microscopy to follow the development of the synchronized first larval stage (L1) progeny from heterozygous parents. Homozygous *lin-6* mutant larvae initiated post-embryonic blast cell divisions at the same time as their heterozygous and wild-type siblings (data not shown). The 12 P precursor cells produced on average 27 $\pm$3.1SD daughters, confirming that some even went through a second round of division in the absence of DNA replication. Together, these observations confirm that post-embryonic blast cells omit DNA replication but initiate mitosis without delay in *lin-6* mutant animals.

lin-6 is required for the checkpoint that couples M phase entry to S phase completion

Replication defects are expected to trigger a checkpoint that delays mitotic entry (Hook et al., 2007). Thus, it is remarkable that somatic cells in *lin-6* mutants enter mitosis at the normal time in the apparent absence of DNA replication. We considered two alternative explanations for this aspect of the Lin-6 phenotype: *lin-6* is required for DNA synthesis and also to activate the checkpoint that monitors completion of DNA replication, or alternatively, incomplete S phase cannot be sensed by a checkpoint in *C. elegans* larvae. To discriminate between these possibilities, we added the DNA replication inhibitor hydroxyurea (HU) to synchronously growing cultures of L1 animals. Subsequently, we fixed and stained animals for the mitosis specific phospho-histone H3S10 epitope at various times of L1 development. Treatment of wild-type animals with HU delayed onset of mitosis for prolonged times (Figure 2C, compare with A), demonstrating that initiation of mitosis is indeed dependent on the completion of DNA synthesis in *C. elegans.* Initiation of mitosis was not delayed in *lin-6* mutants treated with HU, indicating that mitotic entry is not coupled to DNA synthesis in these mutants (Figure 2B, D). Thus, *lin-6* is required for DNA replication as well as for the checkpoint that restrains mitosis until completion of S phase.

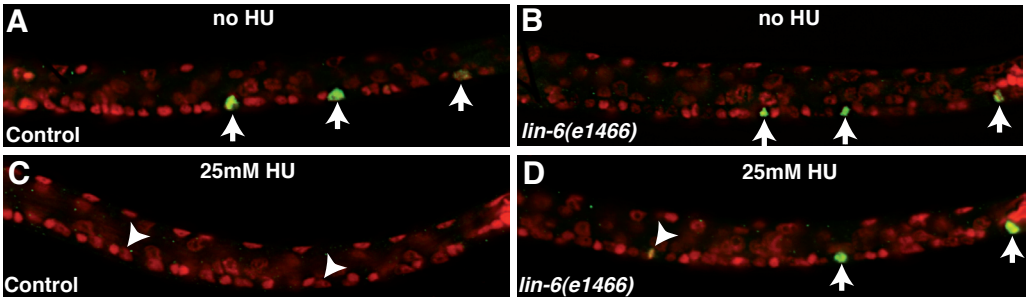


Figure 2. Absence of a replication checkpoint response in *lin-6* mutants. Several P-cells in the ventral cord stain positive for the mitosis-specific phospho-H3S10 antibody in both wild-type (A, arrows) and *lin-6* animals (B, arrows) at 10 hrs of L1 development. In the wild-type animal treated with the DNA-replication inhibitor hydroxyurea (HU), P-cells migrated into the ventral cord, arrested before mitosis and remained phospho-H3S10 negative (C, arrowheads). In the HU-treated *lin-6* animal, P cells failed to arrest before mitosis and show phospho-H3S10 positive staining (D, arrows) and a metaphase plate (D, arrowhead).

lin-6 is not required for G1 progression and differentiation

Similar to *lin-6, cdk-4* Cdk4/6 and *cyd-1* Cyclin D are also required for DNA synthesis during larval development (Boxem and van den Heuvel, 2001; Park and Krause, 1999). Post-embryonic blast cells in *cdk-4* and *cyd-1* mutants arrest in G1 and do not activate the S phase transcriptional reporter *rnr-1::GFP*. We observed *rnr-1::GFP* expression in *lin-6* larvae, but also quite frequently in starvation arrested L1 larvae of the *rnr-1::GFP* control strain (Data not shown). The latter observation indicates that fluorescence may result from ectopic expression of the *rnr-1::GFP* transgene or GFP lingering from previous divisions. To create a more reliable marker for the G1/S transition, we included a CYB-1/Cyclin B1 N-terminal fragment (CYB-1DesBox), which directs APC-dependent protein degradation in mitosis (King et al., 1995). We placed the CYB-1DesBox fragment in tandem with triple Venus (3XVenus) and expressed the translational fusion protein from the G1/S activated *rnr-1* promoter (Materials and Methods). This *Prnr-1::CYB-1DesBox3XVenus* reporter is highly specific for cells in division, as fluorescence appeared during S phase, accumulated in the presence of HU and disappeared at the time of M phase completion or immediately thereafter. Again, wild-type and *lin-6(e1466)* L1 larvae expressed this S phase reporter with similar temporal and spatial control, while the reporter was not induced in *cyd-1(he112)* mutant larvae (Figure 3). We conclude that in *lin-6* mutants cells progress through the G1/S transition, yet fail to replicate their DNA.

Interestingly, *CYB-1DesBox3XVenus* expression disappeared more slowly in *lin-6* mutants as compared to the wild type (Figure S1). This likely indicates that postembryonic blast cells in *lin-6* mutants enter mitosis at the appropriate time but delay exit from mitosis. *Prnr-1::CYB-1DesBox3XVenus* was also detected at later times of development, in particular in the seam and Pn.p cells (Figure S1, data not shown). This expression cannot be explained by perdurance of the fluorochrome and indicate that cells continue their program of cell cycle entry, even when previous divisions failed.

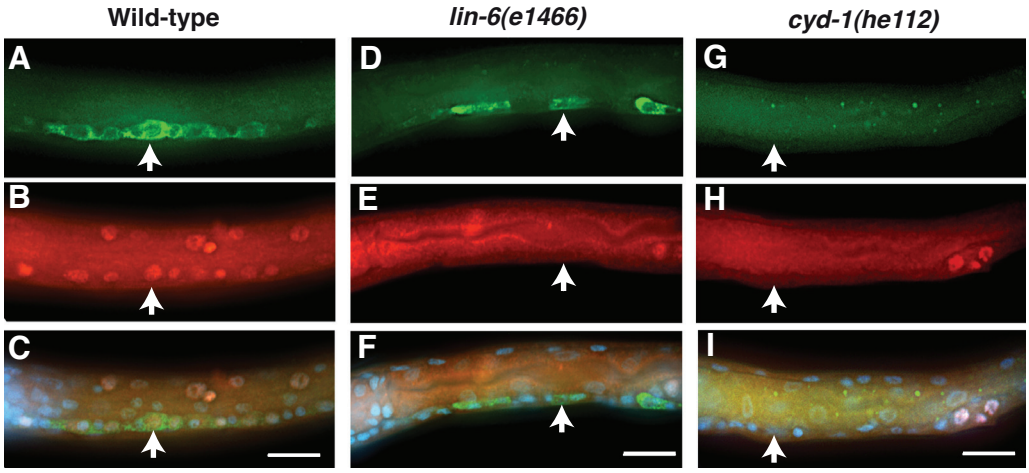


Figure 3. *lin-6* mutant animals express an S-phase entry reporter. Wild-type (left), *lin-6* (middle) and *cyd-1* (right) mid-L1 larvae grown in the presence of EdU, carrying the *Prnr::CYB-1Desbox::3XVenus* marker (see Materials and Methods). A,D,G: Venus expression visualized by antibody detection. B,E,H: DNA replication visualized by EdU staining. C,F,I: Merged images of anti-GFP and EdU staining and DNA staining with DAPI. P cells in the wild-type animal express the marker for S-phase entry and incorporated the thymidine analogue EdU, as indicated by the arrow (A-C). In *lin-6* animals, expression of the S-phase entry marker continues, but DNA replication is absent in these animals except for the gonad (D-F). *cyd-1(he112)* null mutant larvae do not express the S-phase reporter and lack DNA replication outside the gonad (G-I)

At the same time, induction of differentiation also continues in the absence of DNA replication and normal mitosis. Previous work on the characterization of the *lin-6* mutant phenotype demonstrated that *lin-6* mutants do not have a functional postdeirid as assayed by dye filling of this neuron (Sulston and Horvitz, 1981). We introduced the *Pdat-1::GFP* transgene, a reporter for postdeirid fate determination, in the *lin-6(e1466)* background (Nass et al., 2002). Expression of this transgene was seen in 20/20 *lin-6* animals, assayed at the L4 larval stage (Figure S2). Interestingly, although the morphology of the cells at the position of the postdeirid is severely disrupted in *lin-6* mutants compared to wild-type (Figure S1 arrow), the *Pdat-1::GFP*-positive neurons in *lin-6* animals sometimes form axonal projections that look similar to the projections seen in wild-type *Pdat-1::GFP*-positive neurons. Together these data suggest that induction of cell cycle entry and differentiation continues in developing *lin-6* larvae even in cells that went through aberrant division.

lin-6 encodes an MCM4 DNA replication pre-initiation subunit

We identified the *lin-6* gene through a combination of mapping with linked mutations and deficiencies, followed by rescue with a yeast artificial chromosome (YAC) (Materials and Methods, Figure 4A). Using this YAC as a probe, we identified 43 cDNAs and assigned these to 9 individual genes. Based on several criteria, one of these genes is *lin-6.* First, injection of dsRNA from the candidate *lin-6* cDNA caused cell cycle defects that phenocopied *lin-6* loss of function. A few early progeny closely resembled *lin-6(e1466)* mutant larvae, while all subsequent progeny died during embryogenesis. These larvae and embryo's contained sub-diploid cells and apparently went through mitoses in the absence of DNA replication. In addition, genomic DNA corresponding to this cDNA substantially rescued the *lin-6(e1466)* phenotype. Such animals generally showed normal larval development but did not produce viable progeny,

probably because of transgene silencing in the germline (see below). Furthermore, the *e1466* allele contains a nonsense mutation in the predicted *lin-6* open reading frame. We obtained the DNA sequences of candidate *lin-6* cDNAs and genomic clones. All were found to encode a protein of 823 amino acids (Figure 4B). Analysis of the corresponding DNA sequences in *lin-6(e1466)* revealed a G:C to A:T transition, which is predicted to change the Gln88 codon to a stop. Termination of translation this early is likely to result in strong or complete inactivation of protein function, in agreement with the genetic data for *lin-6(e1466)* and further supporting that we identified the *lin-6* gene. Finally, the predicted function of the LIN-6 protein matches the loss-of-function phenotype. Conceptual translation of the open reading frame and database searches revealed that *lin-6* encodes an MCM family member, sharing 54% amino-acid identity with human and *Xenopus laevis* MCM4 (Figure S3). While first discovered in yeast, the MCM genes are essential for DNA replication in all eukaryotes studied (Bell and Dutta, 2002). Six different genes of this family, MCM2 to 7, are present in *S. cerevisiae*, and a single member of each subfamily appears to be conserved in other eukaryotes. The 6 MCM proteins form a hexameric complex, which licenses origins for DNA replication as a critical component of the DNA replication pre-initiation complex and acts as the replicative helicase during DNA replication.

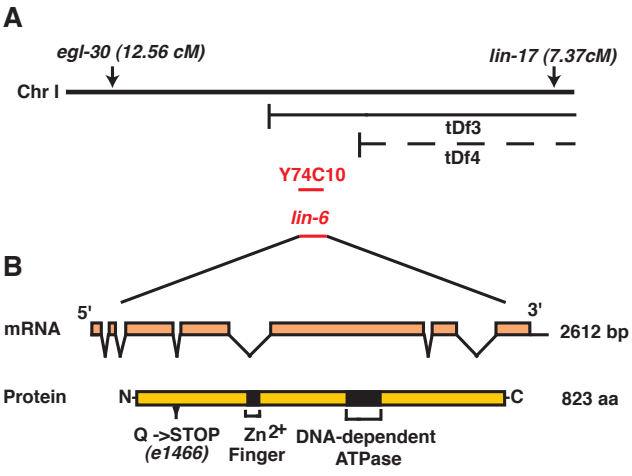


Figure 4. Mapping and cloning identifies *lin-6* as the *C. elegans* MCM4 ortholog. A: *lin-6* was previously mapped between *egl-30* and *lin-17* on the left arm of chromosome I. A combination of mapping with linked mutations and deficiencies as well as germline transformation rescue with yeast artificial chromosomes (YAC) was used to identify the *lin-6* gene (see Materials and Methods for a detailed description). Y74C10 rescued the *lin-6* mutant phenotype and was used as a probe to isolate *lin-6* cDNA. B: DNA sequence analysis of cDNA and genomic clones revealed the structure the *lin-6* gene and mRNA. Conceptual translation predicts a LIN-6 protein of 823 amino acids, which is closely related to members of the MCM4 subfamily in other eukaryotes (Figure S3).

The replication pre-initiation complex is needed to restrain M phase

Analysis of the embryonic RNAi phenotype further confirmed the double function for *lin-6* in DNA synthesis and activation of a replication checkpoint that inhibits progression into mitosis. Previous studies have shown that replication defects delay progression through the cell cycle of early blastomeres (Encalada et al., 2000), through activation of an *atl-1* ATR-dependent checkpoint pathway (Brauchle et al., 2003). In agreement with these studies, inhibition of DNA replication by exposing adults to the ribonucleotide reductase inhibitor HU (100 mM) or *rnr-1* RNAi resulted in delayed mitotic entry of up to 12 minutes in the one-cell embryo. Following the delay, spindle duplication continued without chromosome segregation, and the DNA remained

present in the center of the embryo as a single or two separate masses (derived from the paternal and maternal pronucleus) in 49 out of 50 embryos (Figure 5C, arrows). Following *lin-6* RNAi, the DNA content of the pronuclei was clearly reduced (Figure 5B, compare DNA staining in polar bodies (arrowheads)) yet cell division continued without delay, and the DNA became fragmented (50/50 embryos, none in N2 control). Ultimately, these embryos arrested with up to 30 nuclei and very little DNA in each nucleus (Figure 7) RNAi of another MCM subunit, *mcm-5*, resulted in a phenotype similar to *lin-6* (48/50 embryos). Genome fragmentation has also been reported for *cdt-1(RNAi)* and *cdc-6(RNAi)* embryos (Kim et al., 2007; Zhong et al., 2003). In contrast, inhibition of *div-1*, which encodes the DNA Polymerase α primase B-subunit, resembled *rnr-1* inactivation and triggered mitotic delay (Encalada et al., 2000). We conclude that the MCM replicative helicase, which forms part of the DNA replication pre-initiation complex, is required for DNA synthesis and to delay cell cycle progression when replication is incomplete.

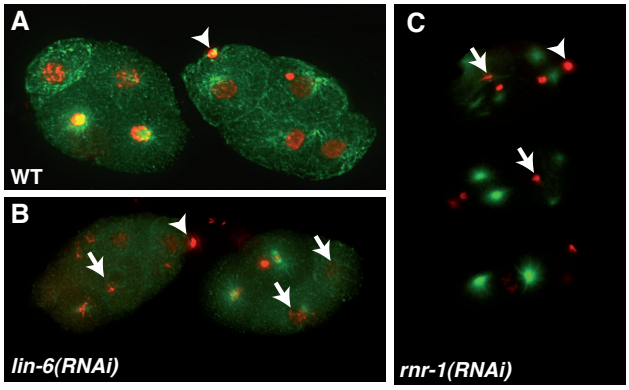


Figure 5. *lin-6/MCM-4(RNAi)* embryos continue DNA segregation in mitosis in the absence of DNA replication. A-C: alpha-tubulin(green) and PI (red) co-staining of wild-type, *lin-6/mcm-4(RNAi)* and *rnr-1(RNAi)* embryo's. Arrowheads indicate polar bodies. A. Wild-type 4-cell stage embryos show nuclei with normal DNA content. B: *lin-6/mcm-4(RNAi)* embryos continue mitotic divisions and DNA segregation without DNA synthesis, resulting in cells with a sub-diploid DNA content (B, arrows, compare with arrowheads: polar bodies). C: Inhibition of DNA replication by RNAi-mediated knockdown of ribonucleotide reductase *rnr-1*, delays mitosis and prevents chromosome segregation. Hence, the zygotic DNA remains present in the center of the embryo, independent of the spindle and cell division (C, arrows).

LIN-6/MCM-4 shows cell cycle dependent expression and localization

How cells commit to and withdraw from the division cycle are important developmental questions. As a subunit of the DNA replication pre-initiation complex, LIN-6/MCM-4 should be present at the time of S phase onset. Moreover, replication licensing is separated in time from activation of origin firing at G1/S, and as such LIN-6/MCM-4 might be expressed well before S phase or even remain present at the end of mitosis. To be able to examine the temporal and spatial expression of LIN-6, we created a *Plin-6::LIN-6::mCherry* reporter construct and generated antibodies that recognize LIN-6.

The reporter transgene contains 2.4 kb of genomic DNA upstream of the predicted ATG translation initiation codon, the predicted *lin-6* exon and intron sequences, and 650 bp downstream of the stop codon, including the predicted poly(A) signal. Coding sequences for mCherry were inserted just before the termination of the *lin-6* open reading frame (Materials and Methods). Expression of this combined promoter and C-terminal translational fusion construct rescued the *lin-6(e1466)* mutation. Specifically, *lin-6(e1466)* animals with the P*lin-*

6::LIN-6::mCherry transgene appeared healthy and viable and showed normal cell division in the ventral cord, intestine and seam, formed a normal vulva and occasionally produced a few embryos (Data not shown). As LIN-6::mCherry functionally substitutes for LIN-6/MCM-4, its expression and localization likely resembles the endogenous protein.

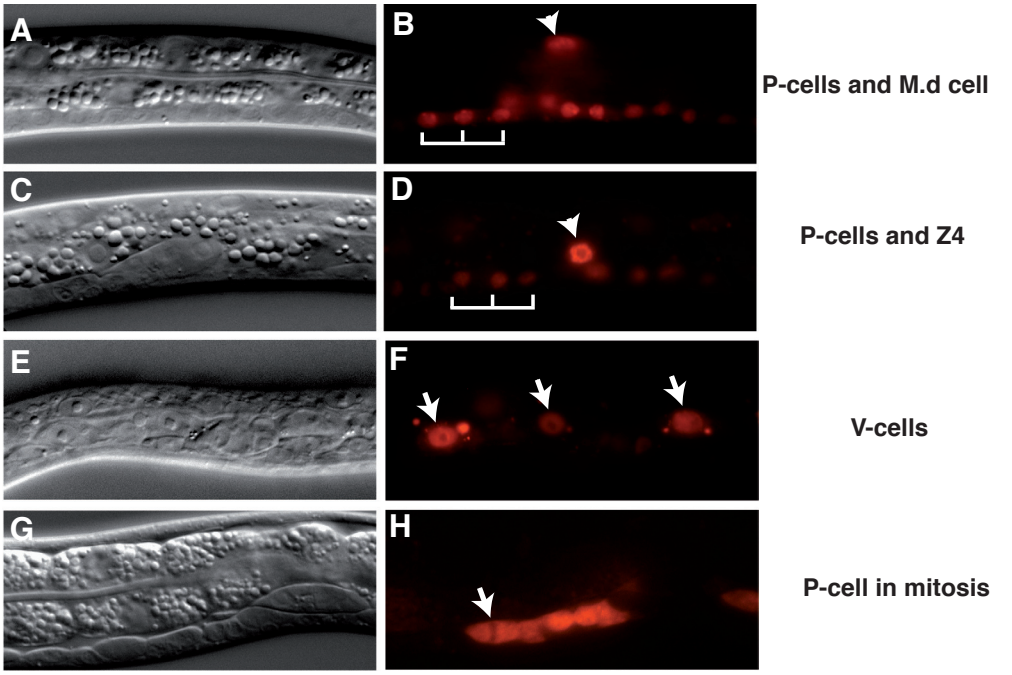
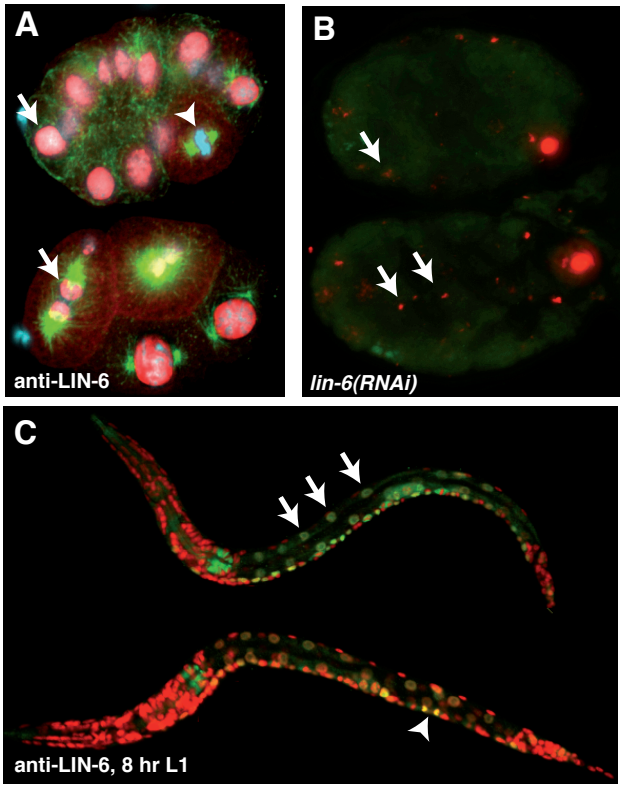
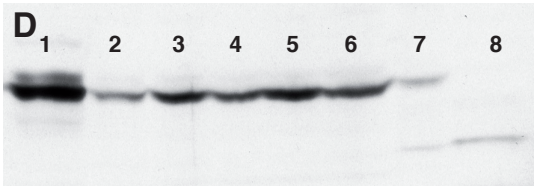


Figure 6. Expression of a LIN-6::mCherry transcriptional/translational reporter marks cells in the division cycle. DIC (A,C,E,G) and fluorescence microscopy images (B,D,F,H) of L1 animals carrying a *Plin-6::LIN-6::mCherry* expression construct (see Materials and Methods). This transgene, which rescues the *lin-6(e1466)* mutant, is highly expressed in the nuclei of dividing post-embryonic blast cells, including P-cells (A-D, brackets, G,H), M-daughter cells (A,B, arrowhead), the somatic gonad precursors (C,D, arrowhead) and V-cells (E,F, arrows). LIN-6::mCherry is nuclear in interphase and absent from the DNA in metaphase (G,H, arrow), resembling LIN-6 detection with antibodies (Figure 7).

We did not detect LIN-6::mCherry in starvation arrested L1 animals. However, expression was specifically induced in all post-embryonic blast cell lineages well before mitotic entry, at the expected time of S phase induction (Figure 6A-H). The fusion protein localized to the cell nucleus, yet upon degradation of the nuclear envelope was not chromatin-associated in mitotic prophase and metaphase (Figure 6H, arrow). LIN-6::mCherry did not disappear upon completion of mitosis and even cells that permanently withdrew from cell division, such as the motor neurons of the ventral nerve cord, initially retained LIN-6::mCherry expression. However, this expression subsequently disappeared in differentiated cells as well as in cells that temporally arrested cell division, such as the Pn.p vulval precursor cells in the ventral cord. These experiments indicate that *lin-6* is transcriptionally activated at approximately the time of G1/S transition, and that LIN-6 protein is segregated to both daughter cells during each mitosis.





1 Embryo
2 Starved L1
3 L2
4 L3
5-L4
6 Adult
7 Dauer
8 *lin-6(e1466)*

Figure 7. LIN-6/MCM-4 is dynamically localized at different stages of the cell cycle and expressed at different levels during development. A: Early *C. elegans* embryos stained for alpha-tubulin (green), LIN-6/MCM-4 (red) and DNA (DAPI, blue). LIN-6/MCM-4 is present in the nucleus in interphase (A, top arrow), does not co-localize with DNA in metaphase (A, arrowhead), but associates with chromatin in late anaphase (A, bottom arrow). *lin-6(RNAi)*-treated embryo's (B, arrows mark fragmented DNA). C: L1-stage larvae at 8 hours of L1 development immunostained with LIN-6/MCM-4 antibodies and DNA-stained with Propidium Iodide. LIN-6/MCM-4 is visible in the intestinal nuclei (arrows), nucleus and cytoplasm of the gonad precursors, and P-cells in the ventral cord (arrowhead indicates a P-cell in late anaphase). LIN-6/MCM-4 does not overlap with DNA in metaphase, but does co-localize with DNA in late anaphase (C, arrowhead) D: LIN-6/MCM-4 protein levels at different developmental stages. Levels are highest in the highly proliferative embryo and lowest in the developmentally arrested starved L1 and Dauer larvae.

Detection of endogenous LIN-6/MCM-4 confirmed these observations. We generated rabbit polyclonal antibodies against an extended C-terminal LIN-6 fragment, as well as mouse polyclonal antibodies recognizing an N-terminal domain. Antisera directed against either fragment recognized proteins of ±105 kD apparent MW in total worm lysates (Figure 7 and data not shown). This is most likely the LIN-6 protein, based on the MW and lack of detection in *lin-6* mutants. We found LIN-6 expressed during all stages of development in wild-type animals, at levels that roughly correlate with cell proliferation. Embryo's showed the highest levels of LIN-6 expression, in agreement with the fact that more than half of the somatic cells are formed during embryogenesis (Figure 7D, lane 1). Interestingly, LIN-6 was reduced but clearly detectable in developmentally arrested L1 animals that were hatched in the absence of food

(Figure 7D, lane 2). Even dauer larvae, which arrested cell division for two weeks, still contained detectable LIN-6 protein levels (Figure 7D, lane 7). These results suggest that some LIN-6/MCM-4 is retained during prolonged periods of quiescence, which may be needed for initiation of DNA synthesis when conditions improve.

Upon immunostaining of wild-type animals, the anti-LIN-6 antibodies showed strong nuclear staining in the gonad, embryos and postembryonic lineages (Figure 7A,C). This staining was LIN-6-specific, as RNAi of *lin-6* eliminated the nuclear signal in the germline and embryo's, and *lin-6(e1466)* larvae did not shown staining (Figure 7B). LIN-6 was detectable in sperm and accumulated during oocyte maturation in the nucleus, but did not show overlap with the condensed chromosomes in diakinesis of meiotic prophase. (Figure S3 and data not shown). LIN-6 was not chromatin-associated during Meiosis I of the fertilized oocyte, and the first polar body did not receive LIN-6 (Figure 7A). This is consistent with absence of S phase in between Meiosis I and -II. The second polar body and maternal pronucleus received LIN-6. Subsequently, embryonic cells in interphase showed strong nuclear staining (Figure 7A, Figure S4 left panel). In prophase, LIN-6 localization did not overlap with the condensing chromosomes (Figure 7A Figure S4). Upon nuclear envelope degradation, LIN-6 became diffusely localized throughout the cell and clearly did not co-localize with the metaphase-aligned chromosomes (Figure S4, arrowheads). LIN-6 remained cytoplasmic at the onset of anaphase, however, chromatin association became apparent in late anaphase (Figure 7A, Figure S4A,C,E, compare arrows with arrowheads). These data show that chromosome association of LIN-6/MCM-4 is tightly controlled, consistent with origin licensing taking place at the end of mitosis and disappearing during S phase.

Similar observations were made during larval divisions. Matching the LIN-6::mCherry reporter, endogenous LIN-6/MCM-4 expression was detectable prior to and during mitosis (Figure 7C). Staining of synchronized L1 animals revealed the timing of LIN-6 expression, which in general preceded mitosis by 1-to-2 hours. After 5 hours of L1 development at 20°C, staining was predominantly detected in the epithelial seam cells, Q neuroblast daughters and gonad primordium. The somatic gonad precursor cells Z1 and Z4 showed nuclear staining, while the mitotically arrested germline precursor cells Z2 and Z3 showed diffuse cytoplasmic staining. At six hours of L1 development, the mesoblast (M) also stained strongly as well as the most anterior ventral cord precursors cells (W and P1/2). Subsequently at 7 hours, additional P cells showed nuclear LIN-6/MCM-4 expression, which became apparent prior to migration of the nucleus into the ventral nerve cord (data not shown). At eight hours of L1 development, the intestinal nuclei showed LIN-6/MCM-4 expression (Figure 7C, arrows), which preceded nuclear division by at least 4 hours. At subsequent time points, daughter cells that continued division, such as the Pn.a and M descendants retained strong nuclear staining. L2 animals stained at 16 hours of larval development showed strong LIN-6/MCM-4 expression in the gonad, the H1.a, H2.p, V1-6.p and Tap seam cells and, weakly, the intestinal nuclei (Data not shown).

Importantly, LIN-6/MCM-4 staining did not overlap with DNA in prophase and metaphase, while in late anaphase co-localization with the chromosomes was clearly detectable (Figure S4, 7C arrowheads). We did not observe any asymmetry in LIN-6 segregation, hence cells received LIN-6 in mitosis whether or not they undergo an additional S phase. Also, the LIN-6/MCM-4 signal became undetectable during quiescence, even in the Pn.p daughter cells in L2 that resume DNA replication in the L3 stage. However, based on detection of LIN-6 in the Western blot experiments described above, we expect that low amounts of LIN-6/MCM-4

remain chromatin-associated in Go/G1 arrested cells. However, cells coming out of quiescence probably also use MCM-4 that has been newly synthesized in late G1 or early S for origin licensing and DNA replication (see Discussion).

Rescue of lin-6 in the hypodermis reveals an important role for LIN-6 in organismal growth and viability

How cell division is coordinated with organismal growth is another important question in developmental biology. *lin-6* mutants grow slowly and remain smaller and slimmer than wild-type animals (Figure 1A-D, Figure 8A,B). In addition, *lin-6* mutants quite frequently arrested at the larval molts and subsequently died. In contrast, mutations in *cyd-1* or *cdk-4* cause slow growth without associated lethality. As *cyd-1* and *lin-6* mutants both fail to replicate DNA in postembryonic somatic cells, we assumed that DNA replication is needed for normal growth, while the lack of an S phase checkpoint in *lin-6* mutants probably underlies the lethality. To examine in which cell type *lin-6* is required to promote growth and viability, we expressed *lin-6* specifically in the hypodermis or intestine. These two tissues go through endoreduplication cycles during larval development, which have been implicated in growth (Hedgecock and White, 1985); (for review see (Edgar and Orr-Weaver, 2001).

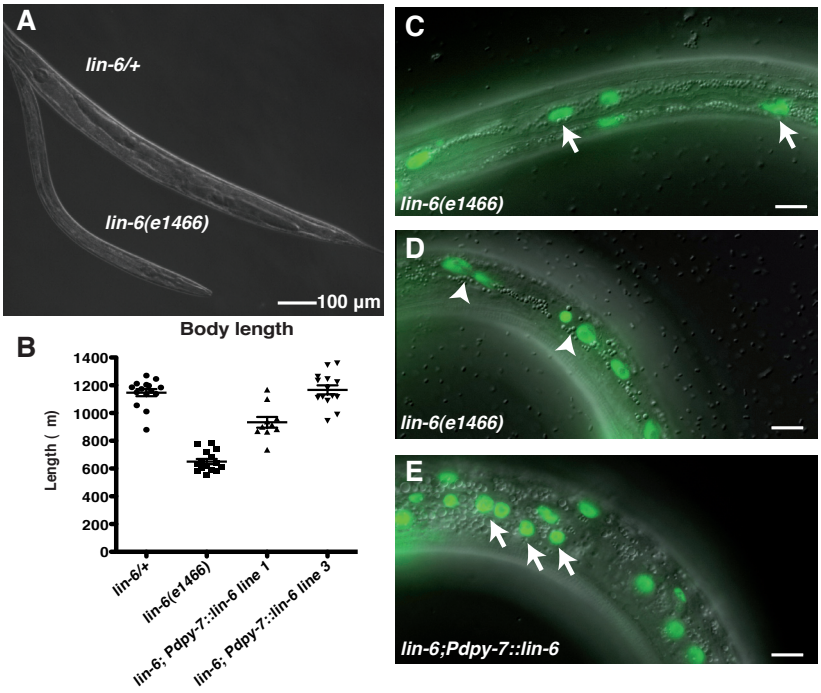


Figure 8. Epidermal expression of LIN-6 rescues the growth defect of *lin-6(e1466)* mutants. A: Image of a heterozygous *lin-6(e1466)/+* and similarly staged homozygous *lin-6(e1466)* animal. B. Quantification of the growth defect of *lin-6* animals in different genetic backgrounds. C-E. Combined DIC and fluorescence images of *lin-6* mutant animals and *lin-6* animals carrying the *Pdpy-7::LIN-6::mCherry* rescue construct, which is specifically expressed in the epidermis, together with a *sur-5::GFP* marker, which marks all nuclei. *lin-6* mutant animals have very few cells in their epidermis (C, arrows) and many cells seem to have undergone aberrant mitosis (D, arrowheads). Mutants carrying the *Pdpy-7::LIN-6::mCherry* rescue construct have additional epidermal nuclei (E, arrows) as well as an increased body size compared to *lin-6* mutant animals without the rescue construct. (E, compare with C,D, quantification in B).

Expression of LIN-6::mCherry under the control of the intestine specific *elt-2* promoter (Hawkins and McGhee, 1995) restored both nuclear divisions and endoreduplication cycles in the intestine of *lin-6* animals (Figure S5). As expected, this confirms that *lin-6* acts cell-autonomously in DNA synthesis and endoreduplication cycles. However, expression of LIN-6::mCherry in the intestine did not rescue the size or lethality of the *lin-6* mutant. In contrast, expression of LIN-6::mCherry from the hypodermal specific *dpy-7* promoter did recue the growh and viability of *lin-6(e1466)* mutant larvae. Control *lin-6* transgenic animals expressing the *sur-5::GFP* marker without the *Pdpy-7::LIN-6::mCherry* rescue construct contained few hypodermal nuclei (Figure 8C, arrows). Often, such nuclei were often small and fragmented, probably because of aberrant mitosis without DNA replication (Figure 8D arrowheads, compare with figure 1F). In transgenic lines carrying the *Pdpy-7:: LIN-6::mCherry* transgene, we noticed a population of sterile transgenic animals with a vulval protrusion but which looked phenotypically normal otherwise. Closer examination of these animals revealed that these animals had no post-embryonic divisions, similar to the *lin-6* mutant (Data not shown). Moreover, this population of transgenic animals together with the non-transgenic animals that displayed a LIN-6 phenotype formed 25% (17/59) of the progeny from heterozygous *lin-6(e1466)* parents with an extrachromosomal transgenic array. We concluded that these transgenic animals were in fact *lin-6* mutant animals. Expression of the *Pdpy-7:: LIN-6::mCherry* transgene efficiently rescued both the lack of hypodermal nuclei as well as the small body size and width of the *lin-6* mutant (Figure 8B,E and data not shown). These data indicate that *lin-6* is strictly required in the hypodermis for normal growth and viability of the whole animal. This illustrates that that even general cell cycle components may have surprisingly tissue specific developmental functions.

Discussion

Studies in model organisms such as *C. elegans* allow for the functional analysis of cell division control in a developmental context. We started such an approach with the characterization and cloning of *C. elegans lin-6*. The *lin-6(e1466)* mutant phenotype combines absence of DNA replication with continued mitosis, substantial development of the gonad and germline, variable growth and partly penetrant larval lethality. Our finding that *lin-6* encodes the MCM4 subunit of the putative MCM2-7 replicative helicase explains several aspects of the phenotype. However, other aspects remain surprising, in particular the continued DNA replication in the germline and the growth defects and lethality that are fully rescued by expression in one tissue.

lin-6/mcm-4 plays a key role in DNA replication and the replication checkpoint, but mutants continue gonad development

Absence of DNA synthesis in *lin-6* mutants is in line with the critical function of MCM-4 in DNA replication licensing and origin unwinding (Aparicio et al., 1997; Ying and Gautier, 2005; You et al., 1999). However, DNA replication and cell division continue in the somatic gonad and germline during much of larval development, even allowing the formation of sperm. We expect that perdurance of maternal product in combination with flexibility in the number of active replication origins permits continued division cycles in the *lin-6* gonad. The precursors of the somatic gonad and germline (Z1/Z4 and Z2/Z3, respectively) are formed and set aside in early embryogenesis (Sulston et al., 1983). Hence, Z1-Z4 may use residual maternal product to continue replication in larval development. Similarly, *cyd-1* and *cdk-4* mutants show fully penetrant arrest of post-embryonic somatic blast cell divisions, while some divisions of the gonadal precursors continue (Boxem and van den Heuvel, 2001; Park and Krause, 1999).

However, proliferation in the gonad of *lin-6* mutants is far more extensive. At the same time, gonad development is severely retarded compared to wild-type. We expect that this reflects a gradual decrease in the number of active replication origins. In *Xenopus*, it has been estimated that 10-20 times more MCM2-7 molecules are loaded onto DNA in G1 than the number of origins that are used for DNA replication (see (Edwards et al., 2002; Takahashi et al., 2005). This excess of MCM proteins is expected to license origins that are normally dormant, but allow full DNA synthesis during replicative or genotoxic stress (Ibarra et al., 2008; Woodward et al., 2006). This process is likely to play a role in *C. elegans* replication control as well. Partial inactivation of *C. elegans* MCM proteins by RNAi made animals hypersensitive to an otherwise non-inhibitory dose of HU (Woodward et al., 2006). Because of the normal excess of MCM proteins, depletion of maternal LIN-6/MCM-4 pools may not be harmful for several rounds of replication, if the reduction in origin number is compensated by prolonged time in S phase. Paradoxically, such a delay in cell cycle progression would be expected to use checkpoint mechanisms that are absent in *lin-6* mutants. The functioning of this checkpoint may be an important distinction between the somatic blast cells and gonadal precursors.

Our data show that *lin-6/mcm-4* is required for replication checkpoint activation in somatic cells. During larval development of *lin-6* mutants, somatic blast cells entered mitosis without delay and independent of the presence of HU. In addition, divisions in *lin-6(RNAi)* embryos continued without DNA replication, resulting in a fragmented genome. These results agree with work in other model organisms, which clarified the requirement of the MCM complex in activation of the DNA damage and replication checkpoints. Critical in checkpoint activation is the recruitment of Replication Protein A (RPA) to single stranded DNA (Zou and Elledge, 2003). The helicase activity of MCM proteins generates ssDNA, through unwinding the DNA at the replication fork. When replication forks are stalled, e.g. because of treatment with HU, the MCM helicase activity becomes uncoupled from DNA polymerase activity (Byun et al., 2005). Consequently, fork stalling leads to an accumulation of ssDNA, which recruits additional RPA and causes activation of the checkpoint kinases ATR and Chk1. Both replication fork formation and ssDNA generation require *lin-6/mcm-4* function; when this function is absent the replication checkpoint cannot be activated.

In contrast to the spindle assembly checkpoint, the replication checkpoint is activated by a quantitative signal, the amount of ssDNA. The critical amount may depend on the cell type, and the germline may be particularly sensitive. Gonadal cell divisions in *lin-6(e1466)* mutants did arrest upon HU treatment (Figure 2), thus sufficient ssDNA was generated to trigger the checkpoint. Brauchle et al. (2003) showed that the difference in cell cycle length of blastomeres in the early embryo is partly due to differential checkpoint activation. The anterior blastomere AB normally initiates mitosis approximately two minutes before the posterior blastomere P1. Disruption of *atl-1* ATR and *chk-1* Chk1 in otherwise normal embryos reduced the time difference by half (Brauchle et al., 2003). Interestingly, P1 is the precursor of the germline and somatic gonad and the time difference may reflect more stringent replication control in the lineage that produces the germ cells. An advanced ability to deal with replication stress may allow the gonad to continue replication cycles, albeit at a slower rate.

LIN-6/MCM-4 is dynamically regulated throughout development, supporting the presence of different pools of MCM proteins

In agreement with observations in other eukaryotes, our data indicate that DNA licensing takes place in late M phase and possibly continues in G1. In embryonic as well as larval cell divisions, LIN-6/MCM-4 started to colocalize with the DNA in late anaphase (Figure 6, 7, S4). However, upon temporal arrest of cell division or terminal differentiation LIN-6/MCM-4 decreased below detection by immunostaining or LIN-6::mCherry fluorescence. In contrast, Western blotting experiments showed reduced but clearly detectable LIN-6/MCM-4 levels in arrested L1 animals and dauer larvae (Figure 7). Thus, a relatively low amount of origin-bound MCM2-7 may drive DNA synthesis in cells that come out of arrest.

We observed strong induction of LIN-6/MCM-4 expression around the time of S phase onset. An important question is whether this newly synthesized MCM-4 contributes origin-licensing and helicase activities in the same cell cycle. The transcription of MCM2-7 genes is inhibited by *lin-35* Rb/E2F and activated by CYD-1/CDK-4 and CYE-1/CDK-2 (JK and SvdH, unpublished, (Kirienko and Fay, 2007). Thus, MCM expression is likely induced at the G1/S transition, close to the switch from origin licensing to origin firing. In mammalian cells released from quiescence, MCM loading has been shown to occur in late G1 (Mukherjee et al., 2009). Cdt1 and Cdc6 are essential loading factors for the MCM2-7 complex in various eukaryotes, and their inactivation prevents origin re-firing during S phase (Arias and Walter, 2007). The *C. elegans* orthologs CDT-1 and CDC-6 are both inactivated during S phase in a CUL-4/DDB-1 E3 ubiquitin ligase dependent manner (Kim et al., 2007; Kim and Kipreos, 2007; Korzelius and van den Heuvel, 2007; Zhong et al., 2003). Based on analogy with other systems, degradation of the presumed critical targets of the CUL-4/DDB1 complex, CDT-1 and CKI-1 Cip/Kip may depend on interaction with PCNA (Arias and Walter, 2006; Senga et al., 2006). This may couple CDT-1 and CDC-6 inactivation to DNA replication initiation. If this is the main limitation to origin licensing, MCM proteins expressed in late G1 should be able to act in the ensuing S phase.

Loss of lin-6 reveals a remarkable uncoupling between cell cycle progression and organismal development and growth

One of the striking aspects of the *lin-6* phenotype is that it exposes an uncoupling between cell cycle progression and developmental processes such as differentiation. During cell cycle progression, checkpoints ensure that earlier events are completed before later events initiate. Such feedback mechanisms do not appear to exist for cell division and cell fate acquisition. Based on reporter-gene expression and cell morphology, successful mitosis is not needed for induction of subsequent S-phases or induction of differentiation (Figure 3, S1, S2). In this light, it is surprising that growth, which can occur quite independently of cell division, was severely retarded in *lin-6* mutants. Furthermore, *lin-6* mutants animals frequently died during the larval molt. *cyd-1* and *cdk-4* mutants also lack DNA replication and grow slowly, but these mutants do not display any larval lethality (Boxem and van den Heuvel, 2001). Thus, DNA replication is needed for growth, while continued mitosis without DNA replication may cause reduced viability.

Surprisingly, restored *lin-6* function in the hypodermis was sufficient for normal growth and viability. In *C. elegans*, the intestine and hypodermis undergo rounds of endoreduplication during larval development (Hedgecock and White, 1985). Endoreduplication has long been thought to promote growth. In fact, a positive correlation between ploidy of the *C. elegans*

hypodermis and volume of the adult animal has provided a strong argument for growth control by endoreduplication (Flemming et al., 2000; Lozano et al., 2006). Intestinal expression of LIN-6 restored DNA replication and nuclear division in *lin-6* larvae, but did not rescue the growth and viability defects. In contrast, hypodermal expression of LIN-6 rescued the larval lethality as well as the reduced length and thin appearance of *lin-6* mutants. DBL-1 TGFβ has been shown to control postembryonic growth through regulation of SMA-3 in the hypodermis (Wang et al., 2002). This effect has been linked to the control of DNA replication (Lozano et al., 2006; Wang et al., 2002). Mutation of *cye-1*, *cyd-1* and *cdk-4* mutants all show reduced growth, in contrast to *cdk-1* mutants that arrest cell division in G2 (Boxem et al., 1999; Boxem and van den Heuvel, 2001; Lozano et al., 2006). Hence, it is likely that the TGF-β/SMA-3 pathway induces G1 cyclin expression to promote DNA replication, which in turn supports growth of the animal.

The lethality associated with the *Lin-6* phenotype, but not the *Cyd-1* and *Cdk-4* phenotype, was rescued by hypodermal *lin-6* expression. Expression of *lin-6* from the *dpy-7* promoter increased the number of hypodermal nuclei. Therefore, we expect that aberrant cell cycles with continued mitosis in the absence of DNA replication compromise the function of the hypodermal cells. This might explain why the lethality coincides with the molt, which is driven by the hypodermal cells. Taken together, our data show that *lin-6/mcm-4* is required for DNA replication and replication checkpoint activation. Inactivation of these functions leads to uncoupling of cell division form developmental progression, reduced growth and lethality. Remarkably, expression of *lin-6* in a single tissue is sufficient to restore growth and survival.

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Supplementary figures

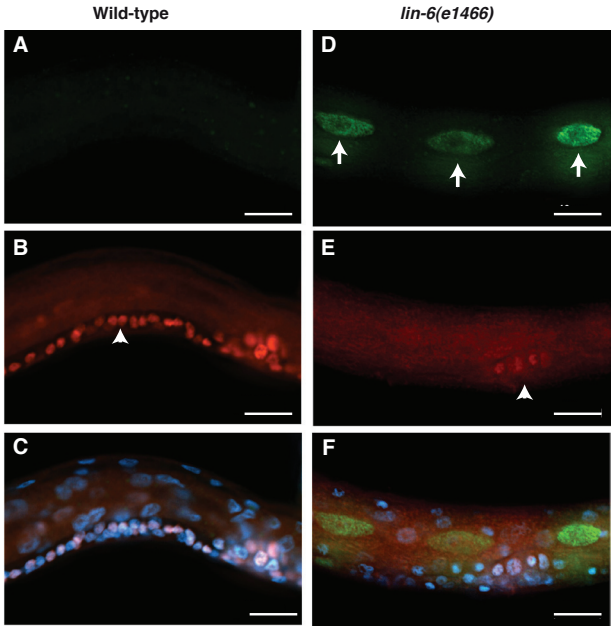


Figure S1. Animals synchronized at the late L2 stage show persistent expression of the S-phase entry marker *Prnr::CYB-1DesBox::3XVenus*. Wild-type (left) and *lin-6* (right) larvae grown in the presence of EdU and carrying the *Prnr::CYB-1Desbox::3XVenus* marker (see Materials and Methods). A,D: Venus expression visualized by antibody detection. B,E: DNA replication visualized by EdU staining. C,F: Merged images of anti-GFP and EdU staining and DNA staining with DAPI. Wild-type late L2 animals show widespread incorporation of EdU in the ventral cord (B, arrowhead), but no expression of the S-phase entry marker at this stage (A). In contrast, many *lin-6* animals had persistently high levels of the *rnr*-reporter transgene at this stage (D, arrows), while EdU incorporation was still virtually absent, except for the gonad (E, arrowhead).

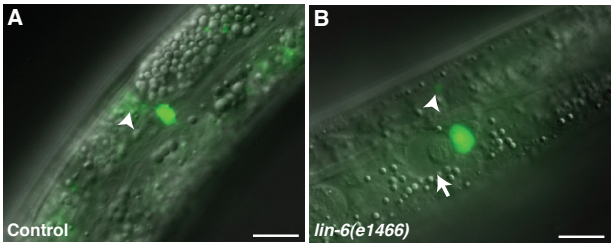


Figure S2. *lin-6* mutant animals express the postdeirid marker Pdat-1::GFP. A,B: Combined DIC and fluorescence images of the postdeirid in a wild-type L4 animal (A) and a similarly staged *lin-6(e1466)* animal. Even though the cell divisions that give rise to a normal postdeirid is incomplete in *lin-6* animals (B, arrow, note the large nucleus), the *Pdat-1::GFP* marker is expressed, with cells even producing axonal protrusions similar to wild-type (A,B, arrowheads)

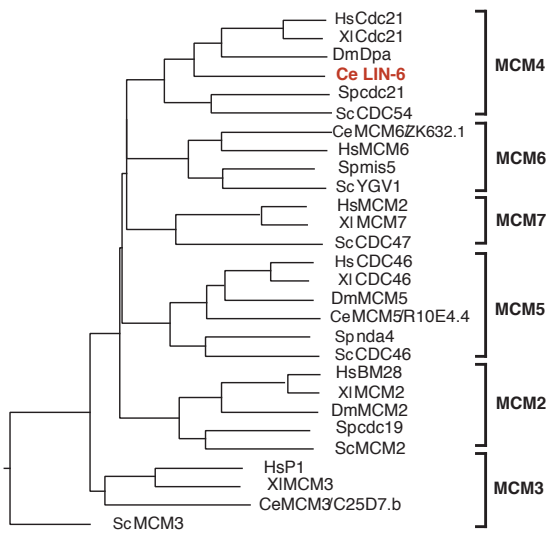


Figure S3. Phylogenetic tree of MCM2-7 complex members. The LIN-6 protein is highly related to the MCM4 subfamily, which includes *Drosophila* Disc Proliferation Abnormal (Dpa), *Homo sapiens*, *Saccharomyces pombe* and *Xenopus laevis* Cdc21/MCM4 and *Saccharomyces cerevisiae* CDC54.

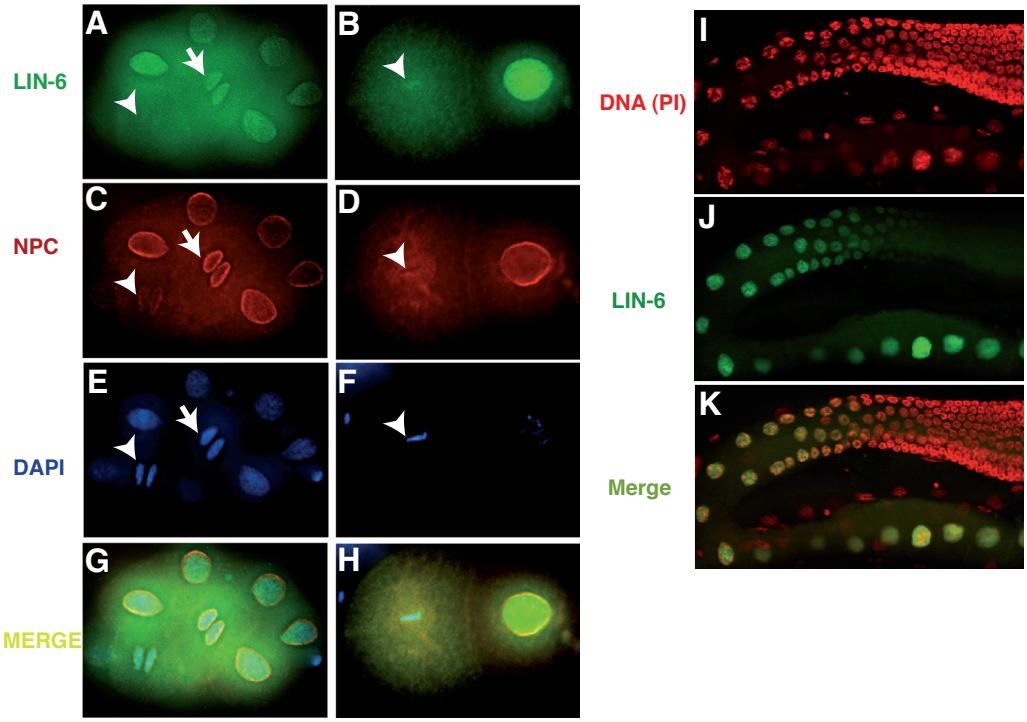


Figure S4. Subcellular localization of LIN-6/MCM-4 during mitosis and germline development. Embryos were co-stained for LIN-6 and the Nuclear Pore Complex to ascertain the timing of LIN-6 co-localization with DNA in mitosis. B,D,F,H: 2-cell stage embryo. Note that LIN-6 staining is almost completely absent from the DNA during metaphase (B,D,F arrowheads). A,C,E,G: ~16-cell stage embryo. LIN-6 is still absent from the DNA in early anaphase (A,C,E, arrowheads), but locates to the nucleus in late anaphase/telophase (A,C,E, arrows). I-K. LIN-6/Propidium Iodide (PI) co-staining in the germline of a wild-type adult animal. LIN-6 staining becomes more prominent as the cells in the germline start to cellularize. Note that LIN-6 staining is nuclear, but does not show substantial overlap with the DNA.

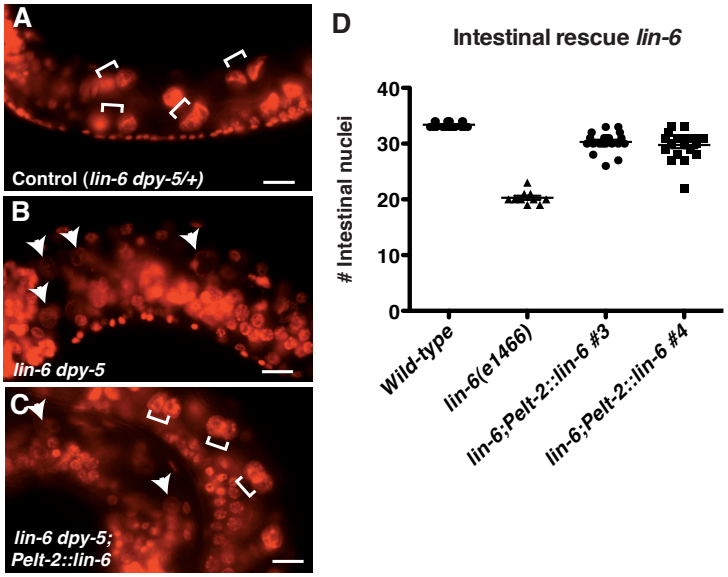


Figure S5. Intestinal expression of LIN-6::mCherry rescues nuclear division and endoreduplication in the *lin-6(e1466)* mutant. A-C: DNA staining (PI) of wild-type and *lin-6(e1466) dpy-5(e61)* larvae, with and without intestine specific LIN-6::mCherry expression. A: Control heterozygous L3 larvae have pairs of intestinal nuclei with bright PI staining (A, brackets). B: *lin-6* mutants lack intestinal divisions as well as endoreduplication cycles, resulting in large undivided intestinal nuclei with a 2N DNA content. C: Two *lin-6* mutant larvae, on the left without and to the right with the *Pelt-2::LIN-6::mCherry* rescue construct. Note the difference in intensity and number of intestinal cells in the *lin-6* mutant (C, arrowheads) and rescued animal (C, brackets). D: Quantification of intestinal rescue in L3 stage larvae for 2 independent *Pelt-2::LIN-6::mCherry* rescue lines. Note that a Dpy background is used, which causes close colocalization of intestinal nuclei.

Chapter 3

Cell cycle re-entry of terminally differentiated muscle cells in *C. elegans*

Jerome Korzelius [1], Inge The [1], Mike Boxem [1], Vincent Portegijs [1] Teije Middelkoop [1], Marian Groot-Koerkamp [2], Frank Holstege [2] and Sander van den Heuvel [1]

[1] Developmental Biology, Utrecht University,
Padualaan 8, 3584 CH Utrecht, The Netherlands.

[2] Department of Physiological Chemistry, University Medical Center Utrecht,
Utrecht, The Netherlands.

'Jogging in the morning, go man, go!
Workouts in the health spa, muscles glow'

Village People - 'Macho Man'

Abstract

The permanent arrest of proliferation in association with terminal differentiation is thought to involve Cdk inhibition, transcriptional repression by the pRb family and permanent gene silencing. Here, we use the *C. elegans* bodywall muscle as an *in vivo* model for cell cycle arrest in association with terminal differentiation. Inactivation of *lin-35*/Rb and knockdown of *cki-1*/Cip/Kip did not prevent terminal differentiation and permanent arrest of muscle cells. However, expression of G1 Cyclin/Cdk combinations triggered gradual cell cycle re-entry of bodywall muscle during larval development. Specifically, we observed S-phase reporter expression, DNA replication, chromosome condensation and nuclear division in post-embryonic muscle cells. Importantly, induction of cell cycle entry did not interfere with the differentiated state. Muscle-specific gene expression profiling revealed that *cye-1* Cyclin E/*cdk-2* Cdk2 expression activates a specific cell cycle transcriptional program enriched in E2F target genes. Expression of *cyd-1* Cyclin D/*cdk-4* Cdk4/6 induced an overlapping but larger number of genes, and also triggered DNA replication more efficiently. Our results indicate that a cell cycle transcription program can be activated without reprogramming the chromatin, and can coincide with the differentiated state. At the same time, quiescent intestinal cells were much more efficiently induced to re-enter the cell cycle, which emphasizes the multiple levels of cell cycle inhibition in differentiated cells. A forward genetic screen identified two mutants with specifically enhanced mitosis in the bodywall muscle. Deciphering the reverse relationship between differentiation and proliferation can contribute valuable insights with relevance to cancer biology and regenerative medicine.

Introduction

Metazoan development displays a remarkable inverse relationship between cell proliferation and differentiation. Embryogenesis starts with many rounds of cell division, which continue until developmental cues cause cell division to halt. The division arrest can be temporal, if cells enter a quiescent state while retaining the ability to re-enter the cell cycle. However, the transition to a differentiated state usually coincides with irreversible exit from the cell division cycle. The question what distinguishes temporal arrest from a permanently arrested state is of great fundamental and clinical importance.

The decision whether or not to go through another round of division is made in the G1 phase of the cell cycle. Negative regulators of G1 progression include the pINK and Cip/Kip protein families that associate with and inhibit Cyclin dependent kinases (Cdk's) (Besson et al., 2008; Sherr and Roberts, 1999). In addition, members of the Retinoblastoma (Rb) protein family inhibit cell cycle entry, at least in part by repressing E2F-regulated promoters (Cobrinik, 2005; van den Heuvel and Dyson, 2008). These two levels of regulation are connected, as phosphorylation by Cdk's overcomes G1 inhibition by pRb. The importance of these mechanisms is highlighted by the common presence of *Rb* mutations or alterations that increase Cdk activity in G1 phase in human cancers (Sherr, 2004). Differentiation factors often activate the negative regulators of G1 progression to coordinate cell cycle withdrawal with differentiation (Buttitta and Edgar, 2007). While Cyclin/Cdk inactivation by inhibitors may be reversible, pRb has been implicated in temporal as well as irreversible cell cycle exit. This later function involves establishment of repressive histone modifications at the promoters of cell cycle genes, (Blais et al., 2007; Jacobs et al., 1999). In parallel with other chromatin modifiers, such as Polycomb group repressor complexes, this is thought to cause stable and permanent repression of cell cycle genes in terminally differentiated cells.

There are, however, observations that put into question if cell cycle exit is truly irreversible and if proliferation is excluded by the differentiated state. Perhaps the most striking example of differentiating cells re-entering the cell cycle is limb generation in certain amphibians (Tanaka, 2003). While this process involves dedifferentiation, recent work in the Axolotl shows that the differentiated state is not fully lost (Kragl et al., 2009). In mammals, fully differentiated hepatocytes divide extensively in response to tissue damage, but this cell type never shuts down its proliferation ability (Duncan et al., 2009). Differentiated skeletal muscle cells do not re-enter the cell cycle, but in vitro muscle differentiation and arrest requires pRb function (Blais et al., 2007). Interestingly, conditional knockout of *Rb* in the mouse inner ear resulted in cell cycle re-entry of post-mitotic hair cells that maintained their differentiated appearance (Sage et al., 2005). In humans, differentiated cone precursor cells in the eye appear to give rise to retinoblastoma upon loss of pRb (Xu et al., 2009). Furthermore, conditional pRb family knockout mice develop retinoblastoma-like tumors that arise from a population of fully differentiated neurons in the retina (Ajioka et al., 2007). Thus, in a few specific cases, loss of the pRb tumor suppressor allows proliferation of terminally differentiated cells.

In this study, we use the small nematode *C. elegans* to further examine the mechanisms that restrict proliferation of terminally differentiated cells. Cell fate specification in *C. elegans* happens in a highly reproducible fashion and the fate of each cell has been traced (Sulston and Horvitz, 1977; Sulston et al., 1983). In addition, *C. elegans* has a reduced level of complexity, as many regulators involved in progression, such as the pRb family, G1/S promoting Cdk's and Cyclins D

and E, are encoded by single genes (Boxem and van den Heuvel, 2001; Hong et al., 1998; Lu and Horvitz, 1998; Park and Krause, 1999). Animals that lack *cki-1* p27 or *lin-35* Rb show some cell division defects, but proliferation and differentiation in the majority of cell lineages remains unaffected (Boxem and van den Heuvel, 2001; Hong et al., 1998; Kostic et al., 2003; Ouellet and Roy, 2007). In fact, truly uncontrolled proliferation has not been observed in *C. elegans*, with a possible exception of germline tumors that result from failure of the mitotic stem cells to enter a meiotic cell cycle (Berry et al., 1997). Somatic precursor cells in *C. elegans* lose totipotency within the first few embryonic divisions and rapidly progress towards a differentiated fate. We hypothesized that the mutually exclusive nature of proliferation and differentiation prevents uncontrolled proliferation in *C. elegans*.

Here, we study the *C. elegans* bodywall muscle (bm) as a model for cell cycle arrest in association with terminal differentiation. The *C. elegans* larva is born with 81 bodywall muscle cells that are fully differentiated (Sulston and Horvitz, 1977). We set out to identify critical brakes on the cell cycle by examining which manipulations trigger cell cycle re-entry in the post-embryonic bodywall muscles. We found that forced expression of *C. elegans* Cyclin/Cdk combinations was sufficient to induce cell cycle re-entry. Muscle-specific expression of CYD-1/CDK-4 or CYE-1/CDK-2 triggered S-phase entry, mitosis and nuclear division. Moreover, transcriptional profiling of bodywall muscle cells revealed that CYD-1/CDK-4 and CYE-1/CDK-2 induce highly overlapping sets of cell cycle-related genes, while the expression of muscle-specific genes remained unchanged. Compared to differentiated muscle, quiescent intestinal cells were much more efficiently induced to enter the cell cycle. This confirms the presence of additional controls that restrict the proliferation of terminally differentiated cells. We identified two mutants that enhance the level of cell cycle re-entry specifically in the bodywall muscle. This work demonstrates that induced Cdk/Cyclin function activates a cell-cycle specific transcriptional program in differentiated cells, and that additional levels of control may be genetically identified.

Materials and Methods

C. elegans strains and culturing

C. elegans strains were cultured on NGM plates seeded with *E. coli* strain OP50 as described (Stiernagle, 2006). Experiments were conducted at 20°C unless indicated otherwise. Feeding RNAi was performed on NGM plates supplied with 50 µm/ml Ampicillin and 2 mM IPTG. Animals were synchronized by hypochloride bleaching and hatching the eggs in M9 medium with 0.05%Tween-20. Animals were then allowed to develop for the appropriate amount of time. The following strains were used in this study: N2 Bristol wild-type, SV326 *rtIs14[elt-2::GFP; osm-10::HT150Q]*, SV822 *heEx288[Pelt-2::cye-1; Pelt-2::cdk-2AF::Venus;Pmyo-2::GFP]*, SV1030 *rtIs14; heEx345[Pelt-2::cdk-4::Venus; Pmyo-2::TdTomato]*, SV1031 *rtIs14; heEx346[Pelt-2::cyd-1;Pelt-2::cdk-4::Venus;Pmyo-2::TdTomato]*, SV837 *ccIs4251[myo-3::mtGFP;myo-3::4XNLSGFP]; heEx302[Pmyo-3::cye-1; Pmyo-3::cdk-2AF::venus;pRF4 rol-6(+)]*, SV857 *heIs9[Pmyo-3::GFP::H2B; Pmyo-3::cyd-1;Pmyo-3::cdk-4::Venus]*, SV858 *heIs10[Pmyo-3::GFP::H2B; Pmyo-3::cye-1;Pmyo-3::cdk-2AF::Venus]*, SV859 *heIs11[Pmyo-3::GFP::H2B]*, SV860 *heIs12[Pmyo-3::GFP::H2B;Pmyo-3::cyd-1;Pmyo-3::cdk-4::Venus]*, SV861 *heIs44[Pmyo-3::GFP::H2B;Pmyo-3::cye-1;Pmyo-3::cdk-2::Venus]*, SV852 *heIs10;heIs1[Prnr-1::cyb-1DesBox::TdTomato; pRF4 rol-6(+)]*, SV911 *heIs10;gaIs146[Pmyo-3::FLAG::PAB-1; Psur-5::GFP]*, SV912 *heIs11;gaIs146[Pmyo-3::FLAG::PAB-1; Psur-5::GFP]*. Integrated strains were created by γ-irradiation and strains were backcrossed with N2 a minimum of 2 times before analysis.

Molecular cloning, transgenesis and dsRNA injection

Expression constructs for *cye-1*, *cyd-1*, *cdk-4*, *cdk-2* and *cdk-2AF* were created by amplifying and cloning the respective cDNAs into derivatives of the Fire lab pPD49.26 expression vector, carrying either a 2.4 Kb *myo-3* promoter (PCGS1 (*Pmyo-3),* bodywall muscle expression) or a 5 Kb *elt-2* promoter (intestinal expression (Fukushige et al., 1998)). CDK-2, CDK-2AF and CDK-4 were all fused to the *C.elegans*-optimized coding region of the Venus YFP (A kind gift of Yuichi Iino). The CDK-2AF mutant was created by mutating nucleotide 73 (A=>G) and nucleotide 77 (A=>T) using the QuickChange site-directed mutagenesis kit (Stratagene). This results in a Thr25 to Ala(A) and a Tyr26 to Phe(F) in the *C. elegans* CDK-2 protein, removing these highly conserved putative Wee1 phosphorylation sites. The *Pmyo-3::GFP::H2B* marker was created by re-cloning the GFP::H2B fusion protein from pAZ132(*Ppie-1::GFP::H2B*) into the PCGS1(*Pmyo-3) vector.* The *Prnr::CYB-1DesBox::TdTomato* marker was created by using the *rnr-1* promoter from pVT501 (Hong et al., 1998) to drive the TdTomato fluorophore coupled to an N-terminal part of *C. elegans* CYB-1 (N-CYB-1) which harbors a KEN box sequence that allows targeting by the APC. This results in the destruction of the fluorophore after each round of cell division. To avoid co-suppression issues, we designed a synthetic coding sequence for the N-CYB-1 fragment in which each of the 100 codons was changed. Detailed cloning information including sequence maps and primers are available upon request. Transgenic lines were created by micro-injection as described (Mello et al., 1991). For our analysis of cell cycle re-entry in bodywall muscle and intestine, multiple transgenic lines were analyzed for each combination. Representative lines with 40-70% F2 transmission were selected for γ-irradiation. Integrated lines were backcrossed a minimum of 4 times before analysis.

Double strand RNA (dsRNA) for injection of *cki-1* and *lin-35* dsRNA was made by PCR amplification from library clones mv_To5A6.1 (*cki-1*) and sjj_C32F10.2 (*lin-35*). These PCR fragments were used as DNA template for the RNA synthesis reaction using an *in vitro* transcription kit (Megascript, Ambion). dsRNA was injected at a concentration of 500 ng/µl (*lin-35*) and 750 ng/µl (*cki-1*). Injected Po animals were allowed to lay eggs for 18 hours and were when washed off plates and washed an additional 4-5 times with M9 + 0.05%Tween-20. Animals were then transferred to a Falcon tube with M9 + 0.05%Tween-20, supplied with 10mM serotonin to stimulate egg laying. The hatched L1's and embryo's were then examined 24 hours later.

Immunostaining and co-immunoprecipitation

Immunostaining for phospho-H3S10/GFP was done by fixing L3 stage animals by freeze-cracking the worms on Poly-L-Lysine coated slides in liquid nitrogen and subsequent fixation in methanol (5 min. at -20 °C) and acetone (20 minutes at -20 °C) (Duerr, 2006). Primary antibodies used: rabbit anti-phospho-H3S10 (1:200, Abcam), mouse anti-GFP (1:100, Sigma). Secondary antibodies used: Donkey anti-mouse FITC and Donkey anti-rabbit TexasRed (1:200, Jackson Immunolaboratories). Immunostaining for CYE-1 and GFP in L1 animals was performed similarly. Primary antibodies used: mouse anti-CYE-1 (M.Krause, 1:200), rabbit anti-GFP (1:100, Molecular Probes). Secondary antibodies used: Donkey anti-mouse FITC and Donkey anti-rabbit TexasRed (1:200, Jackson Immunolaboratories). Worms were mounted in Prolong Anti-Fade Gold (Invitrogen), supplied with 2 µg/ml DAPI (Sigma). BrdU staining was performed as described (Boxem et al., 1999). EdU labeling and staining was performed according to a protocol developed by S. Crittenden and J. Kimble, using the Click-IT EdU Alexa Fluor 594 kit (Invitrogen). In short, MG1693 (Thymidine deficient) bacteria were grown in 100 ml of minimal medium containing 20 µM EdU. Worms were fed on NGM+Ampicillin plates

with these bacteria for the appropriate time. Worms were fixed by freeze-cracking on Poly-L-Lysine coated slides in liquid nitrogen and subsequent fixation in methanol (5 min. at -20 C) and acetone (20 minutes at -20 C). The Click-IT reaction was subsequently performed on slides according to manufacturers instructions. Antibody staining then proceeded as described (Duerr, 2006). Primary antibodies used: Monoclonal mouse anti-GFP (1:100, Sigma). Secondary antibodies used: Donkey anti-mouse FITC (1:200, Jackson Immunolaboratories) was used as a secondary antibody. Slides were mounted in Prolong Anti-Fade Gold (Invitrogen) supplied with 2 µg/ml DAPI (Sigma). For UNC-15 Paramyosin staining on L4/adult worms, we used a modified version of the Finney-Ruvkun whole-mount staining protocol (Bettinger et al., 1996). Animals were tumbled overnight in primary antibodies: mouse monoclonal antibody 5-23 to UNC-15 (1:3, tissue supernatant, Developmental Studies Hybridoma Bank) and rabbit anti-GFP (1:200, Molecular Probes), secondary antibodies used: Donkey anti-mouse TexasRed (1:200, Jackson Immunolaboratories) and Donkey anti-rabbit FITC (1:200, Jackson Immunolaboratories). For co-immunoprecipitation of the CYE-1/CDK-AF::Venus complex, N2 and SV837 (see *C. elegans* strains and culturing) animals were homogenized by grinding in liquid nitrogen and transferring the worm debris into 4 ml chilled lysis buffer (20mM Tris-HCl (pH 8.3), 137mM NaCl, 1% Nonidet P-40, 2mM EDTA, protease inhibitor cocktail (Roche Diagnostics), 0.14% β-mercapto-ethanol), followed by treatment of the lysate in a French pressure cell. 1 mg of protein was used for the IP experiments and 50 µg was used for direct lysate samples. Immunoprecipitation was performed by incubating 500µl lysate with either anti-CYE-1 (M. Krause) and anti-GFP (Molecular Probes) covalently coupled ProtG and ProtA beads respectively. For negative control immunoprecipitation 5µl mouse anti-SD15 (a kind gift of A. Thomas) was coupled to protG beads and rabbit anti-EiF4E (a kind gift of A. Thomas) was coupled to protA beads. Following immunoprecipitation, 20 µl of each sample was loaded on an 8% polyacrylamide gel. Proteins were blotted on PVDF membrane (Amersham Bioscience). Blots were incubated with either mouse anti-CYE-1 (M.Krause, 1:750), rabbit anti-GFP or monoclonal mouse anti-actin (MP Bio Medicals). Secondary antibodies used: goat anti-mouse-peroxidase and goat anti-rabbit peroxidase (Jackson Immunolaboratories). The chemiluminescent detection reaction was performed using a Biorad ECL kit (Biorad) and the blot was subsequently exposed to hypersensitive film (Amersham Hyperfilm, GE healthcare).

Motility assays, live imaging and quantification

Motility assays were performed by determining the average number of body bends per minute in a three-minute interval for each animal as described (Robatzek and Thomas, 2000). For live imaging, animals were synchronized by hypochloride bleaching, washed off with M9+ 0.05% Tween-20, anaestisized with 10mM Sodium Azide and subsequently mounted on slides with a 2% agar pad. Extra division events were quantified at L3 and L4 stages by counting the number of cells that had mitotic phenotypes (including cells with extra nuclei, nuclei with condensed DNA and nuclei in the various stages of mitosis) anterior of the prospective vulva. Pictures were taken with an Axioplan 2 microscope mounted with an Axiocam mRM camera (Zeiss Microscopy). Quantitive data and graphs were produced using GraphPad Prism Version 5 for Mac (GraphPad Software) and Excel 2008 for Mac (Microsoft).

Tissue-specific microarray analysis

Tissue-specific mRNA isolation was performed as described in (Roy et al., 2002). 4 idependently grown biological samples were used for each different line: SV911 (control) and SV912 (CYE-1/CDK-2AF). Microarrays used were *C. elegans* Gene Expression Microarrays (Agilent Technologies,

Belgium) representing 60-mer probes in a 4x44K layout. RNA amplifications and labeling were performed as described (Roepman et al., 2005) on an automated system (Caliper Life Sciences NV/SA, Belgium) with a minimum of 50 ng IP'd RNA from each sample. Hybridizations were done on a HS4800PRO system supplemented with QuadChambers (Tecan Benelux B.V.B.A.) using 500 ng of labeled cRNA per channel according to (van de Peppel et al., 2003). Hybridized slides were scanned on an Agilent scanner (G2565AB) at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery), printtip Loess normalization was performed on mean spot-intensities (Yang et al., 2002). Dyebias was corrected based on a within-set estimate according to (Margaritis et al., 2009). Data was analysed using MANOVA (Wu et al., 2002). In a fixed effect analysis, sample, array and dye effects were modeled. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes with $p < 0.05$ after family-wise error correction were considered significantly changed. Manual annotation of genes > 2-fold upregulated was done in Excel 2008 by using WormBase (Version 207). GO-term enrichment was determined using Funcassociate (Berriz et al., 2003).

Results

Knockdown of cki-1 Cip/Kip results in extra division of bodywall muscle during embryogenesis, but not during later development

We first examined to what extent the differentiated state of *C. elegans* bodywall muscle depends on *cki-1* Cip/Kip and *lin-35* Rb, the two major negative regulators of G1 progression (Boxem and van den Heuvel, 2001; Hong et al., 1998) In addition to its role in cell cycle arrest, a previous study implicated *cki-1* in differentiation and morphogenesis of the pharynx and epidermis during embryogenesis (Fukuyama et al., 2003). We extended this analysis to the bodywall muscles, making use of the muscle specific *Pmyo-3::GFP::H2B* nuclear marker. Control animals that express the marker hatch with the normal 81 body muscle cells, arranged in 4 quadrants alongside the longitudinal axis of the animal (Figure 1A,B,E) (Sulston and Horvitz, 1977). We injected adult animals with a high amount of *cki-1* dsRNA to remove maternal and zygotic *cki-1* Cip/Kip function. This resulted in a high percentage embryonic lethal progeny as well as arrested L1 animals with a Vab (Variable Abnormalities) phenotype. Both the dead eggs and L1 larvae had several extra muscle nuclei (Figure 1C-E). However, the bodywall muscle appeared fully differentiated and did not show signs of cell cycle entry in animals that continued larval division (data not shown). Thus, *cki-1* RNAi results in formation of extra bodywall muscle during embryogenesis, probably by allowing extra division at the time of normal cell cycle exit. Importantly, terminal differentiation of these cells still occurs and the differentiated muscle cells remain cell cycle arrested.

Single *lin-35*/Rb inactivation or combined knockdown of *lin-35* and *cki-1* did not change the number or pattern of the embryonic bodywall muscle (Figure S1D,E). In the same *lin-35;cki-1* double RNAi animals we did see supernumerary divisions in the larval Mesoblast (M) lineage (Figure S1F, arrows). Again these extra cells appeared at the time of normal cell cycle delay, indicating temporal failure in cell cycle arrest. As was shown previously (Boxem and van den Heuvel, 2001), double RNAi of *lin-35* and *cki-1* also resulted in supernumerary divisions of the intestinal nuclei, visualized by *Pelt-2::GFP* expression (Figure S1A-C). Thus, *lin-35* Rb and *cki-1* Cip/Kip appear to be strictly required for temporal arrest of cells that are not fully withdrawn from the division cycle. Knockdown of the negative G1/S regulators *cki-1* and *lin-35* does not prevent terminal differentiation and does not allow cell cycle re-entry of terminally differentiated bodywall muscle cells.

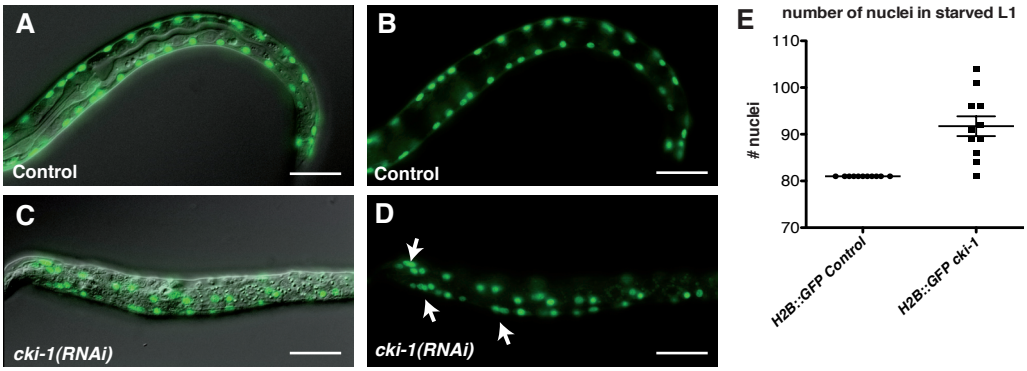


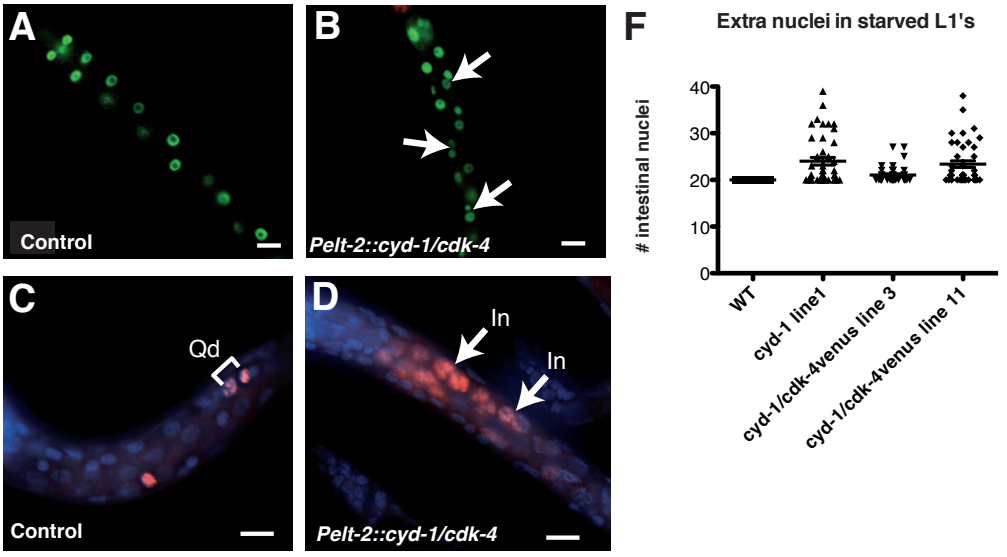
Figure 1. Knockdown of the p27 orthologue *cki-1* leads to supernumerary divisions in the bodywall muscle of arrested L1 larvae. A-D: DIC and GFP fluorescence (A,C) or fluorescence only (B,D) images of a control animal carrying the *Pmyo-3::GFP::H2B* marker (A,B) or an arrested *cki-1(RNAi)* L1 animal that has too many bodywall muscle nuclei (C,D). Arrows indicate extra nuclear divisions. E: Quantification of the number of BWM nuclei in developmentally arrested L1 animals in control and *cki-1(RNAi)* animals. Each dot represents a single animal.

Expression of G1 Cyclin/Cdk complexes leads to extra nuclear divisions and DNA replication in the intestine

We wondered whether *cki-1* and *lin-35* show a limited requirement in cell cycle arrest upon terminal differentiation in bodywall muscle cells because Cyclin/Cdk activity is efficiently shut down by other means. To test if transcriptional activation of G1 Cyclin/Cdk combinations can trigger cell cycle entry, we first examined the *C. elegans* intestine. Wild-type animals hatch with 20 intestinal nuclei, 12-14 of which go through nuclear division at the end of the L1 stage. In addition, all nuclei complete an endoreduplication cycle at each of the four larval transitions, resulting in 32-34 nuclei with a 32n DNA content in the adult intestine (Hedgecock and White, 1985). Previous studies have shown that the intestine is susceptible to cell cycle deregulation, e.g. to downregulation of *cki-1* and *lin-35* or hyperactivity of the *cdc-25* phosphatase (Boxem and van den Heuvel, 2001; Clucas et al., 2002; Kostic and Roy, 2002; Ouellet and Roy, 2007).

We used the promoter of the intestine-specific GATA transcription factor *elt-2* to express Cyclin/Cdk constructs specifically in the intestine (Fukushige et al., 1998). These constructs were used to generate transgenic strains that express a single Cyclin or Cdk, or the combination: CYD-1/CDK-4, CYE-1/CDK-2, and CYE-1/CDK-2AF, a mutated form of CDK-2 that lacks the negative Tyr14 and Thr15 phosphorylation sites (Gould and Nurse, 1989; Krek and Nigg, 1991; Norbury et al., 1991). For each strain, we examined whether Cyclin/Cdk expression overcomes starvation induced quiescence and developmental cell cycle arrest.

Embryos from each transgenic line were hatched in liquid medium without food. The starved L1 animals remain developmentally arrested, with quiescent post-embryonic blast cells. Expression of any of the three Cyclin/Cdk combinations in the intestine resulted in extra nuclei and incorporation of the thymidine analogue BrdU (Figure 2A-E). For example, animals expressing CYD-1/CDK-4 have extra nuclei in their intestine, regularly exceeding the maximum number of 34 nuclei seen in normal adults (Figure 2B,F). Hence, expression of Cyclin/Cdk complexes can bypass the L1 arrest in cell division. Though some of the extra nuclei were formed during embryogenesis, the observed BrdU incorporation demonstrates that DNA synthesis also occurs



E

Strain	n	% BrdU-positive
N2	many	0
Pelt-2::cdk-4::Venus	50	0
Pelt-2::cyd-1	43	12
Pelt-2::cyd-1/cdk-4::Venus	51	51
Pelt-2::cye-1/cdk-2AF::Venus	37	46

Figure 2. Expression of G1 Cyclin/Cdk combinations in the intestine of arrested L1 larvae leads to extra nuclear divisions and DNA synthesis. A-B: L1 animals carrying an integrated *Pelt-2::GFP* marker alone (A) or in combination with CYD-1/CDK-4 expressed from the intestinal *elt-2* promoter (B). Arrows indicate clusters of extra nuclei. C-D: BrdU incorporation in the intestine of starved L1 wild-type control animals (C) or animals expressing CYD-1/CDK-4 in the intestine (D). Control L1 arrested animals have few BrdU positive cells, only the Q neuroblast daughters (C, brackets) and some epidermal V-cells occasionally escape arrest. The intestine of the *Pelt-2::CYD-1/CDK-4* animal shows an extensive amount of intestinal cells that have undergone DNA replication during starvation induced quiescence (D, arrows). E: Quantification of the percentage of animals staining positive for BrdU in the gut in representative lines of each Cyclin/Cdk combination. F: Quantification of the number of intestinal nuclei in arrested L1 animals. Each dot represents a single animal. Note that CYD-1 expression in the gut alone is sufficient to trigger cell cycle progression in these animals.

post-embryonically in starved L1 animals. In addition, we found that expression of each of the two Cyclins alone is sufficient to trigger nuclear division and DNA replication, both in arrested L1 animals and during subsequent larval development, whereas Cdk expression alone is not (Figure 2 E,F, Figure S2). This confirms the notion that Cyclin expression is often rate limiting in cell cycle entry. Together, expression of G1 Cyclins or Cyclin/Cdk combinations is sufficient to trigger cell cycle entry in the intestine, even in starvation induced quiescent cells.

Cyclin/Cdk expression in the bodywall muscle leads to cell cycle re-entry during larval development

We next set out to test whether Cyclin/Cdk expression could trigger cell cycle re-entry in the terminally differentiated bodywall muscle. The *myo-3* promoter induces expression late in muscle differentiation and was chosen for these experiments. We expressed the three different Cyclin/Cdk combinations together with a reporter construct, *Pmyo-3::GFP::H2B*, to facilitate the

quantification of muscle nuclei. Proper expression of the constructs and complex formation were verified by antibody staining and co-immunoprecipitation for CYE-1 and CDK-2AF (Figure S3). In contrast with our findings in the intestine, animals expressing any of the three Cyclin/Cdk combinations hatch with the normal complement of muscle nuclei (Figure 3B,E). Thus, *myo-3* promoter controlled Cyclin/Cdk expression does not lead to extra muscle division during embryogenesis. The difference with the *cki-1* RNAi results (Figure 1) supports that *cki-1* RNAi leads to increased CDK activity before terminal differentiation.

Prolonged times in medium without food did not lead to more than 81 bodywall muscle nuclei, despite the fact that the *myo-3* promoter induced Cyclin/Cdk expression in starved L1 animals (data not shown). We attempted to activate the insulin signaling pathway to overcome a potential nutrition-dependent arrest of cell cycle progression (Baugh and Sternberg, 2006). However, muscle-specific overexpression of *akt-1*/AKT or mutation of *daf-18*/PTEN or *daf-16*/FoxO in combination with CYE-1/CDK-2AF expression did not result in cell cycle re-entry in starved L1 animals (Figure S4). In contrast, when larval development was stimulated by addition of food, clear signs of cell cycle re-entry appeared in the bodywall muscle (Figure 3C,D).

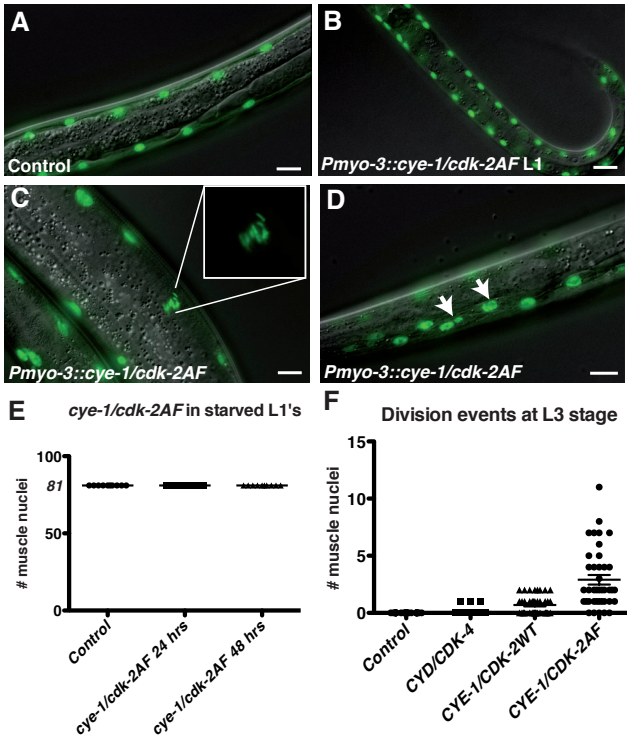


Figure 3: Expression of G1/S Cyclin/Cdk combinations in the terminally differentiated bodywall muscle leads to cell cycle re-entry. A-D: Phenotypes of animals expressing CYE-1 and CDK-2AF in the differentiated bodywall muscle. A,B Starved wild-type animals carrying the integrated *Pmyo-3::GFP::H2B* marker only, or together with *Pmyo-3::cye-1/cdk-2AF* do not display extra divisions in the bodywall muscle. C, D: At later stages, *Pmyo-3::cye-1/cdk-2AF* animals show various signs of cell cycle re-entry, such as mitotic figures (C) and extra nuclei (D). E-F: Quantification of extra division events (see text) anterior of the prospective vulva in starved L1 animals (E) or L3 stage animals (F). Each dot represents a single animal. The cell cycle phenotype does not appear in embryogenesis or L1 animals (E), but becomes increasingly more prominent from the L3 stage (F) onwards.

From the L2 stage onwards, some muscle cells started to show signs of mitosis, including chromosome condensation (Figure 3C), chromosome congression, and nuclear division (Figure 3D), sometimes even resulting in clusters of small nuclei. To quantify these phenotypes, we counted the number of cells with mitotic events anterior to the vulva. We focused on the anterior part of the animals, to exclude descendants of the Mesoblast. As previously mentioned, this cell generates a series of muscle cells during larval development, including 14 bodywall muscle and

the sex muscle cells that aid in egg laying (Sulston and Horvitz, 1977). In contrast to the bodywall muscle formed during embryogenesis, the initial Mesoblast daughters cannot be regarded as post-mitotic muscle cells and are therefore excluded from our analysis. The results, summarized in Figure 3F, show that the combination of CYE-1 and CDK-2AF was the most potent in inducing mitotic events during larval development. CYE-1 in combination with wild-type CDK-2 and CYD-1/CDK-4 also induced extra division events, albeit at a lower frequency (Figure 3F).

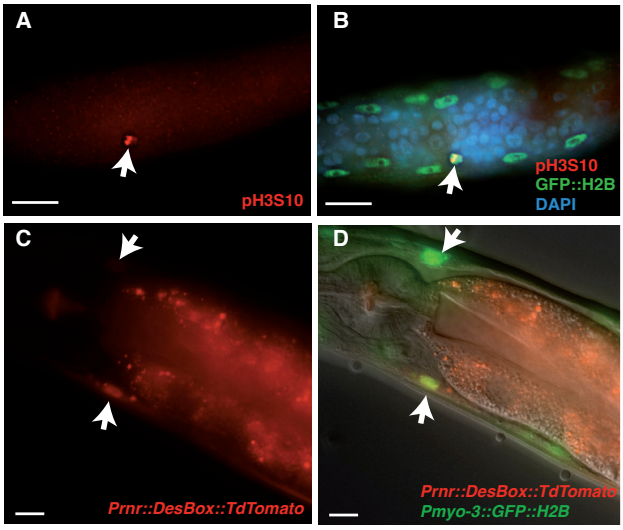


Figure 4. Bodywall muscle nuclei in *Pmyo-3::cye-1/cdk-2AF* animals express markers for both S-phase entry and mitosis. A,B: Staining for the mitosis-specific Histone H3S10 phosphorylated epitope reveals a bodywall muscle nucleus in mitosis. C,D: Expression of the S-phase marker *Prnr::DesBox::TdTomato* (see text) in the bodywall muscle of an adult animal (arrows).

We included additional markers for mitosis and S-phase entry to confirm that the mitotic DNA figures are indeed a result of cells re-entering the cell cycle. Staining for the mitosis specific phospho-histone H3S10 epitope readily detected mitotic nuclei in the bodywall muscle of CYE-1/CDK-2AF animals (Figure 4 A,B arrows). To examine S-phase entry we designed an S-phase reporter that expresses TdTomato coupled to a N-terminal CYB-1 fragment, which directs APC/C-dependent protein degradation in mitosis (King et al., 1995), under the control of the *rnr-1* promoter (see Materials and Methods). The addition of the N-terminal CYB-1 fragment ensures proper degradation of the fluorophore after each cell cycle. Expression of this S-phase marker was seen in bodywall muscle cells from L2 onwards and even in adult animals (Figure 4 C,D). It appears therefore that the process of bodywall muscle cells re-entering the cell cycle can continue even at the adult stage, when normal somatic cell division has been completed. In support of this, analysis of the different Cyclin/Cdk expression lines at later timepoints than the L3 stage showed even higher numbers of muscle cells with mitotic events (data not shown). These data suggest that cell cycle re-entry of differentiated body wall muscle is a continuous process, the effects of which become more pronounced with extended time or as development progresses.

The CYD-1/CDK-4 complex is a more potent activator of DNA replication than CYE-1/CDK-2

To determine the extent of DNA replication in the different Cyclin/Cdk lines, we used EdU, a thymidine analogue that can be stained without the use of antibodies and DNA denaturation of the sample (Salic and Mitchison, 2008). This allowed us to examine DNA replication in combination with GFP antibody staining to detect the nuclei with transgene expression. We stained L4 animals for both EdU and GFP and analyzed muscle cells anterior of the vulva. To our

surprise, only 2 out of 35 CYE-1/CDK-2AF animals had detectible EdU incorporation in a single bodywall muscle, and all other muscle cells were negative (Figure 5 B,D circles). On the other hand, the anterior of more than half (19/35) of the CYD-1/CDK-4 animals contained EdU positive bodywall muscle cells (Figure 5A arrows, C). Hence, even though CYE-1/CDK-2AF animals show obvious signs of mitosis and S-phase entry (Figures 3 and 4), the CYD-1/CDK-4 combination might trigger a more faithful induction of the cell cycle in differentiated muscle cells.

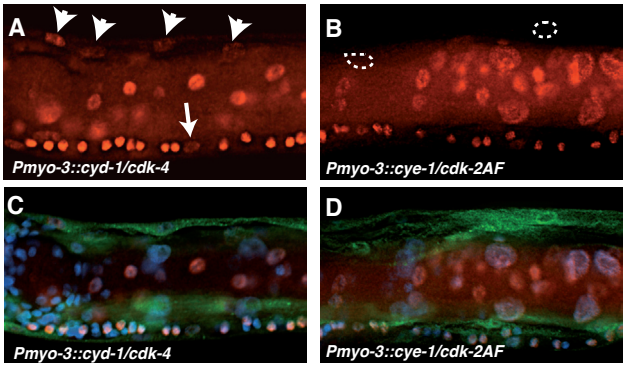


Figure 5. Animals expressing CYD-1 and CDK-4 have more DNA replication in the bodywall muscle than CYE-1/CDK-2AF animals. A-D: Incorporation of the thymidine analogue EdU in the bodywall muscle. A,B: EdU staining, C,D,: merged image of EdU, GFP and DAPI staining. Whereas EdU-positive nuclei can easily be detected in the bodywall muscle of CYD-1/CDK-4 animals (for example in A, arrowheads), CYE-1/CDK-2AF animals rarely show EdU stained bodywall muscle nuclei (B, circles). For comparison, the arrow in A indicates a Pn.p cell, which completed one round of DNA replication in the presence of EdU.

Dividing bodywall muscle activate a highly specific cell cycle transcriptional program while retaining their muscle fate

Our combined data indicate that differentiated bodywall muscle cells can re-enter the cell cycle post-embryonically in response to G1/S Cyclin/Cdk expression. This argues against models that envision the differentiated state as fixed and excluding any possibility of cell cycle initiation. Therefore, we wanted to examine whether induction of the cell cycle coincides with loss of muscle differentiation. Animals expressing G1 Cyclin/Cdk complexes in the bodywall muscle appear phenotypically normal and move normally over the plate. We quantified movement by determining the average number of body bends per minute. This assay did not reveal a significant effect of CYE-1/CDK-2AF-expression on animal motility (Figure 6C). To examine muscle structure, we stained animals for UNC-15/Paramyosin, a component of thick muscle filaments in *C. elegans* (Moerman and Williams, 2006). Muscle cells in L4 and adult CYE-1/CDK-2AF animals displayed a normal pattern of thick muscle filaments, even when nuclei with clear mitotic figures were present (Figure 6A,B, arrows). This further supports the idea that CYE-1/CDK-2AF expression does not change muscle structure and function. However, these methods cannot exclude subtle changes in gene expression and muscle fate.

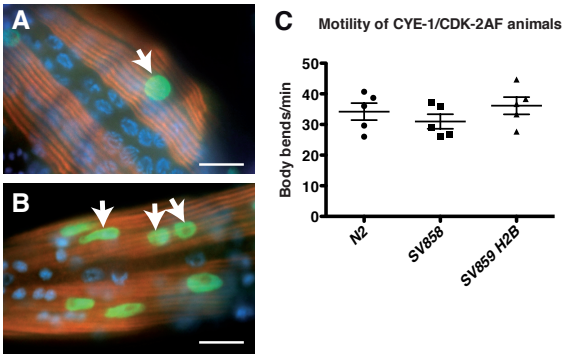
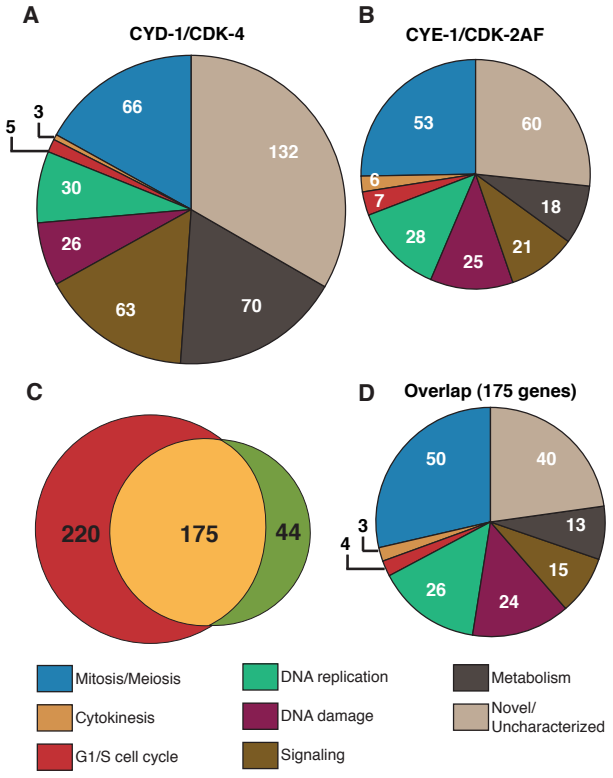


Figure 6: Animals with dividing bodywall muscle retain normal motility and muscle structure. A,B: UNC-15/Paramyosin staining of a control (*Pmyo-3::GFP::H2B*) line (A) and a line with muscle expression of CYE-1 and CDK-2AF (B). Bodywall muscle in both animals show thick filament structures, despite the signs of mitosis in B (arrows). C: Motility assay of L4 larvae: N2 is wild type, SV858 contains *Pmyo-3::cye-1/cdk-2AF*, and SV859 is a *Pmyo-3::GFP::H2B* control. Each dot represents a single animal.

We set out to obtain a comprehensive picture of the transcriptional changes induced by Cyclin/Cdk expression in bodywall muscle and additionally, gain understanding of the differences between CYE-1/CDK-2AF and CYD-1/CDK-4 in the induction of cell cycle gene expression. We performed tissue-specific mRNA profiling on bodywall muscles using a method developed in the Kim lab (Roy et al., 2002). In this approach, mRNA is extracted by immunoprecipitation of a FLAG-tagged poly-A binding protein (PAB-1) expressed specifically in the muscle cells. The mRNA bound to PAB-1 is then isolated and used as a probe for hybridization on microarray chips (Figure S5A). mRNA isolated from *Pmyo-3::FLAG::PAB-1* transgenic animals showed a high enrichment for muscle-expressed genes, as previously described by Roy et al. (2002), confirming our ability to isolate muscle-enriched mRNA with this method. We next compared the mRNA profiles of control L1 animals (carrying the *Pmyo-3::GFP::H2B* and *Pmyo-3::FLAG::PAB-1* transgenes), and L1 animals with muscle specific expression of CYE-1/CDK-2AF or CYD-1/CDK-4 in combination with FLAG::PAB-1. The experiment was repeated four times for each line. After dye correction, MANOVA statistical analysis was performed to determine the genes that were significantly enriched over total RNA (see Materials and Methods for a detailed description). We then compared the mRNA profiles of the control line with our CYE-1/CDK-2AF and CYD-1/CDK-4 lines.



E

Mitosis/Meiosis	DNA replication	DNA damage	G1/S cell cycle
cyb-3/Cyclin B	*mcm-2-7* MCM	*chk-1*/Chk-1	*cki-1*/p27
air-1 Aurora A	*pri-1*/DNA Primase	*scc-1*/RAD21	*cki-2*/p27
plk-1 Polo Kinase	*pri-2*/DNA Pimase	*msh-6*/MUTS	*dpl-1*/DP
bub-1 BUB1	*rfc-3/RFC3*	*brd-1*/BARD1	*skpt-1*/Skp1 related
zen-4/Kinesin 6	*rfc-4/RFC4*	*msh-2*/MSH2	
bmk-1/Kinesin 5	*Y53F4B.3*/DNA Pol-ε	*exo-3*/APEX exonuclease	
spdl-1/Spindly	*pcn-1*/PCNA	*cku-70*/XRCC6	

Figure 7. Microarray analysis reveals specifically induced transcription of cell cycle genes in differentiated muscle cells. A: Pie chart for the annotated functions of the genes that were significantly increased more than 2-fold in *Pmyo-3::cyd-1/cdk-4* muscle cells (see Materials and Methods for a detailed description of data analysis). B: A similar pattern is seen in the mRNA profile of the *Pmyo-3::cye-1/cdk-2AF* animals. A large overrepresentation of genes involved in various stages of the cell cycle is seen in both data sets, although there is a distinct increase in the proportion of metabolism and signaling genes in CYD-1/CDK-4 animals. C: Overlap in more than 2-fold upregulated genes between CYE-1/CDK-2AF and CYD-1/CDK-4 bodywall muscle cells. The majority of the genes induced by CYE-1/CDK-2AF are also induced in CYD-1/CDK-4, but the latter also contains a substantial number of non-cell cycle genes. D: Functional annotation of the genes present in the overlap between CYD-1/CDK-4 and CYE-1/CDK-2AF reveals a strong (60%) representation of known cell cycle genes. E: Table of representative cell cycle genes and their orthologs that are induced by both CYD-1/CDK-4 and CYE-1/CDK-2AF (>2-fold upregulated).

A set of 219 genes was significantly upregulated ($p < 0.05$) more than 2-fold in CYE-1/CDK-2AF animals compared to the control line. Manual annotation and GO-term enrichment analysis (FuncAssociate, see Materials and Methods) of these induced genes revealed a large overrepresentation of genes involved in various aspects of the cell cycle, including G1/S regulation, DNA replication, DNA damage response, mitosis, and cytokinesis (Figure 7B). In contrast, the majority of the genes, including all muscle-specific genes, did not show a significant change in expression levels in CYE-1/CDK-2AF animals, confirming that these muscle cells retain a muscle-specific transcriptional program. A substantial number of the induced cell cycle related genes has ascribed functions in DNA damage or checkpoint control. This probably indicates that cells re-entering a cell cycle activate genes that are potentially needed for dealing with DNA damage or chromosome alignment defects. Knockdown of *chk-1* caused a significant increase in the number of division events in CYE-1/CDK-2AF expressing muscle cells (Figure S5C, arrow). Thus the DNA replication/DNA damage checkpoint may in fact prevent abnormal mitosis of muscle cells with incompletely duplicated DNA.

Similar to CYE-1/CDK-2AF, CYD-1/CDK-4 expression in the bodywall muscle induced a set of 395 genes with a strong cell cycle signature (Figure 7A). The majority of the genes upregulated in CYE-1/CDK-2AF are also upregulated in CYD-1/CDK-4 (Figure 7C,D). The overlapping gene set has a particularly strong cell cycle signature, with over 60% of the genes placed in a cell cycle category (Figure 7D,E). Interestingly, CYD-1/CDK-4 also induces a large set of 220 genes that are not cell cycle-related, but are highly enriched for cellular growth and metabolism GO annotation terms (Figure 7C, data not shown). This suggests that CYD-1/CDK-4 has additional functions in G1 in stimulating cellular growth and metabolism. A major function of G1/S Cyclin/Cdk complexes is the inactivation of the pRb-family of transcriptional co-repressors. Inhibition of pRb proteins allows activator E2F transcription factors to turn on expression of genes required for S-phase (van den Heuvel and Dyson, 2008). We analyzed the promoter sequences of the > 2-fold upregulated genes in both CYD-1/CDK-4 and CYE-1/CDK-2AF (Materials and Methods) and found that these sites were significantly overrepresented in the promoters of induced genes in both datasets ($p < 0.001$, Fisher's Exact test). In summary, differentiated bodywall muscle cells that are stimulated by Cyclin/Cdk expression to re-enter the cell cycle activate a highly cell cycle-specific transcriptional program, while retaining muscle specific gene expression.

Identification of novel mutations that lead to extra division events in bodywall muscle cells

Animals expressing CYD-1/CDK-4 in the bodywall muscle show few extra division events at the L3 stage, but our EdU analysis indicates more normal cell cycle induction than CYE-1/CDK-2AF expressing muscle. The low amount of extra division events in the CYD-1/CDK-4 background made this an attractive sensitized background in which to screen for mutants with an enhanced extra division phenotype in the bodywall muscle. We used *Pmyo-3::CYD-1/CDK-4* expression in combination with a *daf-16* null allele as a sensitized background. *daf-16*/FoxO is an important factor in regulating growth and division, in *C. elegans* as well as mammals (Baugh and Sternberg, 2006; Burgering and Kops, 2002). We screened approximately 8000 genomes and found 5 mutants with significant enhancement of the extra mitosis phenotype. Two of the mutations, *he228* and *he229*, could be recovered and maintained as homozygous strains. *he228* mutants show the most dramatic increase in extra mitotic events, with many bi- or multinucleated bodywall muscle cells (Figure 8A,E). Quantification at the L4 stage revealed

a threefold increase in the amount of extra mitotic events in this mutant compared to the starting strain (Figure 8E). *he229* mutants have a less pronounced increase in mitosis, and the phenotype may not be fully penetrant (Figure 8E). Nuclei in the *he229* mutant are often abnormal with chromosome bridges (Figure 8B arrows,C). Preliminary analysis has revealed that the post-embryonic blast cell lineages, such as the intestine and ventral cord precursor (P) cell lineage, are unaffected in these mutants. These tissues are susceptible to cell cycle deregulation by single gene mutations, such as *lin-35* Rb, *cdc-14* and *cki-1* Cip/Kip (Boxem and van den Heuvel, 2001; Saito et al., 2004). Vice versa, we have not observed enhancement of the extra mitotic events in an RNAi screen that included known cell cycle regulators and 263 transcription/chromatin related genes. Hence, it will be of great interest to identify the genes defined by the *he228* and *he229* mutations, in order to clarify their contribution in restricting proliferation of differentiated cells.

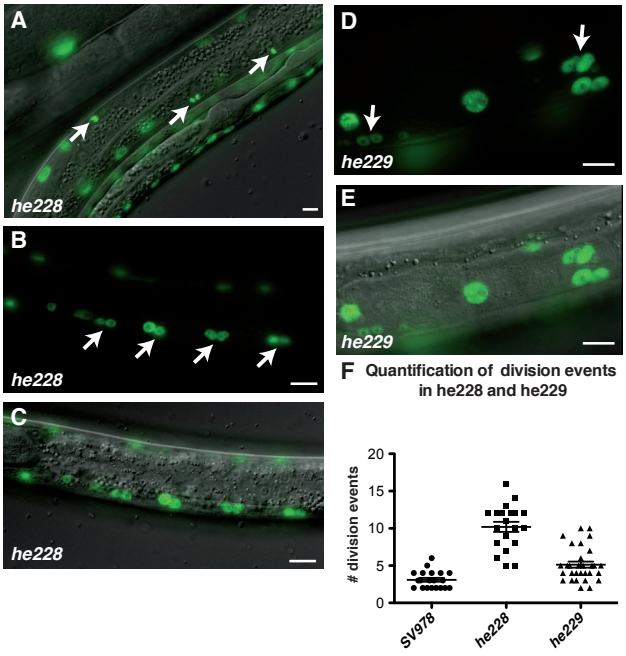


Figure 8. A forward EMS mutagenesis screen identified 2 mutants with enhanced mitosis phenotypes. A-E. Pictures of phenotypes in *he228* (A-C) and *he229* (D,E) mutants. A: Mutant *he228* has a strong increase in mitotic events, resulting in many bi- or multinucleated bodywall muscle cells (A,B arrows). The *he229* mutant often has bodywall muscle cells with clustered nuclei, which may derive from incomplete chromosome segregation (D, arrows). F. Quantification of the number of division events in the control starting line SV978 (*Pmyo-3::cyd-1/cdk-4;daf-16(mu86)*) and mutants *he228* and *he229*. Nuclei with condensed chromosomes, metaphase or anaphase figures and the two nuclei after completed division are counted as one event. Each dot represents a single animal.

Discussion

Cell cycle induction in differentiated bodywall muscle cells by expression of G1/S Cyclin/Cdk complexes

In this study, we analyze the cell cycle arrest of differentiated muscle cells, in comparison to quiescent intestinal cells. In many systems, quiescence and cell cycle exit during differentiation depends on pRb family proteins and Cyclin/Cdk inhibitors such as $p27^{Kip1}$ and $p57^{Kip2}$ (Buttitta and Edgar, 2007; Zhu and Skoultchi, 2001). The same inhibitors are involved in cell cycle exit in *C. elegans*, but they are not critical in establishment and maintenance of the differentiated muscle fate.

Our previous analysis of quiescent vulval precursor cells emphasized the importance of the *cki-1* pathway, with an additional contribution of *lin-35* Rb (Clayton et al., 2008; Saito et al., 2004). Removal of *lin-35* Rb or *cki-1* Cip/Kip also results in extra nuclear divisions in the intestine (Boxem and van den Heuvel, 2001; Ouellet and Roy, 2007; Park and Krause, 1999). Similarly, we found that intestinal expression of Cyclin/Cdk combinations, and even single Cyclins, efficiently induced extra nuclear divisions and DNA replication in the intestine. Furthermore, intestinal expression of CYD-1/Cyclin D or CYE-1/ Cyclin E bypassed the starvation induced quiescence of L1 larvae that are maintained in the absence of food (Baugh and Sternberg, 2006).

Expression of CYD-1/CDK or CYE-1/CDK-2AF also induced a significant cell cycle program in bodywall muscle cells. This conclusion is based on observed expression of an S-phase reporter gene, DNA replication, mitosis, nuclear division, and transcriptional activation of a large set of cell cycle genes. Despite these prominent effects, bodywall muscle cells are clearly quite resistant to cell cycle entry. This is indicated by the partially penetrant mitotic entry and lack of complete division. Moreover, removal of *lin-35*/Rb and/or *cki-1*/Cip/Kip did not lead to cell cycle re-entry of differentiated muscle cells, in contrast to the intestine. In addition, muscle expression of Cyclin/Cdk did not induce cell cycle re-entry in starved L1 animals. Finally, in contrast to the intestine, expression of G1/S Cyclins alone did not trigger cell cycle re-entry in the bodywall muscle (data not shown). Thus, cell cycle arrest in terminally differentiated muscle cells is firm but not fully fixed.

Additional controls act to prevent widespread proliferation of differentiated bodywall muscle cells

As cell cycle re-entry in the bodywall muscle remains limited after Cyclin/Cdk expression, additional safeguards probably inhibit their proliferation. Progression from temporary arrest to irreversible cell cycle exit are thought to depend on epigenetic silencing of cell cycle promoters (e.g. (Jacobs et al., 1999). Nevertheless, a candidate RNAi screen for 263 transcription and chromatin-associated factors did not identify any gene that could induce or increase the ability of differentiated bodywall muscle to enter mitosis (data not shown). While negative RNAi results are little informative, we did pay substantial attention and included mutants for *C. elegans* Polycomb-related genes, Histone deacetylases and a few other good candidates. Based on the data from the gene expression profiling, many cell cycle genes can be efficiently induced in differentiated muscle. Hence, repressive chromatin does not appear irreversible at these promoters.

Interestingly, starvation-induced arrest prevented cell-cycle entry of bodywall muscle cells. This suggests that nutrition-dependent signaling affects the proliferation of muscle cells and may act in parallel to Cyclin/Cdk induction. An insulin-related signaling pathway regulates cell cycle arrest of post-embryonic blast cells in starved L1 animals (Baugh and Sternberg, 2006). Activating mutations in this pathway did not affect the resistance to cell cycle entry in the muscle of starved animals. To examine whether the effect was determined by the larval stage, we examined heterochronic mutations that disrupt the normal timing of stage dependent cell division (reviewed in (Moss, 2007). However, bypassing the L1 stage by using a *lin-14(n179ts)* mutant (Ruvkun and Giusto, 1989) did not affect the absence of cell cycle re-entry in starved L1 larvae. Vice versa, cell-cycle entry was observed in a *lin-4* mutant, which repeats L1 stage development (data not shown). Cyclin or Cyclin/Cdk expression stimulated intestinal cells to incorporate BrdU and undergo nuclear division during starvation, illustrating

that these conditions do not exclude cell cycle progression. Together, these results indicate that a currently unknown nutrition-dependent pathway inhibits cell cycle entry in terminally differentiated cells of starved L1 animals.

In addition, we identified a role for the DNA damage response pathway in Cyclin/Cdk stimulated muscle cells. Various DNA damage response genes are upregulated in muscle cells by expression of CYD-1/CDK-4 as well as CYE-1/CDK-2AF. We expect that the transcriptional induction of these genes forms part of a cell cycle program that is induced upon S-phase entry. We found that removal of *C. elegans chk-1* substantially enhanced the number of mitotic events in CYE-1/CDK-2AF muscle cells, which probably reflects the limited replication. The Chk1 kinase mediates cell cycle arrest in response to DNA damage by inactivating Cdc25 phosphatases (Furnari et al., 1997; Sanchez et al., 1997). Cdc25 removes inhibitory Cdk phosphorylations at sites corresponding to Tyr14 and Thr15 in Cdk1 (Gould and Nurse, 1989; Krek and Nigg, 1991; Norbury et al., 1991). As both of these sites are mutated in CDK-2AF, an endogenous Cdk, probably CDK-1, may be the target of CHK-1 control. However, we cannot rule out other targets, as Chk1,2 kinases also phosphorylate proteins involved in DNA replication such as MCM proteins (Bailis et al., 2008; Ishimi et al., 2003).

Obviously, additional mechanisms that inhibit the cell cycle in differentiated cells remain to be discovered. An EMS enhancer screen identified 2 viable mutants that specifically enhance cell cycle re-entry in the bodywall muscle. These mutants may define novel mechanisms that specifically restrict the proliferation of differentiated cells. In addition, these mutants demonstrate the viability of a systematic genetic approach in identifying the controls that act to restrict proliferation of differentiated cells

Cyclin/Cdk expression in the bodywall muscle induces a highly cell cycle-specific transcriptional program

Bodywall muscle cells expressing G1/S Cyclin/Cdks induce a transcriptional program that is highly enriched in cell-cycle-related genes (Figure 7). These findings indicate that the normally permanent silencing of cell cycle genes at the chromatin level can be reversed. Importantly, CYD-1/CDK-4 induces a wider array of genes than CYE-1/CDK-2AF, including many genes required for metabolism and signaling. Promoter analysis revealed significant enhancement of E2F sites in both CYD-1/CDK-4 and CYE-1/CDK-2AF. Importantly, genes upregulated by CYD-1/CDK-4 and not by CYE-1/CDK-2AF did not show enrichment for E2F sites. This suggests that the CYD-1/CDK-4 complex in *C. elegans* might have growth-promoting functions that are unrelated to Rb/E2F function, as has been reported for Cyclin D and Cdk4 in *Drosophila* (Datar et al., 2000). Such additional functions may explain increased induction of DNA synthesis by CYD-1/CDK-4 as compared to CYE-1/CDK-2AF.

Other studies on Rb/E2F function in *C. elegans* produced somewhat inconsistent results. A clear overrepresentation of E2F binding was found in the promoters of genes differentially expressed in *lin-35* Rb mutants (Kirienko and Fay, 2007). However, this study also revealed that *lin-35* has distinct, non-E2F-related functions in gene expression, especially during embryogenesis. Microarray analysis of *lin-35* Rb, *efl-1* E2F and *dpl-1* DP function in the gonad suggested that *efl-1/dpl-1* function as a conserved activator E2F/DP heterodimer and induce expression of differentiation-related genes (Chi and Reinke, 2006). In contrast to our results and the results for the *lin-35* mutant, these genes did not have a strong cell cycle signature. Furthermore,

similar to *lin-35*, *efl-1* genetically acts as a SynMuv B gene in vulval development and *efl-1(RNAi)* causes extra intestinal divisions (Boxem and van den Heuvel, 2002; Ceol and Horvitz, 2001). These data suggest that *efl-1* acts as a repressor E2F in S-phase gene induction. It is possible that another, as yet unidentified, activator E2F induces the cell cycle-specific transcriptional program in the mitotic bodywall muscle cells.

Deciphering the controls that act to restrict proliferation of differentiated cells

We have shown that terminally differentiated bodywall muscle cells can be triggered to exit the post-mitotic state in response to G1/S Cyclin/Cdk expression. In contrast, removal of *lin-35* Rb and/or *cki-1* Cip/Kip is not sufficient for cell cycle re-entry in the bodywall muscle. This is different from the situation in mammalian tissue culture systems of muscle differentiation. Removal of p27 or pRb, but not p107 or p130, alone is sufficient to trigger cell cycle re-entry in both mammalian and newt muscle myotubes that were differentiated *in vitro* (Blais et al., 2007; Pajalunga et al., 2007; Tanaka et al., 1997). However, loss of pRb family members in these differentiated myotubes not only causes cell cycle re-entry, it also leads to de-differentiation of the muscle fate (Blais et al., 2007). In our *in vivo* model, the differentiated state and cell cycle arrest are maintained in the absence of *Rb* family members, and muscle-specifc expression of Cyclin/Cdk triggers cell cycle re-entry while the differentiated state is maintained.

Data from other model systems suggest that the cell cycle re-entry capability of differentiated cells differs between species and even between the various tissues within one species. For instance, cell cycle re-entry is more strictly regulated in *Drosophila*: activation of both G1/S Cyclin/Cdk complexes as well as the activator E2F, dE2F1, is necessary to induce cell cycle re-entry in post-mitotic differentiated cells of the eye and wing pupal tissue (Buttitta et al., 2007). Interestingly, cells in the pupal eye show more extensive cell cycle re-entry than cells in the pupal wing, illustrating a difference in the arrest of these tissues. This is confirmed by pRb knockout phenotypes in mammalian systems. Acute removal of Rb in quiescent mouse embryonic fibroblasts *in vitro* induces cell cycle re-entry, DNA replication and cell division (Sage et al., 2003). *In vivo* disruption of pocket protein function in mice can lead to cell cycle re-entry of differentiated cochlear hair cells and neurons in the retina (Ajioka et al., 2007; Sage et al., 2005). However, the majority of differentiated cells is unaffected by perturbation of pRb protein function. The difference in susceptibility to cell cycle deregulation is probably determined by other cellular factors that restrict or promote proliferation. Evidence for the latter is seen in the case of human retinoblastoma, where the cells of origin for this tumor express high levels of the proto-oncogenes MDM2 and N-Myc (Xu et al., 2009).

Our work and the examples described above indicate some potential of terminally differentiated cells to resume cell division. Although we are still far from understanding the controls that normally prevent cell cycle re-entry, ultimately manipulation of such controls could contribute to regenerative therapies. Furthermore, delineating these controls might provide valuable insights into tumor ontogenesis. To what extent can a tumor evolve from a differentiated cell and which genetic or epigenetic alterations are necessary to accomplish this? The answer to these questions might depend on the individual tissue. Continuation of our genetic approach is likely to reveal additional controls that restrict proliferation in differentiated cell types and expose tissue-specific differences in proliferative capability.

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Supplementary figures

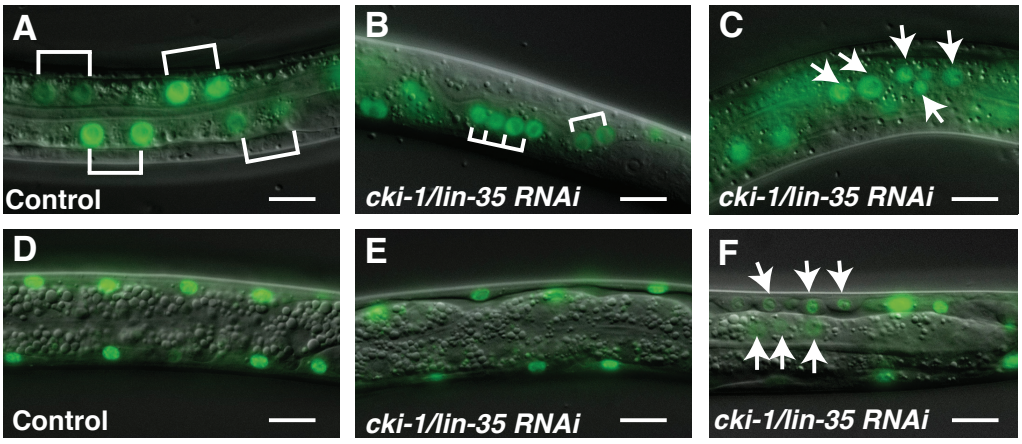

Figure S1. RNAi knockdown of *cki-1* and *lin-35* causes extra division in the intestine, but not in differentiated bodywall muscle cells. A-C: intestinal nuclei marked with *Pelt-2::GFP*. A: Wild-type animals have 2 intestinal nuclei in each intestinal cell at the L3 stage (brackets). B,C: In *lin-35;cki-1* double RNAi animals, nuclei undergo additional divisions (B, brackets), sometimes leading to disorganized clusters of intestinal nuclei (C, arrows). D-F: Bodywall muscle nuclei, marked with *Pmyo-3::GFP::H2B*. D,E: Bodywall muscle nuclei of *lin-35;cki-1* double RNAi animals do not show any signs of cell cycle re-entry anterior of the vulva. Extra divisions do occur in the posterior of the animals, in the post-embryonic cell lineage of the Mesoblast (F, arrows).

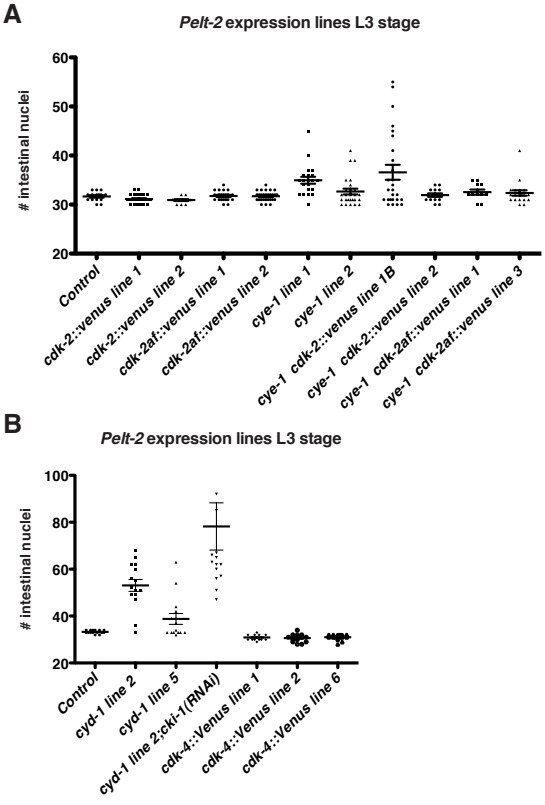

Figure S2. Quantification of different G1 Cyclin/Cdk combinations at the L3 stage. Quantification of intestinal nuclei in different Cyclin/Cdk lines. Each dot represents a single animal. A: CYE-1/CDK-2 combinations trigger extra nuclear divisions in the intestine. Note that CYE-1 alone can induce extra division whereas CDK-2 alone does not. B: CYD-1/CDK-4 shows a similar pattern. Knockdown of the Cip/Kip inhibitor orthologue *cki-1* acts synergistically with intestinal CYD-1 expression in inducing extra divisions. Each dot represents a single animal.

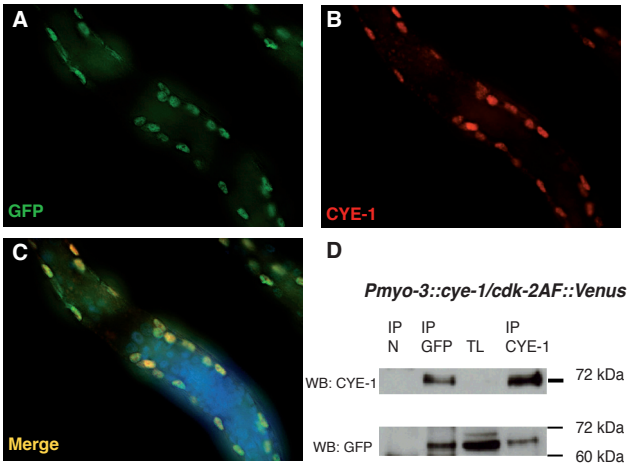

Figure S3. Expression of CYE-1 and CDK-2AF::Venus in the bodywall muscle. A-C: Immunostaining of SV858 (*Pmyo-3::GFP::H2B;Pmyo-3::cye-1/cdk-2AF::Venus*) L1 larvae for CYE-1 and GFP. GFP antibody staining visualizes the bodywall muscle (A), the CYE-1 staining shows nuclear localization of CYE-1 protein in the bodywall muscle (B,C). D: Immunoprecipitation (IP) of the CYE-1/CDK-2AF::Venus complex. CYE-1 migrates with an apparent molecular weight of ~72 kDa. The CDK-2AF::Venus fusion protein is detected at 66 kDa with anti-GFP antibodies. The CYE-1/CDK-2AF::Venus interaction was detected in both the CYE-1 and GFP immunoprecipitations.

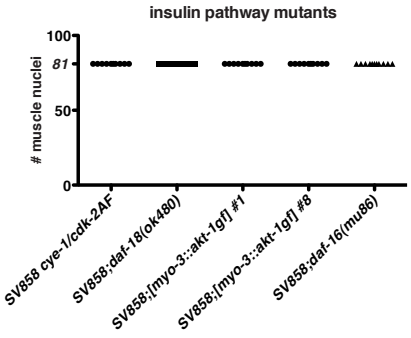

Figure S4. Quantification of extra divisions in starved L1 animals carrying Pmyo-3::cye-1/CDK-2AF (SV858) in combination with various insulin signaling pathway disruptions. Each dot represents a single animal.

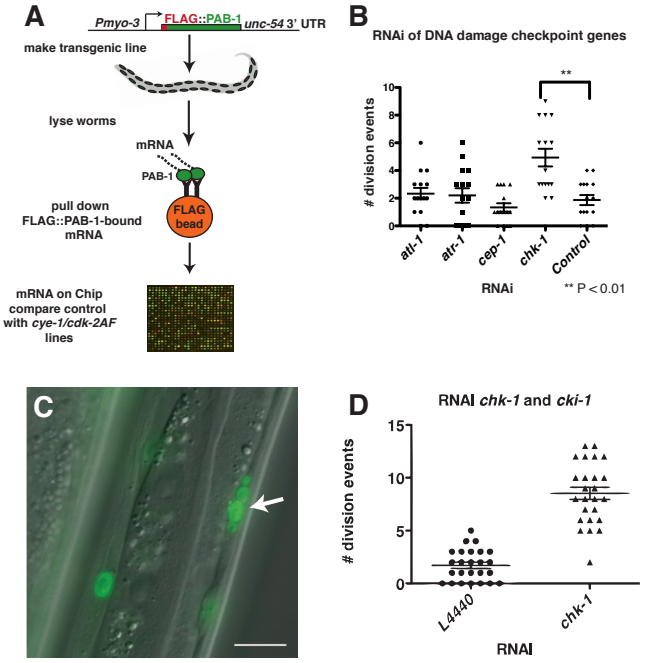

Figure S5. Tissue-specific micro-array analysis reveals a role for the DNA damage checkpoint kinase *chk-1*. A: Outline of the tissue-specific microarray approach. Adapted from Roy et al., 2002. B. RNAi analysis of several DNA damage checkpoint genes. The number of division events was quantified in SV858 (*Pmyo-3::CYE-1/CDK-2AF*) L3 animals at 15 °C. *chk-1(RNAi)* causes a highly significant increase in extra division events. C, D: Further analysis of the RNAi phenotype of *chk-1* in *CYE-1/CDK-2AF* L4 animals grown at 20°C. C: *chk-1(RNAi)* animals often had clusters of small nuclei in their bodywall muscle, as illustrated in the figure (arrow). D: Quantification of extra division events in L4 animals at 20°C. Each dot represents a single animal.

Chapter 4

Deregulation of G1/S progression leads to loss of cell division timing, frequency and fate during asymmetric division in the *C. elegans* seam lineage

Jerome Korzelius [1], Marjolein Wildwater* [1], Tessa Gaarenstroom [1] and Sander van den Heuvel [1]*

[1] Developmental Biology, Utrecht University,
Padualaan 8, 3584 CH Utrecht, The Netherlands.

** These authors contributed equally to this work*

'She's lost control again
She's lost control...'

Joy Division – 'She's Lost Control'

Abstract

During animal development, cells must continuously decide whether to proliferate or differentiate. Across species, this decision is often achieved through asymmetric cell division. During asymmetric cell division, a cell differentially segregates cell fate determinants over its two daughters, resulting in daughter cells with correspondingly different fates. In this study, we use the *C. elegans* seam cell lineage as a model for asymmetric cell division. Seam cells divide asymmetrically to give rise to a posterior cell, which retains the seam cell fate, and an anterior differentiated cell that fuses with the hypodermis, or skin, of the nematode. Previous work has suggested that deregulation of G1 to S-phase progression might influence both seam cell division and fate (Fujita et al., 2007; Hong et al., 1998). In this work, we characterize the effect of G1/S deregulation on seam cell division and timing as well as seam cell fate in detail. Deregulation of G1/S progression by *cki-1(RNAi)* or seam-cell specific expression of *cye-1* Cyclin E and *cdk-2* Cdk2 results in a complete loss of seam cell division timing and frequency. Furthermore, lineage analysis of seam cell division reveals that G1/S deregulation frequently results in a loss or reversal of seam cell fate, with anterior daughter cells that undergo extra division instead of fusing with the hypodermal syncytium. Our results reveal an unexpected role for conserved G1/S regulators in cell fate specification during asymmetric cell division in the *C. elegans* seam cell lineage.

Introduction

Cell division and differentiation are tightly linked during animal development and homeostasis. Many cells will initially divide rapidly during embryonic development, but will subsequently differentiate and withdraw from the cell cycle. However, certain cells will continue to divide during adult life and replace cells in tissues with a high turnover such as skin, blood and intestine (Fuchs, 2009). These tissue-specific stem cells divide to give rise to another stem cell daughter and a more differentiated daughter cell. The timing and frequency of stem cell division needs to be under strict control. Recently, several different tumors were found to harbor a stem-cell-like compartment, with cells that can continually self-renew and give rise to new tumor mass (Barker et al., 2009; Dick, 2008). Hence, identifying the signals that control stem cell-like divisions is of key importance in our understanding of cancer biology.

The *C. elegans* seam cell lineage provides an elegant system in which to study the control of a stem-cell like division in a genetic model organism. The seam cells are a set of epithelial cells that form a lateral row on each side of the animal (Figure 1). These seam cells divide several times during larval development and contribute to the formation of the *C. elegans* 'skin': the hypodermis. A newly hatched L1 larvae starts with 10 seam cells: H0-H2, V1-V6 and T. V1-4 and V6 undergo an asymmetric division during each larval stage with the anterior daughter fusing with the hypodermal syncytium hyp7 and the posterior daughter retaining the seam cell fate (Sulston and Horvitz, 1977). Additionally, the anterior daughter undergoes one round of endoreplication before fusing with the hypodermal syncytium cell hyp7(Hedgecock and White, 1985). The asymmetric division of seam cells is asymmetric in both cell size and fate, with the anterior cell having a 60% smaller cell surface than the posterior daughter cell (M.G. and M. W., unpublished). Before the asymmetric division in L2, the V1-4 and V6 seam cells go through an additional symmetric division, yielding 2 seam cell-fated daughter cells and effectively increasing seam cell number from 10 to 15 in early L2. When the animal reaches the adult stage, the seam cells will stop dividing and form alae, lateral ridges on the hypodermis that aid in movement of the animal. Alae are also present during the L1 stage but are lost during the molt of the L1-L2 transition.

The asymmetrical division of seam cells is governed by both intrinsic and extrinsic cues. One of the pathways involved in establishing the asymmetry of seam cell divisions is the Wnt/β-catenin asymmetry pathway. WRM-1/β-catenin localizes asymmetrically to the cortex in anterior cells and in the nucleus of posterior cells in dividing V-cell daughters. This asymmetry results in activation of the POP-1/Tcf transcription factor in the posterior nucleus that retains the stem-cell fate (Mizumoto and Sawa, 2007; Takeshita and Sawa, 2005). Apart from the Wnt/β-catenin asymmetry pathway, additional cues are important for correct timing and frequency of seam cell division. A key point in controlling cell division and timing is the progression through G1 into S-phase. G1/S progression is governed by a conserved set of molecules in *C. elegans* (Boxem and van den Heuvel, 2001; Park and Krause, 1999; van den Heuvel, 2005). The G1 Cyclin/Cdk complexes CYD-1/CDK-4 and CYE-1/CDK2 act to promote progression from G1 to S. The Rb homologue *lin-35* and the Cip/Kip family member *cki-1* are two negative regulators that inhibit G1/S progression. The *cki-1* gene forms part of an operon together with its paralogue *cki-2*. In mice and Drosophila, p27 and the Drosophila homologue Dacapo regulate the number and frequency of divisions during embryogenesis (de Nooij et al., 1996; Nakayama et al., 1996). In agreement with this, loss of both *cki-1* and *cki-2* leads to embryonic lethality coincident with overproliferation (Fukuyama et al., 2003) and depletion of *cki-1* by RNAi causes precocious

division in seam cells and P-cells in the ventral cord (Hong et al., 1998). Similarly, deregulation of G1/S progression by removal of both *lin-35* and *cki-1* changes the frequency and timing of cell division in several blast cell lineages (Boxem and van den Heuvel, 2001; Fujita et al., 2007; Saito et al., 2004). Interestingly however, depletion of *cki-1* in *C. elegans* also seems to interfere with alae formation (Hong et al., 1998). This might indicate that, in addition to the timing and frequency of cell division, cell fate might also depend on *cki-1*. Additionally, in *cye-1* mutants some seam cells give rise to two daughter cells that fuse with the hyp7 syncytium (Fujita et al., 2007). These results indicate that G1/S deregulation can cause a change in cell fate as well as in cell division timing and frequency.

In this study we set out to characterize the effect of G1/S deregulation on seam cell division timing, frequency and fate. We investigated the effect of the seam cell-specific expression of *cye-1* and *cdk-2* and the removal of the negative G1/S regulator *cki-1*. Temporal analysis of division frequency and lineage tracing experiments reveal that loss of G1/S control affects the fate of seam cell division as well as the frequency and timing of division. We find that seam cells can divide to give rise to additional cells, but also fuse precociously with the hypodermal syncytium, indicating a fate change in the seam cell daughters. Our results demonstrate that deregulation of cell cycle timing and frequency can lead to aberrant stem cell divisions in *C. elegans*.

Materials and methods

Worm strains and culturing

C. elegans strains were cultured on NGM plates seeded with *E. coli* strain OP50 as described (Stiernagle, 2006). Experiments were conducted at 20° C unless otherwise indicated. The following strains were used in this study: N2 Bristol wild-type, SU159 (*ajm-1(ok160) X*; *jcEx44[ajm::GFP]*), FZ223 (*dlg-1::GFP*), SV928 (*heEx319*[*Pdpy-7::GFP;Plin-48::TdTomato]*), SV126 (*lin-35 (n745)* SV921 (*rde-1(pk3301);heIs23*[*Pwrt-2::rde-1;Pmyo-2::TdTomato*]), JR667 *(unc-119(e2498::Tc1)III;wIs51[Pscm::gfp]*), SV1060 (*wIs51[Pscm::gfp]; he*Ex365[*Pwrt-2::cye-1*]), SV1061 (*wIs51[Pscm::gfp];heEx366[Pwrt-2::cye-1;Pwrt-2::cdk-2AF::Venus]*), SV1062 (*wIs51[Pscm::gfp];heEx367[Pwrt-2::cdk-2AF::Venus]*) SV868 (*Pwrt-2::GFP::PH*), SV878 (*Pwrt-2::GFP::tba-2*), SV926 (*wIs51[Pscm::gfp];heEx317[Pwrt-2::cye-1;Pwrt-2::cdk-2AF::Venus]*), SV927 (*heIs19[Pwrt-2::GFP::tba-2]*; *heEx318[Pwrt-2::cye-1;Pwrt-2::cdk-2AF]*).

Synchronization of larvae and lineage analysis

Animals were synchronized at the start of L1 by hypochloride-treatment of gravid adults and hatching the eggs overnight in 2 ml of M9+0.05% Tween-20 in a tissue culture dish. For lineage analysis, 8 to 12 gravid adults were transferred to a NGM plate without bacteria from which they were washed off and transferred to an Eppendorf tube. To remove any remaining bacteria, the animals were washed 3-4 times with M9+0.05% Tween-20. Animals were left for 24 hours in 10 µl M9-0.05% Tween-20 containing 10 mM serotonin to induce egg-laying. The hatched L1 were then transferred to a NGM plate with OP50. Alternatively, an NGM plate containing many gravid adults with eggs was cleared of all worms by adding 2-3 ml of M9+0.05% Tween-20. Adult worms were then washed off with the liquid and this procedure repeated until only eggs remain on the plate. Subsequently, newly hatched L1 animals could be washed off the plate every 30 minutes.

For quantification of the different phenotypes, synchronized animals were mounted on 2% agarose coated slides in M9 with 10 mM Sodium Azide as a sedative. The number of cells, nuclei or spindles in each animal was examined with DIC and fluorescence optics using a Zeiss Axioplan 2 microscope equipped with an EXFO X-Cite fluorescence illumination system and a Zeiss MrM AxioCam camera. For lineage tracing analysis, animals were synchronized as described and mounted in 4 µl of M9 containing 10 mM muscimol as a sedative on a 2% agarose coated slide. For longer observation of L1 development worms were not sedated, but one or two animals were mounted on a 2% agarose coated slide by 3 µl transfer of a droplet S medium from the plate. The coverslip was then sealed with immersion oil to avoid dehydration.

RNAi by injection

Both *lin-35* and *cki-1* dsRNA was generated by in vitro transcription of a PCR template of cDNA. The cDNA of *cki-1* was obtained from the Vidal RNAi library (clone mv_T05A6.1) and the *lin-35* cDNA from the Ahringer RNAi library (clone sjj_C32F10.2). This PCR fragment was used as DNA template for the dsRNA reaction according to manufacturers instructions (Megascript kit, Ambion). We injected young adults directly in the distal gonad with 400-to 500 ng/µl of dsRNA. The F1 could then be used for phenotypic analyses. Progeny laid in the first 12 hours was discarded.

Molecular cloning of Pwrt-2::cye-1 and Pwrt-2::cdk-2AF

To induce seam cell-specific expression of CYE-1 and CDK-2AF, we cloned the *wrt-2* promoter (a kind gift of Thomas Bürglin) into pCGS1, a modified pPD49.26 expression vector (Fire lab vector kit) to create PCGS1-*Pwrt-2*. For *Pwrt-2::cye-1*, the *cye-1* cDNA was digested out of a *Pmyo-3::cye-1* plasmid with *Acc65I* and *BamHI* and ligated into the PCGS1-*Pwrt-2* expression vector using the same enzymes. CDK-2AF::Venus is a modified version of CDK-2 that has the inhibitory Tyr25 and Thr26 residues modified to Ala25 and Phe26, respectively (Quick Change Site-Directed Mutagenesis Kit (Stratagene)), fused to the *C. elegans*-optimized Venus YFP coding sequence (a kind gift of T. Iino).The coding sequence of *cdk-2AF::Venus* was digested out of a *Pmyo3::cdk-2AF::Venus* plasmid with *BamHI* and *Acc65I* and re-ligated into the PCGS1-*Pwrt-2* expression vector using the same enzymes. *Pwrt-2::GFP::PH* was created by re-cloning the *GFP::PH* coding sequence from pAA1 (*Ppie-1::GFP::PH*) into PCGS1-*Pwrt-2* using HindIII/BamHI. *Pwrt-2::GFP::tba-2* was created by recloning the *GFP::tba-2* (alpha-tubulin) coding sequence from pOD107 (*Ppie-1::GFP::tba-2)* into PCGS1-*Pwrt-2* using HindIII/BamHI. Detailed cloning information is available on request.

Generation of transgenic strains

C. elegans strains were microinjected as described (Mello et al., 1991). All *Pwrt-2* promoter-driven constructs were injected at a concentration of 10 ng/µl, since higher levels of this promoter had a deleterious effect on the animals. *Pmyo-2::TdTomato* and *Plin-48::GFP* were both used as co-injection markers at a concentration of 10ng/µl. Integration of transgenic arrays was accomplished by γ-irradiation with 4000 Gy from a ^{137}Cs source. 200 F3 animals were singled and subsequently the F4 was screened for homozygosity. Integrated strains were backcrossed at least twice with N2 before analysis.

Immunostaining and BrdU labelling

Staining of P-granules and CYE-1 was performed by first synchronizing larvae by bleaching and overnight hatching in M9 + 0.05% Tween-20. The hatched L1 were then transferred to a NGM plate with OP50. After 16 hours of feeding, worms were washed 3 times with M9 + 0.05% Tween-20 and once with dH^2O + 0.05% Tween-20. Larvae were mounted on a slide coated with poly-L-lysine and permeabilized by freeze cracking. Animals were fixed by 10 minutes incubation in methanol, followed by 20 minutes in acetone at -20°C. Slides were rinsed twice in PBS + 0.1% Tween-20, and incubated for 60-90 minutes with 50 µl blocking solution (1% BSA and 10% donkey serum in PBS + 0.1% Tween-20). To prevent the slides from drying out, the worms were encircled with a PAP pen and the slides kept in wet boxes. Subsequently, slides were incubated with primary antibody diluted in blocking solution (1:100 for M17C8 mouse anti-CYE-1, rabbit anti-GFP (Sigma) and rabbit anti-Tubulin (Sigma), 1:1 for mouse anti-PGL-1 clone K67 (Hybridoma database) for 60 to 120 minutes. Three more washes with PBS + 0.1% Tween-20 were followed by 45 minutes incubation with the secondary antibody, Donkey anti-Mouse TexasRed or Donkey anti-Rabbit FITC (both 1/100) (Jackson Immunolaboratories). Subsequently, slides were washed 3-4 times with PBS + 0.1% Tween-20, dried and mounted in 4.5 µl Anti-Fade Gold (Invitrogen) containing 1 µg/ml DAPI. BrdU labelling BrdU labelling and staining were performed as previously described (Boxem et al., 1999). anti-BrdU (Sigma) was diluted 1:200, Donkey anti-Mouse TexasRed was diluted 1:100.

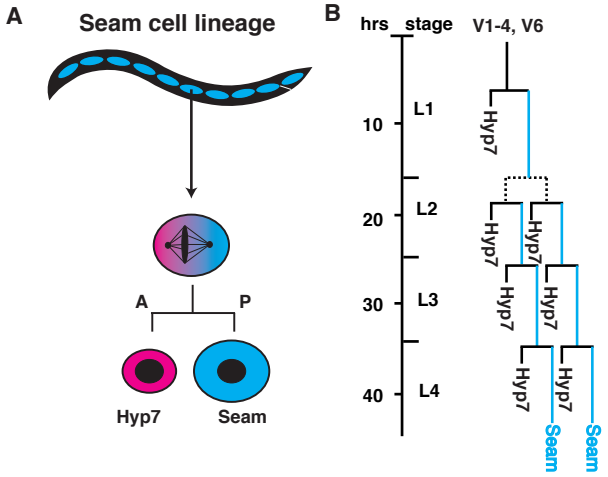

Figure 1. The seam cell lineage in *C. elegans*. A: Position of the seam cells in the worms. The seam cell divides asymmetrically, both in size and fate, to give rise to a hypodermal nucleus and a seam cell daughter. B: Lineage tree of V1-4 and V6 seam cells. Dotted lines indicate the symmetric division that take place during early L2. Blue lines represent the seam cell lineage.

Results

Seam cell-specific overexpression of CYE-1 and CDK-2 results in aberrant cell fates as well as spindle malformation defects.

In *C. elegans*, the Cyclin E homologue *cye-1* and its partner *cdk-2* both have conserved roles in G1 progression. In addition, *cye-1* and *cdk-2* mutants have fate determination defects in the vulval precursor cells and the seam cells (Fay and Han, 2000; Fujita et al., 2007). To examine these defects in fate determination in more detail, we generated overexpression lines of CYE-1, CDK-2AF both under the seam cell-specific *wrt-2* promoter. In the intestine, expression of either a G1/S Cyclin alone or a Cyclin/Cdk combination is sufficient to induce extra division

and S-phase entry, even in developmentally arrested L1 animals hatched in the absence of food (J.Korzelius, unpublished). Therefore, we tested whether seam-specific expression of *cye-1* and *cdk-2AF* resulted in extra division or S-phase in starved L1's. We used incorporation of the thymidine analogue BrdU to visualize S-phase in the seam cells of animals that were hatched in M9 medium. To facilitate the quantification of seam cell number, we injected the *cye-1* and *cdk-2AF* constructs into an integrated *Pscm::GFP* line (JR667, Materials and Methods) and analysed at least 2 independent lines for each construct. Expression of either *Pwrt-2::cye-1* or *Pwrt-2::cdk-2AF* alone did not result in extra division or S-phase entry in developmentally arrested L1's (Figure 2A). However, co-expression of CYE-1 and CDK-2AF resulted in precocious seam cell divisions and DNA replication in starved animals (Figure 2A,B). Staining of control animals revealed that most animals have 2-5 cells that have undergone DNA replication. The Q-blast cell and some V-cells (V5) seem to escape the L1 arrest (Figure 2A,B**)**. However, animals expressing CYE-1/CDK-2AF have significantly more cells that have undergone DNA replication and these BrdU-positive cells sometimes appear in clusters (Figure 2A,C,arrow). Their nuclear morphology and positioning relative to other cells in the larvae strongly suggests that most of these extra BrdU-positive cells are seam cells. The quantification of extra seam cells in starved L1's with the *Pscm::GFP* marker supports these results. In summary, expression of both CYE-1 and CDK-2AF triggers the seam cells to enter the cell cycle, even in arrested L1 animals.

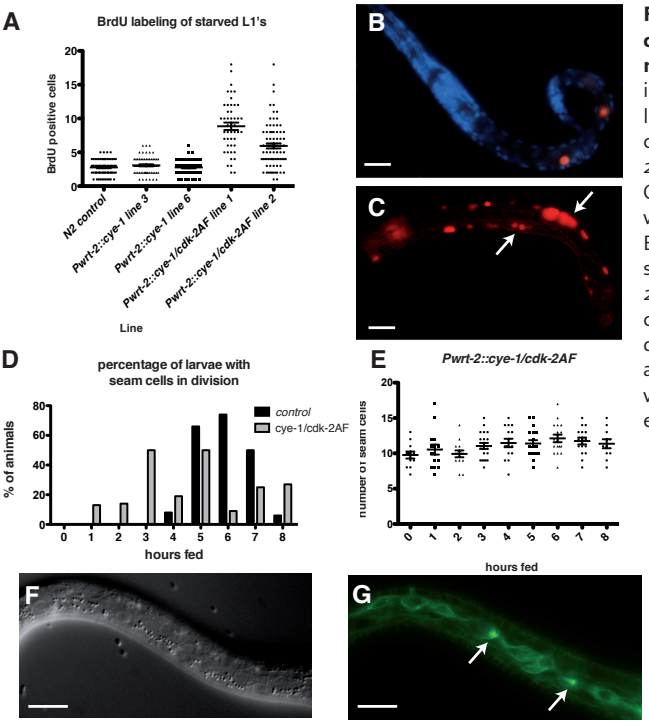

Figure 2. Seam cell-specific expression of *cye-1* and *cdk-2AF* induces DNA replication and cell division. A-C: BrdU incorporation in *Pwrt-2::cye-1/cdk-2AF* lines. A: Quantification of BrdU-positive cells in starved N2, *Pwrt-2::cye-1* and *Pwrt-2::cye-1/cdk-2AF* lines. B. Wild-type N2 and C: *Pwrt-2::cye-1/cdk-2AF* animal stained with BrdU (red) Blue: DAPI. Arrows indicate BrdU-positive seam cells. D: Percentage of seam cells (V1-V6 +T) in division in *Pwrt-2::cye-1/cdk-2AF* lines. E: Number of seam cells (V1-V6 +T) at different time points during L1. F,G: DIC and GFP images of an animal with monopolar spindles, visualized with *Pwrt-2::GFP::tba-2* (G, arrows) in L1's expressing *Pwrt-2::cye-1/cdk-2AF*.

The quantification of the total number of cells, the number of cells undergoing division and the defects seen in dividing cells in *Pwrt-2::cye-1/cdk-2AF* animals all suggest abnormal cell division timing and frequency (Figure 2 D,E). To be able to observe where and when these extra divisions occur during L1 development we followed the seam cell divisions of individual

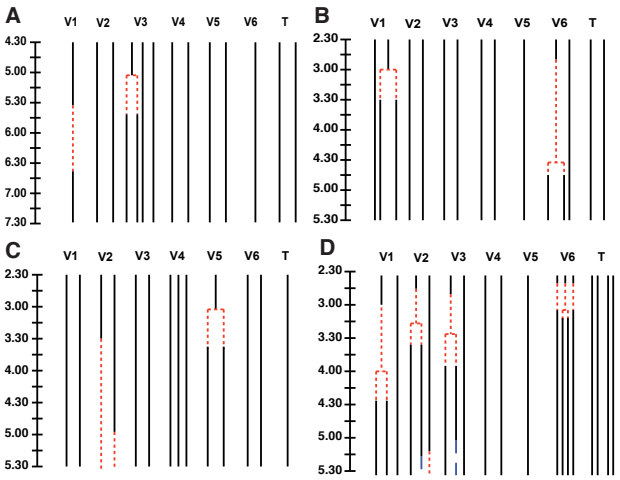

Figure 3. Lineage tracing of 4 different *Pwrt-2::cye-1/cdk-2AF* animals during L1. Animals were followed either from 2:30 hours after hatching (B-D) or from 4:30 hours onwards (A). Dotted lines indicate cells in division. Dashed lines represent cells that have lost the *Pscm::GFP* marker. Note the prolonged period spent in mitosis in many of the V-cells in this background (e.g. in B: V6, C: V2).

animals by lineage tracing. We synchronized the *Pwrt-2::cye-1/cdk-2AF* and control animals by bleaching and hatching in M9 medium. At 4.5 hrs after feeding, when division normally begins, the majority of the seam cells had already divided in the *Pwrt-2::cye-1/cdk-2AF* animals (Figure 3A). Therefore, we decided to start following the divisions from an earlier timepoint, namely 2.5hrs after feeding (Figure 3 B-D). Several seam cells undergo multiple divisions during L1. Curiously, in some cases the anterior daughter cell can undergo an extra division (e.g. Figure 3B: V6). This is unexpected since the posterior daughter is thought to retain the proliferative fate, while the anterior hyp7-fated daughter cell normally differentiates. In addition to this, several seam cells in *Pwrt-2::cye-1/cdk-2AF* animals exhibit a prolonged mitosis, sometimes up to and over one hour (Figure 3A-D, dotted lines). This coincides with a failure to produce a normal bi-polar spindle: sometimes a spindle would form and then disappear without the cell actually having completed a cell division (Figure 3A, V1 cell). Furthermore, the appearance of a monopolar spindle was often observed during our lineage tracing (Figure 2F). Altogether, these results demonstrate that, in addition to deregulating cell division timing and frequency, deregulation of G1/S progression by CYE-1/CDK-2AF expression can lead to changes in cell fate in the seam lineage in *C. elegans*,

cki-1(RNAi) animals have defects both related and unrelated to cell division

Depletion of *cki-1* by RNAi is known to lead to supernumerary divisions in various lineages, including the seam cell lineage (Hong et al., 1998). In the light of our results with CYE-1/CDK-2 expression in the seam cells, we decided to study the defects of *cki-1* RNAi animals in more detail in the seam cell lineage. We injected young adults with *cki-1* dsRNA and analyzed seam cell division in the L1/L2 stages. To track seam cell divisions, we used a *Pscm::GFP* marker that expresses GFP in the nuclei of seam cells. In addition, we use the *Pwrt-2* seam-specific promoter to express the membrane marker *GFP::PH* and the alpha-tubulin marker *tba-2::GFP*. These markers enable us to closely track the seam cell number, fate and division pattern in synchronized control and *cki-1(RNAi)* animals.

We scored the phenotype of the F1 *cki-1(RNAi)* worms 14 hours after feeding (Figure 4). On average, 54.7% of the *cki-1(RNAi)* animals had too many seam cells in comparison to their

staged controls (Figure 4A). This is in agreement with the data from Hong et al. and supports the role of *cki-1* in controlling cell cycle entry. In addition to extra cell division, we also noticed several other defects in the *cki-1(RNAi)* larvae. 12.6% of the animals showed a Vab (Variable Abnormalities) phenotype with characteristic bulges at the tail or head. Often these bulges seemed to contain hypodermal nuclei and coincided with disruption of normal alae formation (Figure 4B, arrow). 20.5% of the *cki-1(RNAi)* animals had clusters of seam cells (Figure 4C). A cluster was defined as a group of 3 or more seam cells at a position in the worm that is usually occupied by just 1 seam cell. This clustering indicates that a seam cell went through several rounds of division and produced daughters that also had the seam cell fate. Another division-related defect we noticed occasionally was the appearance of bi-nucleated seam cells (Figure 4E). By Nomarski optics we could sometimes observe 2 closely apposed nuclei (Figure 4E, arrows), with the *GFP::PH* marker, confirming that these nuclei are in one cell.

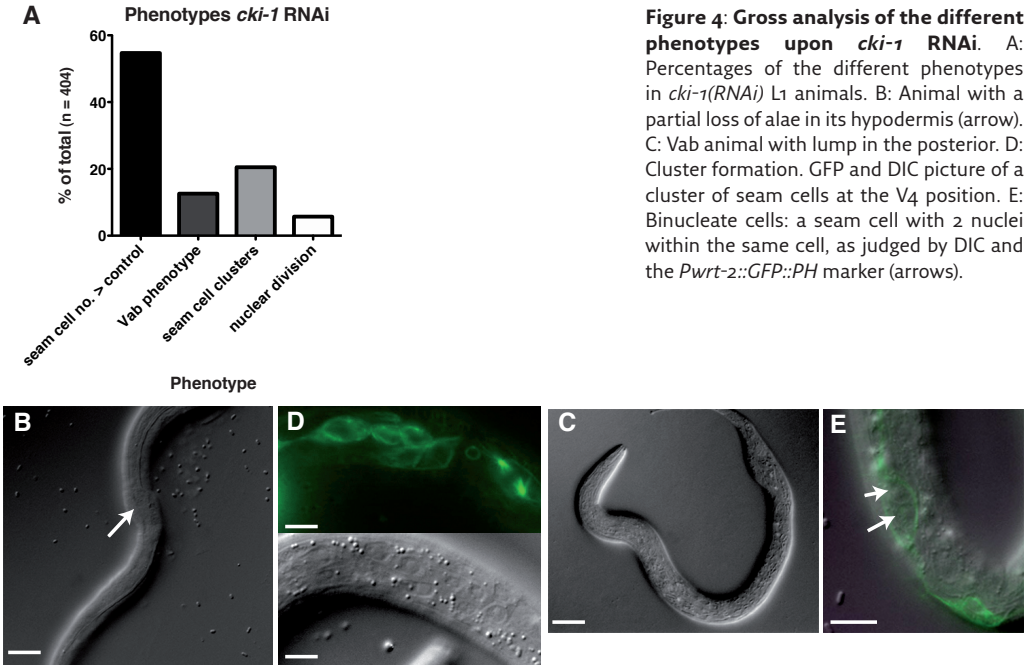

Figure 4: **Gross analysis of the different phenotypes upon *cki-1* RNAi**. A: Percentages of the different phenotypes in *cki-1(RNAi)* L1 animals. B: Animal with a partial loss of alae in its hypodermis (arrow). C: Vab animal with lump in the posterior. D: Cluster formation. GFP and DIC picture of a cluster of seam cells at the V4 position. E: Binucleate cells: a seam cell with 2 nuclei within the same cell, as judged by DIC and the *Pwrt-2::GFP::PH* marker (arrows).

Finally, *cki-1(RNAi)* also resulted in a phenotype that had not been reported previously: silencing of various GFP transgenes, resembling the *tam-1* mutant phenotype (Hsieh et al., 1999). This silencing varied between animals, but also within the same animal, with some cells showing bright GFP expression, some weak, and some no fluorescence at all. The silencing became stronger as the animals progressed through development and became nearly complete by mid-L2 stage. We did not observe any clear correlation between the amount of dsRNA used in the experiments and the level of silencing. Non-seam cell transgenes such as *Pelt-2::GFP* (intestine) and *Pmyo-3::GFP* (muscle) were also silenced in *cki-1(RNAi)* animals (Data not shown). *cki-1* and *lin-35* act in parallel to negatively regulate G1/S progression through inhibition of the CYE-1/CDK-2 complex. Thus, removal of *cki-1* would enable the CYE-1/CDK-2 complex to inhibit *lin-35* function. *lin-35*/Rb mutants also display this transgene silencing defects which is thought to occur through a soma-to-germline fate change in these animals (Wang et al.,

2005). This phenotype is accompanied by ectopic expression of germline P-granules in somatic tissues, especially in the intestine. Since *cki-1* and *lin-35* also act in parallel to control G1/S progression, we tested whether the transgene silencing phenotype in *cki-1(RNAi)* animals uses the same mechanism as in *lin-35* mutants. Although we could clearly detect somatic P-granules in *lin-35(n745)* null mutants, we did not see any P-granules in *cki-1(RNAi)* larvae (Figure S1). Hence, the silencing in *cki-1(RNAi)* animals involves a novel mechanism that is independent of the mechanism in *lin-35* mutants. Taken together, *cki-1(RNAi)* animals do not only display phenotypes related to their role in G1/S progression, but also phenotypes that indicate changes in fate. This warrants a more thorough look at the *cki-1* knockdown phenotype in seam cell development.

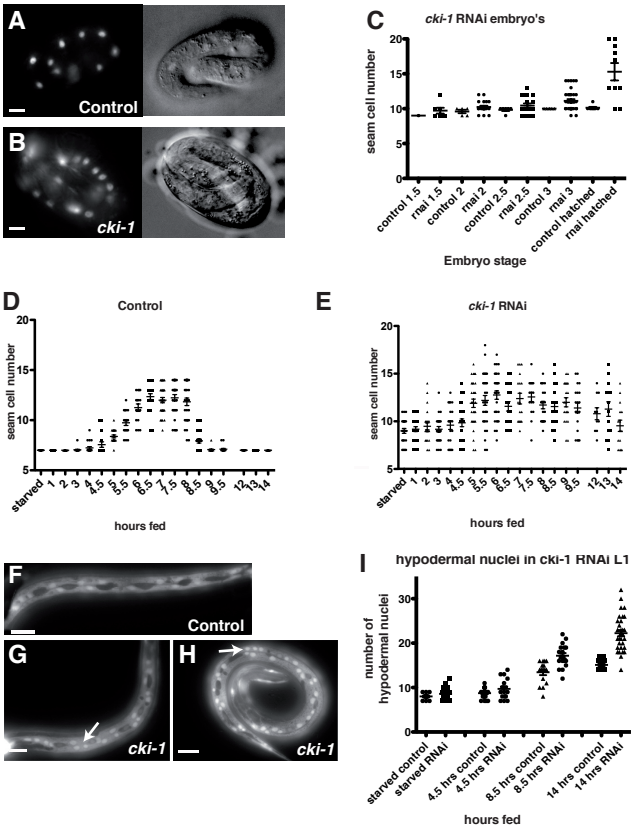

Figure 5. Difference in timing and frequency of division in *C. elegans* embryos and larvae. A, B: Pictures of a 3-fold stage control (A) *and cki-1(RNAi)* (B) embryo expressing *Pscm::GFP*. C: Quantification of seam cell number in embryos from 1.5 fold stage up to hatching. D, E: Number of seam cells (V1-V6 + T) in control (D) and *cki-1(RNAi)* larvae expressing *Pscm::GFP* (E). Each dot represents a single animal. Whereas seam cell number doubles during the 4.5-8 hr period and then sharply decline from 8 hours onwards, this synchronized pattern has disappeared in the *cki-1(RNAi)* larvae. F-I: *cki-1(RNAi)* also affects the number of nuclei in the hypodermis. F: Picture of a wild-type L2 larva expressing *Pdpy-7::GFP*. G,H: *cki-1(RNAi)* animals have extra nuclei in the hypodermis at this stage (arrows). I: Quantification of the number of hypodermal nuclei on 1 lateral side of the animal in wild-type and *cki-1(RNAi)* larvae expressing *Pdpy-7::GFP*.

Extra divisions in cki-1(RNAi) animals start at embryogenesis and continue at a variable frequency during L1

The observation that *cki-1* RNAi resulted in early L1 animals with multiple extra seam cells prompted us to quantify the number of seam cell divisions during embryogenesis to determine when most of the extra cells in the seam lineage first appear. Making use of the *Pscm::GFP* marker, we quantified the number of seam cells on one side of the animal at different stages of embryogenesis, from the 1.5 point elongation stage to larvae immediately after hatching (Figure 5 A,B). Whereas control animals never have more than 11 cells expressing *Pscm::GFP* (10 seam cells and 1 Q-neuroblast that also expressed *Pscm::GFP*), the number of seam cell nuclei

progressively increases during embryogenesis in *cki-1(RNAi)* animals, up to 20 immediately after hatching (Figure 5 A-C). During L1 development, cells normally divide once in asymmetric manner, resulting in one seam cell daughter and one hyp7-fated daughter nucleus, which loses *Pscm::GFP* expression and fuses with the surrounding syncytium (Figure 1). In wild-type animals the seam cells do not divide at the exact same time but within an interval of 4 to 6 ½ hours into L1 development. As seam cell division is followed by fusion of the anterior daughter cells, the number of cells shows a gradual increase and drop during this interval (Figure 5 D). In *cki-1(RNAi)* animals, the synchronization of division was completely lost and *cki-1(RNAi)* animals were observed with a range of 7-18 V1-T seam cells during the course of L1 development (Figure 5E). In addition, the presence of up to 18 V1-T seam cells, which is higher than the wild type amount of 14, suggests that seam cells can actually undergo extra divisions and that the *cki-1(RNAi)* phenotype is not only a precocious division phenotype.

Although additional seam cells were present from early L1 onwards in *cki-1(RNAi)* animals, the average number of seam cells during L1 was only slightly elevated compared to wild-type L1 larvae. These results might indicate precocious division of seam cells and a subsequent delay in their fusion with hyp7, but it might also indicate several rounds of asymmetric division and fusion with hyp7 in these animals. If the latter is true, we would be able to observe extra hypodermal nuclei in the hyp7 of *cki-1(RNAi)* animals. This is indeed the case. Using the marker *Pdpy-7::GFP*, which marks the nuclei in the hypodermis, we observe multiple extra nuclei in the hypodermis of *cki-1(RNAi)* animals (Figure 5G,H, arrows: compare with 5F) indicating that there are indeed extra rounds of asymmetric cell division in these animals. The extra nuclei were usually located anteriorly and ventrally to a seam cell, and in pairs (Figure 5G,H). This might indicate that most seam cells go through one extra round of division and fusion, with a minority going through two or three extra rounds (Figure 5H). Quantification of this phenotype shows a progressive increase in the number of hyp7 nuclei in L1. Whereas control animals have 14-17 hyp7 nuclei in their hypodermis at the L1/L2 molt, the number of seam cells in *cki-1(RNAi)* animals averaged 22 at this stage, with some animals having up to 30 seam cells (Figure 5 I). In summary, these results show that *cki-1* RNAi leads to a loss of both cell division timing and frequency during the L1 stage.

Lineage analysis of cki-1(RNAi) animals reveals a loss of asymmetry in seam cell division

The large spread in seam cell number in L1 larvae, as well as the increased number of nuclei in the hypodermis suggests that the *cki-1* knockdown phenotype is complex: a combination of both loss in division frequency and timing. In addition, several other observations indicate that, similar to the situation in *Pwrt-2::cye-1/cdk-2AF* animals, seam cells in *cki-1(RNAi)* animals may lose their proper seam cell fate. As seam cells are responsible for the formation of alae, the gaps in alae formation that are seen in some L1 animals might be a result of the loss of seam cell fate (Figure 4B). The asynchrony of division in *cki-1(RNAi)* animals makes it difficult to draw conclusions based on observations of a population of different worms. Therefore, we decided to follow the different seam cell divisions in single animals at the L1 stage in both control and *cki-1(RNAi)* animals.

We followed 5 *cki-1(RNAi)* larvae for a course of 3 hours, from 4.5 until 7.5 hours after hatching, the time at which all seam cells divide in wild-type animals (Figure 5 D). Some cells in the *cki-1(RNAi)* larvae had already divided before the start of our analysis at 4.5 hours (e.g. Figure 6B:

V2), indicating a loss of division synchronization. However, other seam cells did not divide over the entire course of our lineage experiment (e.g. Figure 6C: V6). This might indicate that some cells lose the ability to divide as opposed to dividing too frequently as would be expected upon removal of a negative G1/S regulator. Similar to the situation in *Pwrt-2::cye-1/cdk-2AF* animals, we noticed in *cki-1(RNAi)* larvae that the anterior daughter of a seam cell division went through an additional division (e.g. Figure 6C: V2). This abnormal division pattern also indicates that seam cell fate may change upon G1/S deregulation.

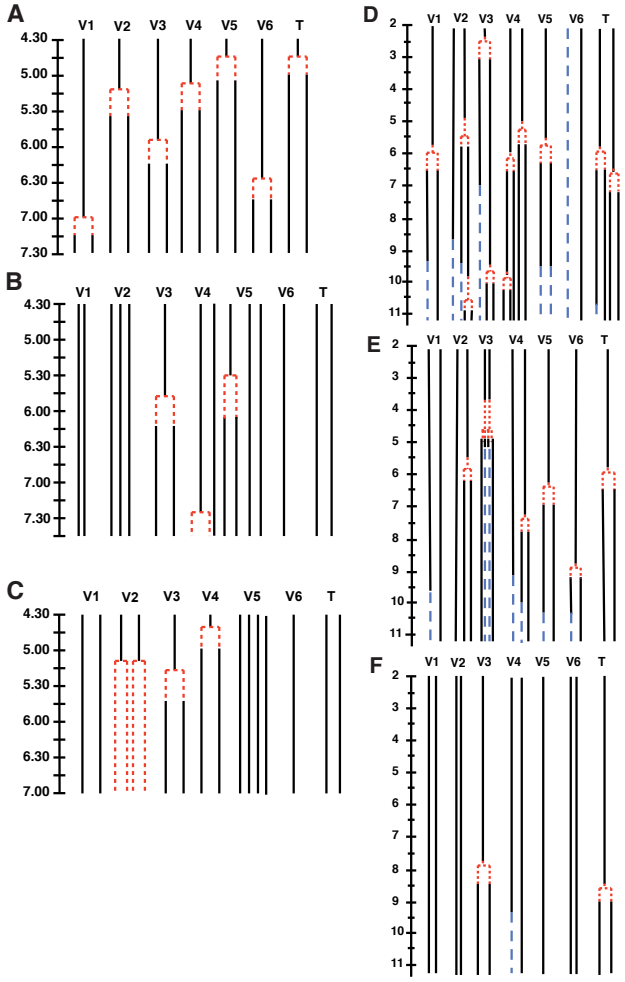

Figure 6. Lineage tracing of *cki-1(RNAi)* larvae reveal loss of seam cell fate and polarity defects. Animals were followed for 3 (A-C) or 9 (D-F) hours at 20°C. Dotted lines indicate cells in division. Dashed lines represent cells that have lost the *Pscm::GFP* marker, indicating a possible loss of seam cell fate. The distance between the lines is indicative for the distance between nuclei A: Wild-type animal followed for 3 hours from 4:30 until 7:30, the normal time of L1 division. B, C: 2 different lineages of *cki-1(RNAi)* animals that were followed for 3 hours. D-F: 9 hour lineages of 3 *cki-1(RNAi)* animals.

We also followed *cki-1(RNAi)* animals for a prolonged period of time, from 2 to 11 hours of L1 development. Even during this extended time course, there were multiple seam cells that did not divide at all. In addition, we observed the formation of a cluster of 4 cells (Figure 6E: V3), multiple divisions from a single parental V-cell (Figure 6D: V2-V4) and division of an anterior daughter cell (Figure 6D: V4). We conclude that RNAi depletion of *cki-1* not only results in supernumerary divisions, but also delays division and changes its normal pattern, possibly caused by a change in seam cell fate.

Discussion

Here we show that knockdown of the cell cycle inhibitor *cki-1*, or expression of both *cye-1* and *cdk-2AF* disrupts the normal timing and frequency of seam cell division. While most cells appear to divide too early in comparison to wild type animals, others divide too late or not at all. Furthermore, although earlier studies have described an inhibitory function for *cki-1* in controlling seam cell division (Hong et al., 1998; Xia et al., 2007), our detailed analysis of G1/S deregulation shows that its function in seam cell division is more complex and likely includes the loss of proper seam cell fate determination. These results shed new light on the connection between cell division control and cell fate determination.

G1/S progression deregulation leads to supernumerary divisions as well as cell cycle delay in the seam cells

We followed seam cell divisions during L1 development in both *cki-1(RNAi)* animals and seam-specific *cye-1/cdk-2AF* expression lines. Extra divisions in *cki-1(RNAi)* embryos only start at later stages in embryonic development. This is in agreement with the fact that *cki-1* expression begins late in embryogenesis, whereas *cki-2* is expressed earlier during embryogenesis (Fukuyama et al., 2003). A complicating factor in the deregulation of G1/S by RNAi is the fact that the knockdown affects *cki-1* mRNA levels throughout the animal. Hence, some of the defects we observe might be due to a cell non-autonomous effect of the *cki-1* knockdown on the seam lineage. To circumvent this issue, we decided to deregulate G1/S progression by expressing the Cyclin E/Cdk2 complex CYE-1/CDK-2AF specifically in the seam lineage (Figure 2,3). The amount of supernumerary divisions in *Pwrt-2::cye-1/cdk-2AF* L1 larvae differed between seam cells and between different animals. Furthermore, a curious observation from our lineage tracing experiments was that G1/S deregulation in seam cells does not only cause them to undergo extra divisions, but can also delay or arrest cell division (Figure 3,6). Although this might indicate variability in the levels of either knockdown or overexpression, it could also mean that there is a positive role for some of these regulators in G1/S progression in *C. elegans*. Studies in mammalian systems have demonstrated that, in addition to its negative role, p27 plays a positive role in G1/S progression by acting as an assembly factor for the Cyclin D/Cdk4 complex (Blain, 2008; Cheng et al., 1999). Although there is no direct evidence of any similar mechanism acting in *C. elegans*, the low levels of *cki-1* might inhibit CYD-1/CDK-4 complex formation early in G1, thereby compromising its ability to trigger entry into S-phase. This explanation cannot be used to interpret the delay in division seen in *Pwrt-2::CYE-1/CDK-2AF* lines, for p27 only has a negative role on the activity of the Cyclin E/Cdk2 complex. The lack of cell division in *Pwrt-2::cye-1/cdk-2AF* animals is often accompanied by a prolonged mitosis, together with the appearance of mono- or multipolar spindles (Figure 2F). A likely explanation for this failure to complete mitosis and cytokinesis is that constitutively high levels of CYE-1/CDK-2 result in a loss of the cyclic activation and inactivation of the CYE-1/CDK-2 complex. It has been shown in mammalian cell culture that overexpression of Cyclin E could inhibit mitotic progression by inhibition of APC^{Cdh1} (Keck et al., 2007). This leads to persistently high levels of mitotic Cyclins like Cyclin B and eventually results in chromosome missegregation. Hence, tampering with the levels of CYE-1 and CDK-2 can disrupt progression through mitosis. Furthermore, the overactivation of CYE-1/CDK-2 might also trigger activation of a G2/M checkpoint in response to aberrant DNA replication during S-phase, which would prevent the proper completion of cell division. More detailed analysis with markers for both mitotic progression and checkpoint activation would be necessary for a comprehensive picture of the effect of G1/S deregulation in the seam cell lineage.

The Wnt/β-catenin asymmetry pathway might be involved in the loss of seam cell fate upon G1/S deregulation.

Our lineage tracking results demonstrate that G1/S deregulation can trigger a fate change in anterior seam cell daughters. Some anterior daughters come to resemble the posterior seam cell daughters and divide several times during the course of L1 development (Figures 3, 6). These anterior daughters also retain the seam cell marker *Pscm::GFP*, a marker that normally disappears from anterior seam cells after their fusion with the hyp7 syncytium. This suggests that the hyp7-fated anterior daughter somehow retains a seam cell fate. A stronger indication of the lack of proper hyp7 fate adoption is the fact that we see *cki-1(RNAi)* animals with a gap in alae formation during L1 (Figure 4 B (Hong et al., 1998). There are several ways through which *cki-1* and *cye-1/cdk-2* might affect cell polarity and fate. It is known that both *cki-1* and *cye-1/cdk-2* are involved in the asymmetric division of the somatic gonad precursor cells Z1 and Z4. The somatic gonad precursors Z1 and Z4 cell generate four cells, one of which will form the Distal Tip Cell (DTC), which serves as a niche and guidance cue for the developing gonad (Kimble and Hirsh, 1979). Loss of *cki-1* results in animals that have either too many or too little DTCs (Kostic et al., 2003). In the absence of either *cye-1* or *cdk-2*, some of the Z1/Z4 daughters, that would normally remain quiescent, will adopt a DTC fate (Fujita et al., 2007). A similar fate change is observed in *cyd-1* mutants (Tilmann and Kimble, 2005). Both studies provide evidence that this phenotype of G1/S regulators correlates with the activity of a Wnt signaling cascade in the somatic gonad precursors. Next to the division of the somatic germline precursor cells, the asymmetric division of the T and V6 seam cells also requires asymmetric localization of Wnt pathway components like WRM-1/β-catenin, APR-1/APC and DSH-2/Disheveled (Mizumoto and Sawa, 2007; Takeshita and Sawa, 2005). Recent work from the Kimble lab has identified a novel β-catenin, SYS-1, which is also involved in the asymmetric division of somatic gonad precursor cells as well as the T cell (Kidd et al., 2005; Phillips et al., 2007). Thus, deregulation of G1/S progression can cause both fate duplication and loss in asymmetrically dividing cells, which is likely to be mediated by a Wnt/β-catenin asymmetry pathway. However, there is still little insight into how G1/S regulators and components of the Wnt/β-catenin asymmetry pathway might interact. Experiments in mammalian cell culture demonstrate that Cdk2 phosphorylates the Wnt pathway components β-catenin and APC in colorectal cancer cell lines (Beamish et al., 2009; Park et al., 2004). Moreover, Wnt signaling is of key importance for the proliferation of a population of stem-cell like progenitor cells, the crypt cells, in the intestine (Radtke and Clevers, 2005). Investigating whether a similar direct interaction exists between G1/S regulators and Wnt pathway components might shed more light on the process of cell fate determination during asymmetric division in *C. elegans*.

In addition, other polarity determinants might be involved in asymmetric seam cell division. Preliminary data from our lab shows that a LIN-5/GPR-1,2/GOA-1 complex, which is important for generating asymmetry in the division of the 1-cell embryo (Srinivasan et al., 2003), plays a similar role in the seam cell lineage (M.Wildwater, personal communication). In summary, the *C. elegans* seam cell lineage provides an excellent system in which to study the intimate, but complex relationship between cell cycle control, polarity and fate determination.

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Supplementary figures

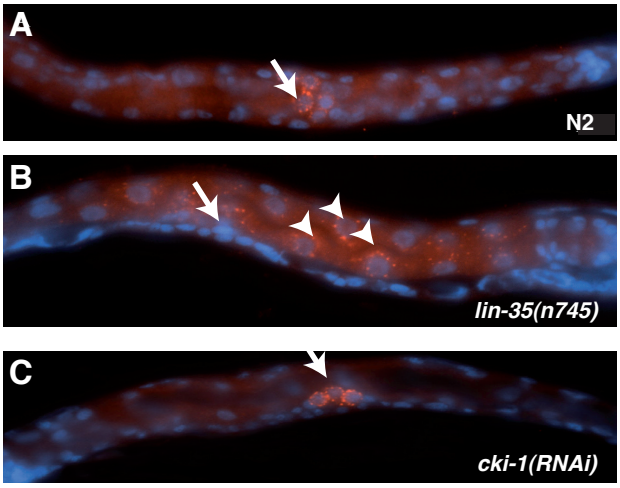

Figure S1. Silencing in *cki-1(RNAi)* animals is not mediated by a soma-to-germline fate change. A-C: P-granule staining of N2, *lin-35(n745)* and *cki-1(RNAi)* L1 larvae. Normally P-granule staining is restricted to the nuclear periphery of the gonadal precursor cells (A-C, arrows). *lin-35(n745)* animals (B) have ectopic expression of P-granules, especially around the intestinal nuclei (arrowheads). This is not the case in *cki-1(RNAi)* L1 larvae.

Chapter 5

Kinase function of *cdk-4* is critical for G1/S progression during *C. elegans* development

Jerome Korzelius [1], Suzan Ruijtenberg* [1], Mike Boxem [1], Inge The [1] and Sander van den Heuvel [1]*

[1] Developmental Biology, Utrecht University,
Padualaan 8, 3584 CH Utrecht, The Netherlands

** These authors contributed equally to this work*

'Slave screams,
Thinks he has something to say'

Nine Inch Nails – 'Happiness In Slavery'

Abstract

How G1/S progression is regulated during the cell cycle is of critical importance for our understanding of both development and cancer. The Cyclin D/Cdk4/6 and Cyclin E/Cdk2 Cyclin/Cdk complexes positively regulate G1/S progression by phosphorylating pRb proteins, which inhibit cell cycle progression in G1. In turn, Cyclin-dependent kinase inhibitors of the pINK and Cip/Kip families negatively regulate progression through G1 by inhibiting G1/S Cyclin/Cdk complexes (Sherr and Roberts, 1999). The Cdk4 kinase has an interesting dual role in promoting G1/S progression. The main function of Cdk4 is the phosphorylation and inactivation of pRb. In addition, p27 is essential for the formation of the Cyclin D/Cdk4 complex. Therefore, Cdk4 is thought to play a secondary, kinase independent role in G1/S progression through inhibition of the Cip/Kip inhibitor p27 by binding and sequestration. Although the effect and mechanism of p27 association with Cdk4 has been extensively studied in mammalian cell culture, the relevance of p27 sequestration in G1/S transition *in vivo* has not been addressed. Here we use *C. elegans* as a model system in which to investigate a possible sequestering role for *cdk-4.* We found that a kinase-dead *cdk-4*, which lacks the ability to phosphorylate *lin-35*/Rb, does not have the ability to rescue the cell division defects in a *cdk-4* null mutant. This indicates that kinase-independent functions of CDK-4 do not contribute greatly to G1/S progression in *C. elegans*. However, it remains to be established whether CKI-1 can bind to the CYD-1/CDK-4 complex and whether this is important for the assembly of this complex.

Introduction

A key decision point in the cell cycle is when a cell decides whether to go through another round of division or withdraw from the cell cycle. This decision is made in the G1 phase of the cell cycle. Entry and progression through G1 is governed by Cyclin D/Cdk4,6 complexes. These are the first Cyclin/Cdk complexes to become active in G1 and many mitogenic factors act directly or indirectly to influence their activity (Ortega et al., 2002). Many tumors have mutations that directly or indirectly disrupt the activity of Cyclin D/Cdk4,6 complexes, underlining the importance of understanding how their activity is controlled (Sherr, 2004). The main target of the Cyclin D/Cdk4 complex is the pRb tumor suppressor gene. pRb phosphorylation by Cyclin D/Cdk4 relieves its suppression of activator E2F transcription factors, thereby allowing S-phase gene transcription (Cobrinik, 2005; van den Heuvel and Dyson, 2008).

Next to the control by mitogenic factors, Cyclin D/Cdk4 complex activity is also regulated by its interaction with 2 different classes of Cyclin Kinase Inhibitors (CKIs). The pINK4 (*IN*hibitor of Cd*K4*) family acts specifically on Cdk4/6 complexes to inhibit their function whereas Cip/Kip family members act on Cdk4 as well as Cdk2 and Cdk1 (Sherr and Roberts, 1999). The main function of CKIs such as the Cip/Kip family member p27, is the inhibition of Cyclin/Cdk complexes by association. Crystallography studies revealed that p27 binds to the Cyclin A/Cdk2 complex and this is most likely to inhibit kinase function in a variety of ways (Russo et al., 1996). In addition, Cip/Kip inhibitors appear to have additional functions next to the negative regulation of Cyclin/Cdk complexes, including inhibition of apoptosis, transcriptional regulation, and regulation of cytoskeletal dynamics (Besson et al., 2008). Due to their potent ability to inhibit G1/S Cyclin/Cdk complexes, Cip/Kip members are often upregulated in Go-arrested or senescent cells as well as upon terminal differentiation to prevent re-entry into the cell cycle in these cell types (Alcorta et al., 1996; Guo et al., 1995; Polyak et al., 1994).

An important discovery on p27 is the apparent dual role of this protein as both a tumor suppressor and an oncogene. Numerous data have provided evidence for the inhibitory role that p27 has in Cyclin/Cdk complex activity, both in vitro and in vivo (Nourse et al., 1994; Toyoshima and Hunter, 1994). However, p27 also appears to be positively required for the proper formation and activity of the Cyclin D/Cdk4 (Blain et al., 1997; Cheng et al., 1999; LaBaer et al., 1997) These results were in contrast with the generally accepted inhibitory role that p27 has on Cyclin/Cdk complex activity. The observations on the two roles of p27 have lead to a model in which the Cyclin D/Cdk4 complex sequesters the p27 protein as G1 progresses, promoting activity of Cdk2/Cyclin complexes and entry into S-phase (Blain, 2008). Hence, next to the kinase-dependent function of pRb phosphorylation, Cdk4 might have a kinase-independent role in sequestering p27. However, the redundancy among G1 and S Cyclin/Cdk's as well as Cip/Kip proteins makes it difficult to address this model using genetic analysis (Kozar et al., 2004; Sherr and Roberts, 2004).

In contrast to more complex metazoans, the small nematode *C. elegans* has only a single D-type Cyclin and Cdk4/6 protein, as well as a single member of the Cip/Kip family. *C. elegans cyd-1*/Cyclin D, *cdk-4* Cdk4/6 and *cki-1* Cip/Kip all have conserved functions in G1/S progression (Boxem and van den Heuvel, 2001; Fukuyama et al., 2003; Hong et al., 1998; Lu and Horvitz, 1998; Park and Krause, 1999). Loss of either *cyd-1* or *cdk-4* leads to similar phenotypes: cells fail to undergo any post-embryonic cell divisions or DNA replication. This can be partially rescued by removal of *cki-1* Cip/Kip or *lin-35*/Rb (Boxem and van den Heuvel, 2001). The combined

knockdown of *cki-1* and *lin-35* in a wild-type background leads to a substantial number of extra divisions in various cell types, underlining a conserved and partially redundant role for these negative regulators of G1/S transition.

In this study, we have used the *cdk-4* mutant phenotype to address the kinase-independent function of *cdk-4* in *C. elegans*. We use low-copy expression of either a wild-type or kinase-dead version of the CDK-4 protein to rescue a *cdk-4* null mutant. Our data indicate that the kinase function of CDK-4 is essential for its function. In addition, the phenotype of animals expressing *cdk-4* kinase dead appeared to be more severe than the *cdk-4* null mutant, indicating a dominant-negative effect. Furthermore, we do not find any proof for CKI-1 sequestration by CDK-4. These data reveal novel functions for CDK-4 kinase activity as well as question the long-standing model of Cip/Kip sequestration by the Cyclin D/Cdk4 complex.

Materials and methods

Strains and culture

C. elegans strains were cultured on NGM plates seeded with *E. coli* strain OP50 as described (Stiernagle, 2006). Experiments were conducted at 20° C unless otherwise indicated. The following strains were used in this study: wild-type Bristol N2, KM48 *(+/szT1[lon-2(e678)] I; cdk-4(gv3)/szt1 X)*, KM122 *(cdk-4(gv3) X; gvEx122[cdk-4::GFP::flag; Rol-6])*, MV17 *(cdk-4(gv3) X; vmEx3[cdk-4wt::flag;sur-5::GFP])*, MV20 *(unc-119(ed3) III; vmIs9 [cdk-4wt::flag unc-119])*, SV882 heEx*364[cdk-4wt::flag;sur-5::GFP]*, SV883 *heEx311[cdk-4kd::flag; myo-2::TdTomato];rtIs14elt-2::GFP]*, SV885 *(cdk-4(gv3) X; vmEx3[cdk-4wt::flag;sur-5::GFP];heEx311[cdk-4kd::flag;Pmyo-2::TdTomato])*, SV915 *(unc-119(ed3) III; heIs22[cdk-4kd::flag; unc-119 (+)])*, SV913 *(cdk-4(gv3)/+; heIs22[unc-119::cdk-4-kd::flag])*, SV929 *(cdk-4(gv3)/+; vmIs9[unc-119::cdk-4::flag]*, *SV1030 rtIs14[Pelt-2::GFP]; heEX345[Pelt-2::cdk-4::Venus;Pmyo-2::TdTomato]*, *SV1060 rtIs14[Pelt-2::GFP]; heEX363[Pelt-2::cyd-1;Pmyo-2::TdTomato]*.

Molecular cloning

The *cdk-4wt::flag* and *cdk-4kd::flag* constructs were made by cloning the genomic cdk-4 ORF together with 3.2 KB of upstream promoter sequence into the pBluescript vector (Stratagene). A linker was used to place a FLAG tag at the C-terminal end of the cdk-4 ORF. For the kinase-dead construct, site-directed mutagenesis was used to create a TG to AA basepair substitution using the primer CGTGAAATTGGCAAATTTTGGATTGTCAAAAG. This resulted in an Asp/Asn substitution at amino-acid position 187, leading to a kinase-dead version of the *cdk-4* construct. *cdk-4wt::flag* and *cdk-4kd::flag* were then re-cloned into a PBluescript vector carrying a *unc-119* rescuing sequence using NotI sites. These constructs were subsequently transformed into *unc-119(ed3)* worms by microparticle bombardment (Praitis et al., 2001). *The elt-2::cyd-1* construct was made by PCR amplification of the *cyd-1* cDNA using primers containing KpnI restriction sites and ligating this into a modified pPD49.26 vector containing the 5 Kb *elt-2* promoter region, linearized with KpnI. Detailed cloning information is available on request.

Microinjection and transformation

C. elegans strains were microinjected as described previously (Mello et al., 1991). Injected concentrations were: *Psur-5::GFP*: 50 ng/µl, *Pmyo-2::TdTomato*: 10 ng/µl, *pRF4 (rol-6(+)):* 50 ng/µl, *cdk-4kd::flag*: 50 ng/µl, *cdk-4wt::flag*: 50 ng/µl, *Pelt-2::cyd-1*: 30 ng/µl, *Pelt-2::cdk-4::Venus*: 30 ng/µl.

RNAi by feeding and injection

A *lin-35* feeding RNAi clone from the Ahringer RNAi library (clone sjj_C32F10.2) was grown overnight in LB with Ampicillin (100 µg/ml) and Tetracycline (12.5 µg/ul). Cultures were concentrated three times by centrifugation. Feeding RNAi was performed on NGM plates supplied with 50 µm/ml Ampicillin and 2 mM IPTG. Several L4 larvae were put on the plate and the resulting F1 progeny was examined. Double strand RNA (dsRNA) of *cki-1* made by PCR amplification from a Vidal library clone (mv_T05A6.1). This PCR fragment was used as DNA template for the RNA synthesis reaction using an *in vitro* transcription kit (Megascript, Ambion). 0.5 to 1 µg/µl of the dsRNA was injected into young adult animals. Progeny from the first day after injection was discarded and subsequent F1 animals were used for analysis.

Synchronization, immunostaining and quantification of *C. elegans* larvae

Animals were synchronized by hypochlorite bleaching and hatching the L1 animals in M9 medium +0.05% Tween-20. The animals, synchronized at the L1 stage, were then placed on NGM or RNAi plates and allowed to develop for the appropriate time. Propidium iodide staining was performed as described previously (Boxem et al., 1999). For the quantification of intestinal and ventral cord nuclei, we used the Bouins fixation protocol to fix later stage larvae (described in Harlow and Lane "Using Antibodies" CSHLP 1999). Younger animals were fixed by freeze-cracking the worms on Poly-L-Lysine coated slides in liquid nitrogen and subsequent fixation in methanol (5 min. at -20 °C) and acetone (20 minutes at -20 °C) (Duerr, 2006). Primary antibodies used in this study were rabbit anti-CDK-4 (1:50, Biosynthesis Corp. clone 5095, information available on request) rabbit anti-FLAG (1:100, Sigma), Mouse anti-FLAGM2 (1:100, Sigma), rabbit anti-GFP (1:100, Invitrogen) and mouse anti-GFP (1:100, Roche). Secondary antibodies were donkey anti-rabbit FITC (1:100), donkey anti-rabbit Texas Red (1:100), donkey anti-mouse FITC (1:100), donkey anti-mouse Texas Red (1:100) (Jackson Immunolaboratories). Animals were mounted in Pro-long Antifade Gold (Invitrogen) supplied with DAPI (1/1000). Immunofluorescence images were taken using a Zeiss Axioplan 2 microscope equipped with an EXFO X-Cite fluorescence illumination system and a Zeiss AxioCam MrM camera. The Zeiss Axiovision 4.5 software was used to acquire and process the images. Quantification of N2 and the integrated *cdk-4wt* and *cdk-4kd* lines was done by staging the worms at the L3/L4 stage, followed by staining with propidium iodide and counting the number of intestinal nuclei and nuclei in the ventral cord, which can easily be identified. In the quantification of nuclei in the ventral cord, the Po and P12 cells (which are located totally anterior and posterior respectively) were left out of the quantification, since these P-cells are very hard to distinguish from the surrounding nuclei at these positions.

Co-immunoprecipitation and Western blotting

Animals for each strain were grown on 30 9 cm plates, washed off with M9 and washed 3 more times with M9+0.05%Tween-20 to wash away the bacteria. Subsequently, worms were pelleted in 15 ml Falcon tubes and frozen in liquid nitrogen. Worms were homogenized by grinding frozen animals and the obtained powder was dissolved in 4 ml of lysis buffer (20 mMTris-HCl pH 7.8, 250 mM NaCl, 15% Glycerol, 1% Triton X-100, 0.5mM EDTA, 14µl β-mercapto-ethanol, 1 protease inhibitor tablet (Roche), 10 mM naphtyl phosphate sodium, 50 mM sodium fluoride, 10mM sodium pyrophosphate decahydrate, 100 µM sodiumortovanadate) and the lysate was further homogenized using a French pressure cell. 1.5 to 2 mg of protein was used for each immunoprecipitation experiment. For the immunoprecipitation of FLAG-tagged CDK-4WT and CDK-4KD proteins, 10µl packed anti-FLAGM2 resin (Sigma) was used and equilibrated in lysis

buffer before use. The immunoprecipitation was performed by adding 1.5 mg of protein to the beads and complementing the volume to 500ul with lysis buffer. After three washes with lysis buffer, 25µl of 1x Laemmli sample buffer+ β-mercapto-ethanol was added to the beads and samples were loaded onto a polyacrylamide gel. Proteins were blotted onto a PVDF membrane (Amersham Bioscience). Blots were incubated with either rabbit anti-FLAG 1:1000 (Sigma), purified rabbit anti CKI-1 1:500 (E. Kipreos), rabbit anti-GFP 1:1000 (Invitrogen), mouse anti-Tubulin 1:1000 (Sigma) as primary antibodies. Secondary antibodies used were: donkey anti-mouse peroxidase (1:5000 Jackson Immunolaboratories), goat anti-rabbit peroxidase (1:10000 Jackson Immunolaboratories). The chemiluminescent detection reaction was performed using the SuperSignal Femto Kit (Pierce) and the blot was subsequently exposed to hypersensitive film (Amersham Hyperfilm, GE healthcare).

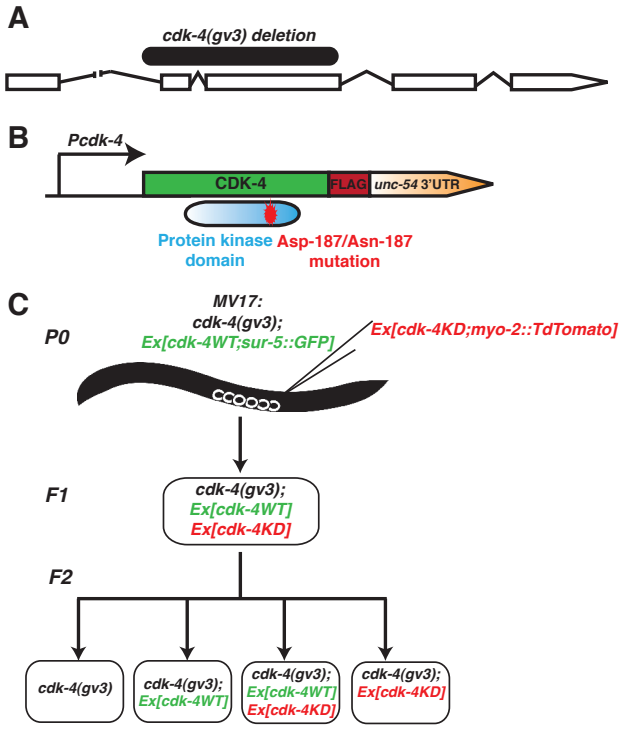

Figure 1: Structure of the *cdk-4* transgene and rescue assay. A: Structure of the *cdk-4* gene. Black bar indicates the *cdk-4* deletion, spanning most of the kinase domain. B: The *cdk-4* transgene used in our rescue assay. Blue bar represents the position of the kinase domain with the Asp/Asn kinase-dead mutation in red. **C**: Transgenic rescue assay performed with the *cdk-4kd* transgene. A line carrying an extrachromosomal array of *cdk-4wt* together with the *sur-5::GFP* marker in the *cdk-4(gv3)* background was injected with the *cdk-4kd* construct together with *myo-2::Tdtomato*, which marks the pharynx of the animal. Single F1 animals carrying both arrays were transferred to different plates. Subsequently, the plates that segregated the 4 classes shown in (C) were selected for analysis.

Results

To examine the contribution of kinase independent functions of CDK-4 we expressed wild-type or kinase dead versions of *cdk-4* in a *cdk-4* mutant background. The *cdk-4* gene in *C. elegans* consists of 5 exons and encodes a protein of 341 amino acids. The *cdk-4(gv3)* mutant used in this study carries a 745 bp deletion, removing most of exons 2 and 3 (Figure 1A). This deletion includes the kinase domain including the catalytic site, making this a null mutant (Park and Krause, 1999). Animals mutant for *cdk-4* lack any post-embryonic division of blast cells and although they undergo a significant amount of cell growth, they arrest as small, sterile, uncoordinated (Stu) larvae. We constructed a wild-type (*cdk-4WT*) and a kinase dead version of *cdk-4 (cdk-4KD)*, coupled to a FLAG-tag under the *cdk-4* promoter and examined whether these

transgenes could rescue the *cdk-4* mutant phenotype. The kinase-dead (KD) version of this construct was made by mutating the catalytic residue at position 187 (Figure 1B). This residue is critical for the positioning of the ATP and removal is predicted to result in a total loss of kinase activity (Taylor and Radzio-Andzelm, 1994). We first tested the ability of the wild-type *cdk-4* construct in rescuing the *cdk-4* mutant phenotype. As expected, we could rescue the *cdk-4* mutant phenotype in the offspring of a heterozygous *cdk-4* animal by expression of a wild-type *cdk-4* construct. Offspring homozygous for the *cdk-4* deletion could be maintained with this transgenic rescue array in the background and showed a full rescue of post-embryonic cell division (data not shown). Next, we used this background to test whether *cdk-4KD* expression could rescue the *cdk-4* mutant phenotype. *cdk-4KD* was co-injected together with a *Pmyo-2::TdTomato* marker and the progeny was examined (Figure 1C). Animals carrying the *cdk-4KD* construct did not show any rescue of the Sterile/Unc phenotype of *cdk-4* mutant animals (Figure 2A-D).

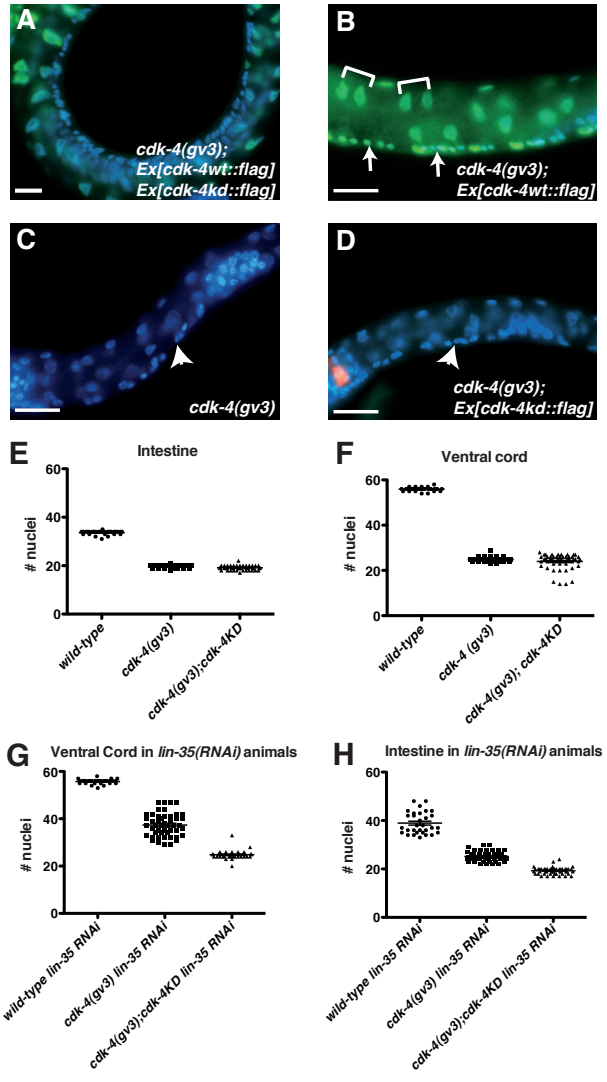

Figure 2: Kinase-dead CDK-4 is not able to rescue the *cdk-4* mutant phenotype in a transgenic rescue assay. A,B: Animals expressing either the wild-type *cdk-4* alone or in combination with *cdk-4::kd* develop into normal larvae. Brackets indicate pairs of intestinal nuclei. Arrows indicate rows of divided P-cell daughters. C,D: animals that only carry the *cdk-4kd* constructs do not show any rescue of larval divisions and have a phenotype similar to the *cdk-4(gv3)* null mutant (D). Arrowheads indicate the gaps in the ventral cord in both *cdk-4(gv3)* and *cdk-4(gv3)* with *cdk-4kd*. E,F: Quantification of ventral cord and intestinal nuclei in cdk-4KD and *cdk-4(gv3)* mutants. G,H: Quantification of ventral cord and intestinal nuclei in *lin-35* RNAi-treated *cdk-4* mutant and rescued animals. Note that the rescue in the *cdk-4kd* line is even less than in the *cdk-4(gv3)* null mutant. Each dot represents a single animal.

To exclude the possibility of missing a partial rescue of the *cdk-4(gv3)* mutant phenotype, we scored the number of intestinal nuclei and P-cells. The *C. elegans* intestine has 20 nuclei at the L1 stage, which increases to 33-34 nuclei after 1 round of division in late L1-early L2. The P-cells are a set of 12 blast cells that migrate ventrally during L1, giving rise to several neuronal and hypodermal cells (Sulston and Horvitz, 1977). *cdk-4* mutant animals arrest with only 20 intestinal nuclei and 12 undivided P-cells in the ventral cord which, together with the nuclei of the 15 juvenile motor neurons, gives a total of 27 cells in the ventral cord. Quantification of *cdk-4KD* animals revealed that they arrest with the L1 number of nuclei in both tissues (Figure 2E,F), indicating that *cdk-4KD* cannot rescue the *cdk-4* mutant phenotype. In mammalian cells, the main kinase function of Cdk4 is the phosphorylation of Rb. Therefore we tested whether removal of *lin-35* Rb could rescue some of the cell division phenotypes in the different backgrounds. Removal of *lin-35* Rb leads to supernumerary divisions in the intestine, but not in the P-cell lineage (Boxem and van den Heuvel, 2001; Ouellet and Roy, 2007). As reported previously, we find that *lin-35(RNAi)* can partially rescue the *cdk-4(gv3)* mutant phenotype (Figure 2 G, H). However, it could not rescue this defect in cell division in *cdk-4(gv3)* animals carrying the *cdk-4KD* construct, again suggesting a possible dominant-negative role of this kinase-dead construct.

parental genotype	% *cdk-4* mutant progeny	n
N2	0.0	many
cdk-4(gv3);cdk-4WT::FLAG	0.36	3/381
cdk-4(gv3)/+	24.51	189/771
cdk-4(gv3)/+;cdk-4KD::FLAG	24.25	131/540
cdk-4(gv3)/+;lin-35(RNAi)	24.18	118/488
cdk-4(gv3)/+;cdk-4KD::FLAG;lin-35(RNAi)	24.72	66/267

Table 1: Scoring of *cdk-4* mutant progeny in wild-type *cdk-4* and *cdk-4kd* integrated rescue lines. Progeny of 3-5 individual animals was scored. *cdk-4(gv3)/+* animals on *lin-35(RNAi)* developed into normal-sized adults, but were all infertile and therefore scored as *cdk-4*. Whereas *cdk-4wt* can almost completely suppress the *cdk-4* phenotype, the *cdk-4kd* line has an almost similar occurrence of the phenotype as the *cdk-4(gv)/+* heterozygote.

It is possible that the excess of kinase-dead CDK-4 expressed in the transgenic array lines acts as a sink that a-specifically sequesters other proteins positively required for cell cycle progression such as S-phase Cyclins. This, in combination with the high levels of expression associated with transgenic array expression in *C. elegans*, could contribute to the dominant negative effect of *cdk-4KD* overexpression. To circumvent the problem of high copy number in transgenic arrays we use the microparticle bombardment protocol to generate low-copy *cdk-4WT* and *cdk-4KD* rescue lines (Praitis et al., 2001). Immunostaining revealed that both constructs were expressed in the proper tissues at similar levels as wild-type (Data not shown). Similar to the situation upon extrachromosomal expression, low-copy *cdk-4WT* expression could rescue the *cdk-4* mutant phenotype and *cdk-4KD* expression could not (Table 1). *cdk-4* mutant animals carrying the *cdk-4WT* rescue construct had similar numbers of intestinal and ventral cord nuclei as wild-type animals, whereas *cdk-4KD* animals do not show any rescue in cell division (Figure 3, figure S1). *cdk-4KD* animals also have a less organized ventral cord compared to *cdk-4(gv3)* mutants (Figure S1F, arrowheads). This suggests that the migration of P-cells into the ventral cord was also affected in these animals similar to the results found for the transgenic arrays. Thus far, we have not found any proof that *cdk-4* has a kinase-independent function in promoting cell cycle transition in *C. elegans*. In fact, our experiments with *lin-35* RNAi suggest that the *cdk-4* kinase might have other substrates next to *lin-35* Rb that need to be phosphorylated to ensure proper G1/S progression.

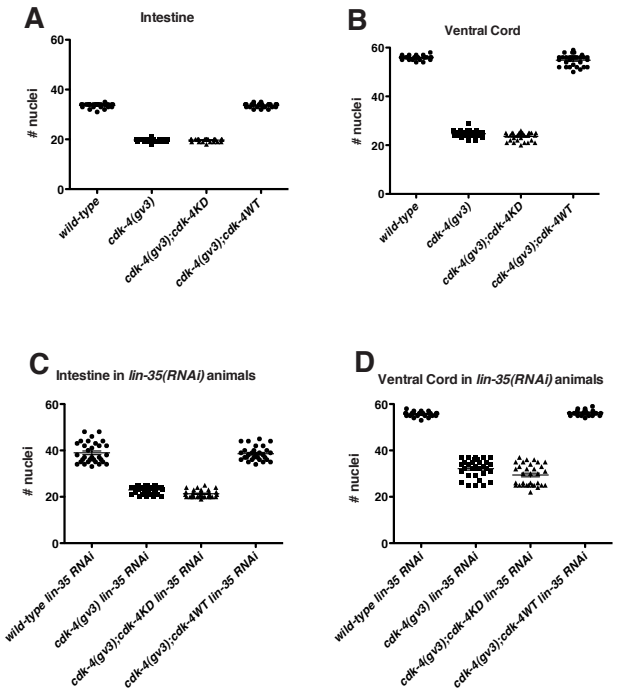

Figure 3. Low-copy integrated kinase-dead CDK-4 is not able to rescue the *cdk-4* mutant phenotype. A-D: Number of nuclei in intestine and ventral cord in WT, *cdk-4(gv3)* and *cdk-4(gv3)* mutants rescued with either WT or KD CDK-4 performed on normal (A,B) or *lin-35(RNAi)* plates (C, D). Note that the dominant negative effect is less pronounced in these integrated *cdk-4kd* lines than in the transgenic array lines.

Expression of a kinase-dead CDK-4 has a dominant-negative effect on cell division

Results with both extrachromosomal and integrated *cdk-4KD* constructs have suggested a dominant-negative role for this construct on cell division and migration of the P-cells (Figures 2,3, figure S1). In order to test the dominant-negative effect of *cdk-4KD* on cell division in the intestine, we expressed either *cdk-4WT* or *cdk-4KD* in a wild-type background and counted the number of intestinal nuclei. Transgenic arrays were created in lines carrying either an integrated *Pelt-2::GFP* transgene that marks the nuclei in the intestine or a *sur-5::GFP* integrated transgene, which marks nearly all somatic cells including the intestine, to facilitate quantification (Yochem et al., 1998). The intestine is a tissue that is prone to cell cycle deregulation by removal of negative G1/S regulators (Boxem and van den Heuvel, 2001; Clucas et al., 2002; Ouellet and Roy, 2007). Expression of *cdk-4WT*, either under its own promoter or specifically in the intestine using the *elt-2* promoter, did not result in an increase in the number of intestinal nuclei. Rather, it seems that there is a slight decrease in intestinal nuclei upon expression of *cdk-4WT*. This result was confirmed by using multiple independent transgenic *Pelt-2::cdk-4WT* lines (Figure 4B,E, data not shown). Intestinal expression of the *C. elegans* only D-type Cyclin together with downregulation of *cki-1* Cip/Kip leads to widespread hyperplasia of intestinal nuclei (Figure 4E,G). In contrast, animals carrying the *cdk-4KD* transgene in an otherwise wild-type background showed a dramatic reduction in the number of intestinal nuclei in several independent lines, confirming the suspected dominant-negative role of *cdk-4KD* on cell division (Figure 4C,D,F).

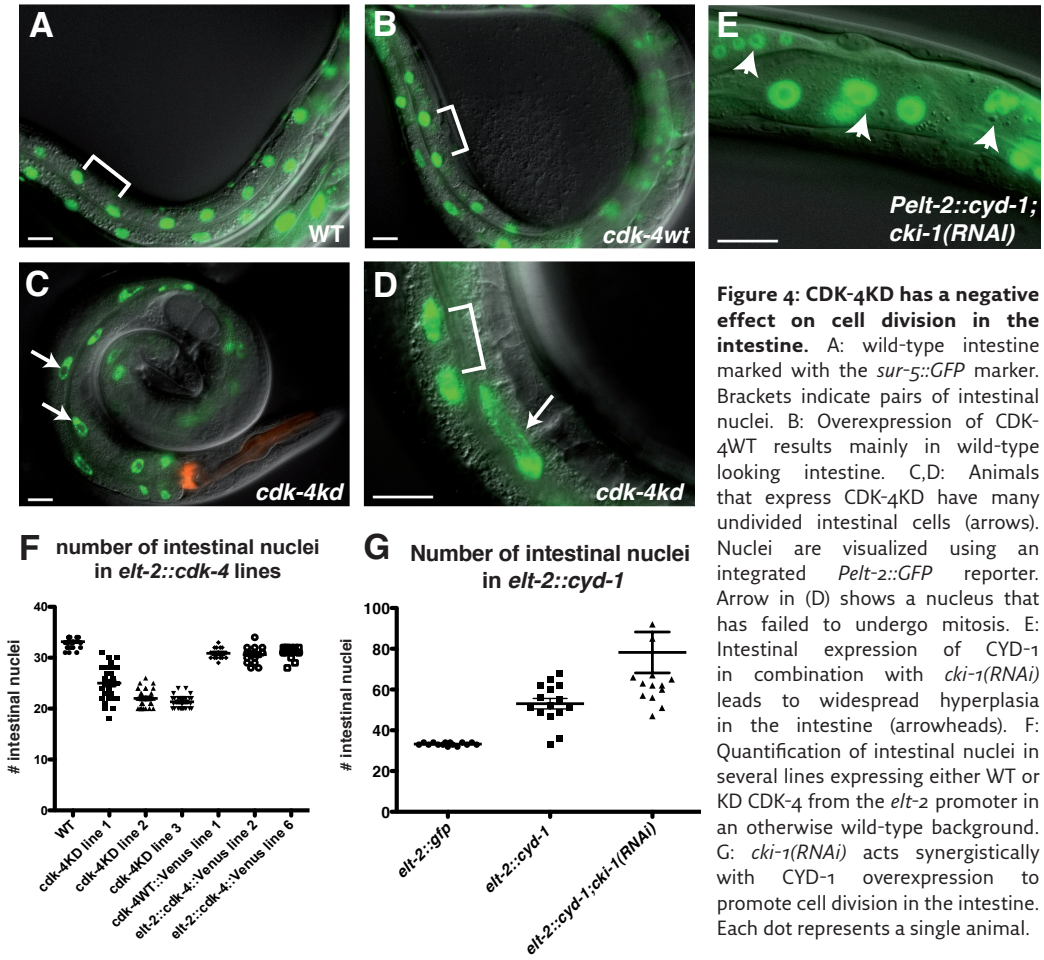

Figure 4: CDK-4KD has a negative effect on cell division in the intestine. A: wild-type intestine marked with the *sur-5::GFP* marker. Brackets indicate pairs of intestinal nuclei. B: Overexpression of CDK-4WT results mainly in wild-type looking intestine. C,D: Animals that express CDK-4KD have many undivided intestinal cells (arrows). Nuclei are visualized using an integrated *Pelt-2::GFP* reporter. Arrow in (D) shows a nucleus that has failed to undergo mitosis. E: Intestinal expression of CYD-1 in combination with *cki-1(RNAi)* leads to widespread hyperplasia in the intestine (arrowheads). F: Quantification of intestinal nuclei in several lines expressing either WT or KD CDK-4 from the *elt-2* promoter in an otherwise wild-type background. G: *cki-1(RNAi)* acts synergistically with CYD-1 overexpression to promote cell division in the intestine. Each dot represents a single animal.

Many *cdk-4KD* -expressing animals had intestinal cells with elongated nuclei, indicating a failure to undergo nuclear division (Figure 4D). Furthermore, these animals had much smaller brood sizes compared to wild-type animals (average 98, compared with 248 for WT). The defect in the brood sizes is likely to be due to the decreased function of the intestine. The intestinal nuclei both divide and endoreplicate during larval stages to accommodate growth of the animal and the intestine is essential for the transport of nutrients to the germline (Hedgecock and White, 1985; Sulston et al., 1983). The lack of division and endoreplication probably prevents the efficient synthesis of the proteins required for intestinal function, resulting in less nutrients being redistributed to the developing germline. Altogether, these data demonstrate that *cdk-4KD* expression cannot rescue the *cdk-4* null mutant but rather has a dominant-negative effect on cell division.

CDK-4 does not interact with CKI-1 in vivo in C. elegans

The hypothesis of a kinase-independent function of Cdk4 is based on the fact that Cdk4 can bind to the Cip/Kip inhibitor p27 and therefore, high levels of Cdk4 sequester p27 away from downstream Cyclin/Cdk complexes. Therefore, we set out to test whether we could find a physical interaction between these two proteins in *C. elegans*. We performed co-

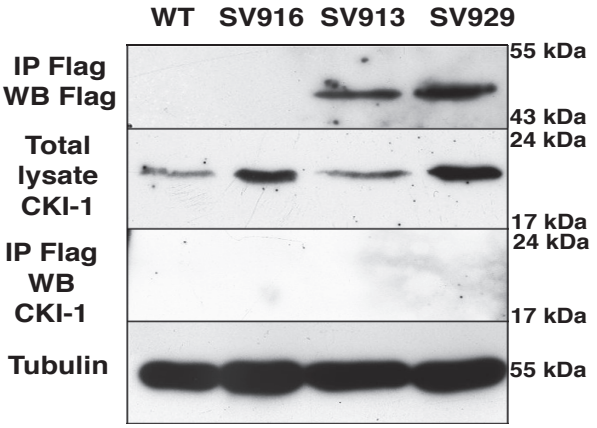

Figure 5. Co-immunoprecipitation between CDK-4::FLAG and CKI-1. Top panel: Flag-tagged CDK-4 could easily be detected in IPs from lysates containing either wild-type (SV929) or kinase dead CDK-4::FLAG (SV913), but not in N2 worms or *cdk-4(gv3)* heterozygous worms (SV916). Middle panels: although the 21 kDa CKI-1 protein is detected in the total lysate, no CKI-1 was co-purified in any of the two CDK-4 IPs. Lower panel: anti-tubulin was used as a loading control for the CKI-1 total lysate blot.

immunoprecipitation on lysates from worms carrying either the wild-type or kinase-dead CDK-4 rescue construct. We affinity-purified the CDK-4WT and CDK-4KD proteins using and antibody directed against the C-terminal FLAG-tag present in these constructs (Figure 1B) and probed for co-purification of CKI-1 using a CKI-1 antibody (a kind gift of Dr. Edward Kipreos). The anti-flag antibody purified proteins of the appropriate molecular weight from lines carrying CDK-4WT or CDK-4KD, while no proteins were purified from N2 or *cdk-4(gv3)/+* lysates. (Figure 5, top panel). However, although we could easily detect CKI-1 protein in the total lysates, no detectable amount of CKI-1 was co-purified with either the CDK-4WT or CDK-4KD protein (Figure 5, middle panels). Hence, there is either no interaction between CDK-4 and CKI-1, or the interaction is too weak to be detected by Co-IP and Western blot.

Discussion

Whether or not Cyclin/Cdk complexes have functions outside of their kinase-dependent roles in cell cycle regulation is a long standing question in the cell cycle field. p27 plays a dual role in the progression of G1 to S-phase as both an activator and a suppressor of Cyclin D/Cdk4 activity (Blain, 2008). Recent observations on the phosphorylation of p27 seem to reconcile both roles of p27. It seems that p27 is phosphorylated in cycling cells by kinases such as Abl and PKB and that this phosphorylation leads to the formation of the active Cyclin D/Cdk-4/p27 complex (James et al., 2008; Larrea et al., 2008). Thus, regulation of p27 phosphorylation upon growth factor stimulation might be able to explain p27's switch from an inhibitor to an activator of Cyclin D/Cdk4 activity. However, it does not provide an answer whether the sequestration role of Cdk4 is important for G1 progression *in vivo*. Cyclin D/Cdk4 complexes accumulate early in G1, before high levels of Cyclin E/Cdk2 activity can be detected (Sherr, 1995). The rise in Cyclin D/Cdk4 complex levels might function as a sink to prevent binding of p27 to nascent Cyclin E/Cdk2 complexes. Studies to test this dose-dependent sequestration model have relied heavily on overexpression and give contradictory results (Cheng et al., 1998; Matsushime et al., 1994).

Studies in model organisms such as *Drosophila*, mice and *C. elegans* have begun to shed some light on the *in vivo* functions of Cyclin/Cdk complexes during development. We have used both transgenic overexpression as well as low-copy microparticle bombardment approaches to rescue *cdk-4* in a null mutant background. We found several arguments that argue against a role for CDK-4 in CKI-1 sequestration in *C. elegans.* First, we did not observe any rescue with the

kinase-dead version of CDK-4, both in high and low copy number transgenic lines. The opposite is seen: animals expressing kinase-dead CDK-4 are more affected than *cdk-4* null mutants, even if the *cdk-4* mutant is combined with *lin-35(RNAi).* Second, the co-immunoprecipitation results reveal that we cannot co-purify significant amounts of CKI-1 with either wild-type or kinase-dead CDK-4. However, we cannot exclude the possibility that we cannot detect any interaction by co-affinity purification in our method or that this interaction is too transient to detect. An additional explanation for the lack of interaction might be that CKI-1 is not required for CYD-1/CDK-4 complex formation in *C. elegans*. Indeed, yeast 2-hybrid data have shown that CKI-1 can interact with CYD-1 (M. Boxem and S. van den Heuvel, unpublished). Therefore, it is possible that CYD-1, and not CDK-4, binds and sequesters CKI-1 to promote G1 progression in *C. elegans*. One argument against this is that lines carrying *cyd-1* under the *elt-2* promoter have an increased number of intestinal nuclei, even in developmentally arrested L1 animals and this could be further enhanced by removal of *cki-1* in this background (Figure 5G, data not shown). This synergy would not exist if high levels of CYD-1 alone were enough to reduce CKI-1 levels. I disagree. The titration may be only a modulatory mechanism, and RNAi would then be much more effective. More elaborate interaction studies, both with yeast-2-hybrid and co-immunoprecipitation, are necessary to provide conclusive evidence for the contribution of either *cyd-1* or *cdk-4* in *cki-1* sequestration in G1/S progression in *C. elegans*.

Another interesting observation is that the *cdk-4KD* construct can act as a dominant-negative inhibitor of cell division and migration, even at low concentrations. Although we cannot find an explanation for the dominant negative effect on cell division and the effect seen on P-cell migration, it does indicate that *cdk-4* might have targets other than *lin-35*/Rb or *cki-1* Cip/Kip in *C. elegans* G1/S progression. In fact, a screen for the suppression of the *cyd-1* phenotype yielded a novel mutation that acts in parallel to *lin-35* Rb. Similar to *cdk-4* null mutants, *cyd-1* null animals arrest as small larvae that lack any postembryonic cell division (Boxem and van den Heuvel, 2001; Park and Krause, 1999). This phenotype is partially rescued by inactivation of either *lin-35* or *cki-1*. The *he121* mutation can rescue *cyd-1;lin-35(RNAi)* animals to fertility and the phenotype does not resemble *cki-1*. Our mapping experiments in fact have placed the *he121* mutation away from known G1/S regulators in the *C. elegans* genome. This work has shown that the study of G1/S progression in *C. elegans* can lead to new insights into the function of well-studied regulators. Furthermore, genetic screens in *C. elegans* can make a contribution in identifying novel ways in which G1 progression is regulated across the animal kingdom.

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Supplementary figures

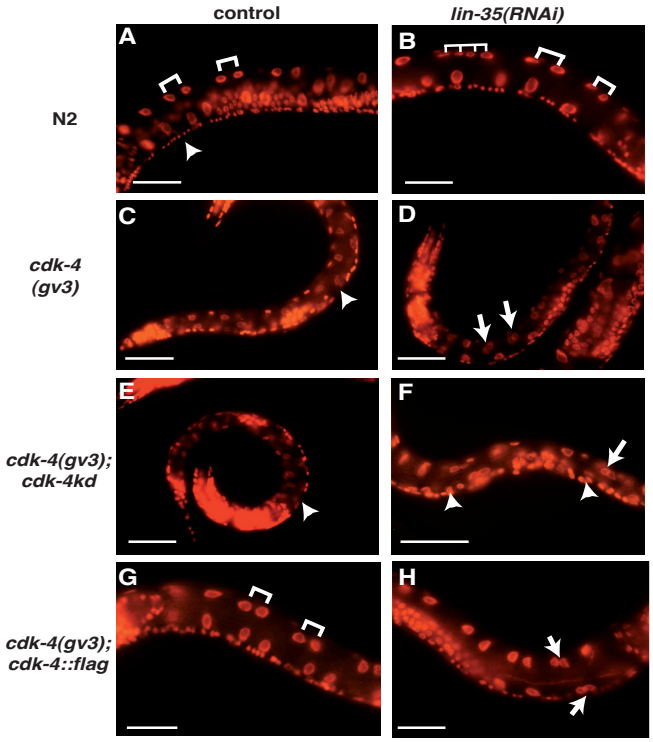

Figure S1. Propidium iodide staining of integrated rescue lines confirms the inability of *cdk-4kd* to rescue cell division and DNA replication in a *cdk-4(gv3)* background. A,B: N2 worms on control (A) and *lin-35 (RNAi)* (B). Brackets indicate pairs of intestinal nuclei, arrowheads point to the P-cell daughters in the ventral cord. *lin-35(RNAi)* results in supernumerary divisions in the intestine (B, upper left). C,D: *cdk-4(gv3)* mutant animals lack cell division in the intestine and ventral cord. Intestinal nuclei also fail to undergo endoreplication, hence the lower intensity of the PI staining in these nuclei compared to wildtype. E,F: *cdk-4kd* animals appear similar to the *cdk-4(gv3)* null mutant animals in their lack of post-embryonic divisions. Note the lack of organization in the ventral cord (F, arrowheads). *lin-35(RNAi)* can rescue some divisions in the intestine in this background. G,H: Wild-type *cdk-4* can rescue both intestinal and P-cell divisions in the ventral cord. Brackets indicate pairs of intestinal nuclei, arrows indicate extra divisions due to *lin-35(RNAi)* in (H).

Chapter 6

Summarizing discussion

'Work it harder, make it better
Do it faster, makes us stronger
More than ever hour after
Our work is never over'

Daft Punk –'Harder, Better, Faster, Stronger'

Developmental biology, the study of the events that happen from the fertilization of the oocyte to the completion of the adult animal body plan, has been crucial for the understanding of how cells divide in a multicellular environment. This understanding is also important in the study of tumor biology. Theodor Boveri was one of the first to recognize the contribution that developmental biology could make to the field of tumor biology. He translated his results on embryonic cell division in sea urchin eggs to the situation in tumor cells, for which he hypothesized that erroneous cell division is instrumental to the development of the malignant phenotype of tumor cells (Boveri, 2008). The advent of molecular biology has lead to a tremendous increase in our understanding of how signaling pathways are involved in patterning, fate determination and subsequent differentiation in the embryo (Fraser and Harland, 2000). Similarly, our knowledge of the molecules and mechanisms that govern cell division has expanded dramatically over the last four decades (Nurse, 2000). It is at the interface between these fundamental processes of fate specification and cell division that there is still substantial progress to be made. The complex relationship between fate specification, or differentiation, and cell division has its roots in the inverse relation between these processes: highly proliferative cells in the embryo often lack any signs of fate determination and differentiation, whereas cells that are in the process of differentiation often withdraw from the cell cycle. This thesis aims to increase our understanding of how cellular differentiation and cell division are linked during animal development

Studying cell cycle regulation in the context of development reveals both conserved and novel roles for cell cycle regulators

C. elegans is an excellent model system to study the function of cell cycle regulators in the context of animal development, in substantial part because of its powerful genetics and conservation of the pathways involved (**Chapter 1**, (Jorgensen and Mango, 2002). This thesis describes several examples of mutations in conserved cell cycle regulators that have interesting developmental consequences.

Chapter 2 describes the phenotype of *lin-6*/MCM-4, a conserved member of the MCM2-7 helicase complex that is necessary for DNA replication licensing and unwinding of the DNA during replication. The *lin-6* mutation resulted in animals with no post-embryonic DNA replication and no S-phase checkpoint function. More interestingly, the *lin-6* mutant revealed a remarkable uncoupling between several processes: S-phase induction still continues during development and the absence of DNA replication and aberrant division does not interfere with fate determination. In addition, *lin-6*/MCM-4 has specific functions in post-embryonic viability and growth. *lin-6* mutants often display larval lethality due to molting defects, which is not seen in other cell cycle mutants with impaired DNA replication such as *cyd-1* and *cdk-4* (Boxem and van den Heuvel, 2001; Park and Krause, 1999). This lethality could be rescued by hypodermal-specific expression of *lin-6*/MCM-4. In addition, the small size and width of the *lin-6* mutant were rescued by hypodermal-specific rescue of *lin-6*. *C. elegans* has 2 tissues that undergo endocycles: the intestine and the hypodermis (the *C. elegans* epidermis) (Hedgecock and White, 1985). Endocyclic tissues often have high metabolic activity and act to provide nutrients in specific areas of the animal (Edgar and Orr-Weaver, 2001).Therefore, it might seem surprising that rescue of *lin-6* in the hypodermis and not the intestine is critical in restoring normal organismal size. However, the hypodermis is important as a site of body size determination and fate patterning in *C. elegans*. A TGF-β-related pathway functions in the hypodermis to control body size (Wang et al., 2002) and the hypodermis is a source of Wnt ligands important

in post-embryonic fate determination (Myers and Greenwald, 2007). Although these results demonstrate that correct cell division in the hypodermis is important for organismal viability, it does not explain why other mutants that do not undergo DNA replication do not show larval lethality. Most likely, the progression through mitosis in the absence of DNA replication in *lin-6* mutants causes this lethality.

Chapter 4 highlights the conserved role for G1/S regulators in the control of cell division timing and frequency *in C. elegans*, as well as identifying an unexpected role for these regulators in cell fate determination. Overexpression of *C. elegans* Cyclin E/Cdk2 (CYE-1/CDK-2) or inhibition of the Cip/Kip family member *cki-1* results in loss of proper seam cell fate determination. Wild-type seam cell divisions are asymmetric both in size and fate outcome, except for the symmetric division in the L2 stage. *cki-1(RNAi)* animals display fate transformation of anterior cells, causing them to divide again instead of fusing with the hypodermal syncytium. Similar fate transformation upon G1/S deregulation has been observed during somatic gonad precursor cell division (Fujita et al., 2007; Kostic et al., 2003; Tilmann and Kimble, 2005). On the other hand, G1/S deregulation in *Drosophila* eye tissue might cause a delay in differentiation, but cells differentiate eventually (de Nooij and Hariharan, 1995; Firth and Baker, 2005). A similar situation is seen in the *C. elegans* body wall muscle (Chapter 3), where *cki-1(RNAi)* causes supernumerary divisions in the muscle (bm) lineage during embryogenesis, but these cells differentiate nonetheless. One possibility is that a shortened G1 phase upon G1/S regulation in the seam cells disrupts the ability of these cells to respond to the signals required for normal seam cell division. As seam cell division is regulated by a complex network of transcription factors and microRNAs (Moss, 2007), it is likely that interfering with the window of response for these determinants would cause a loss of seam cell division timing and fate. A more interesting possibility is that G1/S regulators might directly influence the segregation of fate determinants, i.e. the components of the Wnt/β-catenin asymmetry pathway in the seam cells. In the case of somatic gonad development, it is still unclear how G1/S deregulation influences Wnt/β-catenin asymmetry. The asymmetry of POP-1/Tcf in the somatic gonad precursor is lost in a *cyd-1* mutant (Tilmann and Kimble, 2005). Hence, it could be that POP-1 localization is directly influenced by G1/S regulators, perhaps via direct phosphorylation of POP-1 by the CYD-1/CDK-4 complex. Alternatively, POP-1 transcription might be regulated by the E2F type transcription factors that act during G1/S transition. On the other hand, expression of CYE-1/CDK-2 is dependent on *wrm-1*/β-catenin (Fujita et al., 2007). These results suggest that G1/S regulators can act both upstream and downstream of Wnt components in cell fate determination. Investigating the localization and transcriptional activation of the various Wnt components upon G1/S deregulation and the possible involvement of E2F-dependent transcription of Wnt/β-catenin components in the seam cells will provide us with more insight into the connection between G1/S regulators and Wnt signaling.

Chapter 5 addresses the functions of the *cdk-4* G1 Cdk in *C. elegans* G1/S progression. Next to its main function in phosphorylating pRb pocket proteins to promote G1/S progression, mammalian Cdk4 is thought to sequester p27, thereby relieving its inhibitory effect on the Cyclin E/Cdk2 complex (Blain, 2008). The lack of *in vivo* evidence to support this sequestration role for Cdk4 in G1/S progression prompted us to study the putative sequestration role in *C. elegans*. Our results demonstrate that the kinase function of CDK-4 is critically required for G1/S progression in *C. elegans.* In addition, we did not find evidence that CDK-4 has a kinase-independent role by binding and sequestration of CKI-1. However, we cannot exclude that the

interaction between CDK-4 and CKI-1 is too transient to detect with our assay. Furthermore, the G1 Cyclin partner of CDK-4, CYD-1 can interact with CKI-1 in a yeast 2-hybrid assay. Hence, it might be that the CYD-1/CDK-4 complex does have a pRb-independent function by sequestering CKI-1, but that the interaction occurs via CYD-1 in *C. elegans*. Further biochemical investigation of the binding between the CYD-1/CDK-4 complex and CKI-1 would be necessary to prove this model.

The results presented in this thesis underline the complexity of studying the phenotype of G1/S regulators during development. Assumptions solely based on the function of these regulators during the cell cycle may be misleading, an excellent example of which was the interpretation of the mutant phenotype of *lin-35*/Rb in vulval development. Initially, *lin-35*/Rb was thought to act in the vulval precursor cells, together with other SynMuvB genes, to negatively regulate induction of the vulval fate until the Ras/MAPK pathway relieves this inhibition at the mid-L3 stage (Ceol and Horvitz, 2001). However, tissue-specific rescue experiments convincingly demonstrated that *lin-35* functions in the hypodermis to repress *lin-3*/EGF, thereby preventing ectopic expression (Myers and Greenwald, 2005). Hence, *lin-35* does not only act in an indirect manner in this fate decision in the vulval precursor cells, it does so through a cell cycle-independent function of *lin-35*: transcriptional regulation. We have only begun to scratch the surface of the complexities of cell cycle regulation during development. For instance, many transcription factors and microRNAs are known to be required for the timely control of seam cell division in *C. elegans* (Pasquinelli and Ruvkun, 2002). However, virtually nothing is known about how these factors feed into the cell cycle to control the precisely timed asymmetric division of these cells.

How cell cycle regulators might exert functions unrelated to their role in the cell cycle during development is another important question. *lin-35*/Rb has, like its counterparts in other invertebrate and mammalian model systems, an important role in the establishment of cellular identity through regulation of gene transcription, small RNA metabolism and chromatin structure in *C. elegans* (e.g. (Kim et al., 2005; Wang et al., 2005). Here we show that *cki-1* and *cye-1/cdk-2* might also have a direct effect on cell fate specification, but further investigation is necessary to provide us with a biochemical explanation for this role of G1/S regulators. Lastly, cell cycle studies in *C. elegans* allow us to assess the function of cell cycle regulators in a variety of tissues. *C. elegans* has several variations on the somatic (mitotic) cell cycle, such as endocycles (intestine, hypodermis), embryonic cell divisions lacking G1 and G2 phases and meiotic divisions in the germline (van den Heuvel, 2005). In addition, *C. elegans* has many different blast cells that undergo a specified division pattern during larval development, which allows us to compare the different requirements for cell cycle regulation between tissues.

Cell cycle re-entry of differentiated cells in C. elegans: defining a complex relationship

In **Chapter 3** we investigate the inverse relationship between cell differentiation and proliferation. Cells that rapidly proliferate during development show few signs of cellular differentiation. Conversely, terminally differentiated cells generally do not re-enter the cell cycle. In *C. elegans*, the division of blast cell types, such as the intestine and P-cells, is susceptible to cell cycle deregulation by removal of *cki-1*/Cip/Kip and/or *lin-35*/Rb. However, this removal cannot trigger post-embryonic division in cells that are fully differentiated during embryogenesis, such as the bodywall muscle and neuronal cells. In Chapter 3, we demonstrate

that re-introduction of G1/S Cyclin/Cdk activity can trigger cell cycle re-entry in terminally differentiated cells in the *C. elegans* bodywall muscle. Notably, this is not accompanied by a loss of muscle fate in these cells. Furthermore, expression of G1/S Cyclin/Cdk complexes can induce a highly specific cell cycle transcriptional program. The situation of cell cycle re-entry in body wall muscle cells of *C. elegans* differs from that in mammalian myotubes in several ways. First, removal of *lin-35*/Rb, either by RNAi or by using a genetic null mutant, is not sufficient to trigger cell cycle re-entry in differentiated body wall muscle cells. In contrast, removal of pRb from terminally differentiated muscle myotubes causes cell cycle re-entry of these myotubes, but also causes them to lose their muscle fate (Blais et al., 2007). pRb proteins interact with a wide host of different chromatin regulators, including the Polycomb group (PcG) proteins and Heterochromatin Protein 1 (HP1) to control the chromatin environment and thereby cellular identity (Kuzmichev et al., 2002; Nielsen et al., 2001). Therefore, removing pRb has consequences in addition to the loss of cell cycle control: it also causes the muscle myotubes to lose their differentiated fate. Critically, loss of cell fate does not seem to occur in terminally differentiated body wall muscle in *C. elegans* upon cell cycle re-entry. Hence, our model system might prove more physiological than the mammalian one in identifying the different factors that can affect re-entry in terminally differentiated muscle cells.

Our results with this model for cell cycle re-entry have raised many unanswered questions: Could G1/S Cyclin/Cdk expression in a differentiated neuron trigger cell cycle re-entry? Would it happen to the same extent as in the body wall muscle or would there be tissue-specific differences? Another interesting question is whether timed stimulation of G1/S Cyclin/Cdk combinations in the adult animal could still trigger cell cycle re-entry. It might be that cell cycle re-stimulation works in cells that have recently exited the cell cycle, but not later in development. We have started to use a tissue-specific heat shock-inducible system developed for *C. elegans* to address this question in the body wall muscle (Bacaj and Shaham, 2007), but the results thus far have been inconclusive. As the cell cycle re-entry seen in these body wall muscle cells is limited, enhancer screens can be performed to search for additional controls that act to restrict uncontrolled proliferation in differentiated cells. We have already established that RNAi-mediated knockdown of the checkpoint kinase *chk-1* can modulate the extra division phenotype. Furthermore, we have obtained two mutants that might define additional pathways important in controlling the proliferation of differentiated cells.

Ultimately, this work aims to explore the potential for regenerative therapies that start from differentiated cells, and examine to what extent the development of tumorous growths may arise from terminally differentiated, post-mitotic cells.

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Epilogue

Nederlandse samenvatting

Dankwoord/ Acknowledgements

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Publications

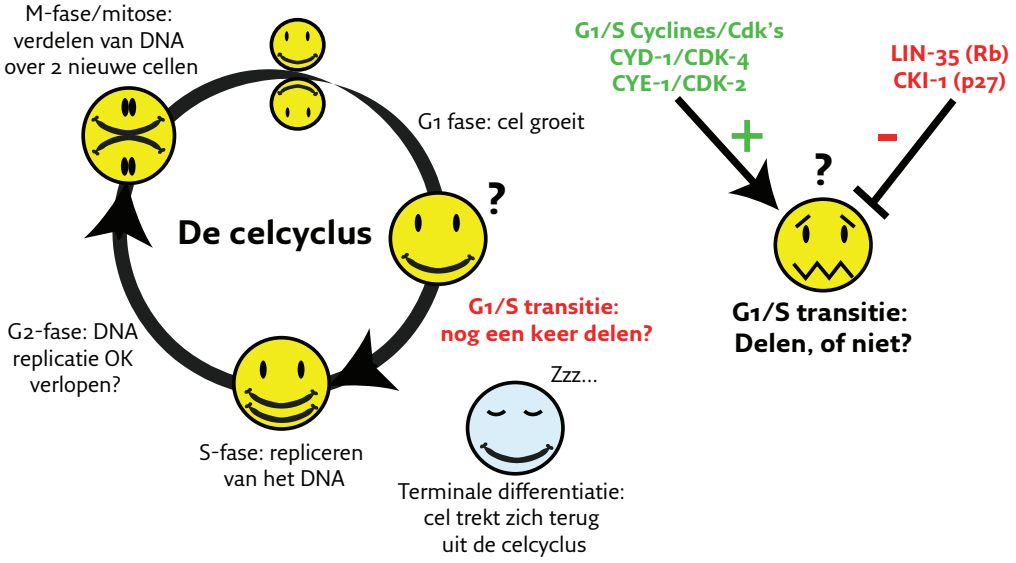
'I'm feeling good, I'm feeling so fine,
Until tomorrow but that's just some other time'

The Velvet Underground - 'I'm Waiting For The Man'

Nederlandse samenvatting

Voor het ontstaan van een organisme uit slechts een enkele bevruchte eicel is een groot aantal celdelingen nodig. Cellen delen zich ook uitgebreid tijdens het volwassen leven van een mens. Dit moet exact gebeuren, want ongecontroleerde celdeling leidt tot het ontstaan van kanker.

Celdeling wordt bestuurd door twee verschillende soorten eiwitten: de Cyclines en de Cycline-gereguleerde kinases (Cdk's). Complexen van Cyclines en Cdk's regelen onder andere het verdubbelen van het erfelijk materiaal en het verdelen ervan in de twee nieuwe dochtercellen (Figuur 1). Tijdens de embryonale ontwikkeling gaan sommige cellen zich na de deling differentiëren: ze ontwikkelen zich tot bijvoorbeeld spier- of zenuwcellen. Die differentiatie betekent voor veel cellen dat ze zich daarna niet meer kunnen delen tijdens het volwassen leven. Alhoewel er veel bekend is over hoe celdeling wordt gereguleerd in cellen in celcultuur en in ééncellige eukaryoten zoals gisten, weten we nog steeds weinig over hoe celcyclus-regulatoren zoals Cyclines en Cdk's functioneren in de context van een meercellig organisme. Ook is er weinig bekend over hoe signaaltransductie-routes de celdeling kunnen beïnvloeden tijdens de ontwikkeling van een organisme.



Figuur 1: De celcyclus en de overgang van G1 naar S-fase. Links: een schematisch overzicht van de celcyclus. In de G1 fase wordt bepaald of een cel nog een keer door een delingscyclus gaat. Dit is o.a. afhankelijk van de hoeveelheid voedingsstoffen en groeifactoren. Vóór dit punt kunnen cellen die zich differentiëren tot bijvoorbeeld spiercellen, besluiten zich terug te trekken uit de celcyclus. Wanneer een cel genoeg positieve groeisignalen krijgt, verdubbelt het DNA en wordt het DNA verdeeld over 2 nieuwe dochtercellen tijdens de mitose. Rechts: Verschillende eiwitten spelen een cruciale rol in de overgang van de G1 naar de S fase. G1/S Cycline/Cdk complexen bevorderen de overgang van G1 naar S, terwijl de Rb (LIN-35 in *C. elegans*) eiwitten en p27 (CKI-1) deze overgang juist blokkeren. Door toename van Cycline/Cdk activiteit tijdens G1, worden deze blokkades uiteindelijk opgeheven en kan de cel opnieuw delen.

In dit proefschrift beschrijven we verschillende aspecten van celcyclus regulatie tijdens de ontwikkeling van de kleine nematode worm *C. elegans*. Dit relatief simpele modelorganisme stelt ons in staat celdeling te bestuderen in een meercellig dier. Doordat deze worm doorzichtig is, is elke celdeling tijdens de ontwikkeling te volgen. Voorts zijn de mechanismes van celdeling

geconserveerd bij alle meercellige dieren, zoals ook de mens. **Hoofdstuk 1** geeft een overzicht van de celcyclus en de belangrijke spelers hierin, met een nadruk op celcyclus regulatie in *C. elegans*. **Hoofdstuk 2** beschrijft het karakteriseren van de *lin-6* mutant. Uit onze analyse blijkt dat het *lin-6* gen een belangrijke rol speelt tijdens de S-fase (DNA Synthese fase) van de celcyclus. Het LIN-6 eiwit is homoloog aan MCM4, één van de 6 leden van het MCM2-7 complex, wat zorgt voor het ontwinden van het DNA tijdens de DNA synthese. Zonder LIN-6/MCM4 is er geen DNA synthese mogelijk en ook de signaaltransductie route die normaliter DNA schade tijdens replicatie beperkt, kan niet worden geactiveerd in wormen zonder functioneel *lin-6* gen (*lin-6* mutant wormen). Aan de andere kant gaan processen zoals celdifferentiatie en de initiatie van de S-fase wel gewoon door. Voorts beschrijven we dat de functie van *lin-6* het belangrijkst is in de hypodermis (huid) van de worm. Het specifiek terugplaatsen van een functioneel *lin-6* gen alleen in dit weefsel is genoeg om de *lin-6* mutanten, die klein blijven en vaak sterven tijdens de overgang naar een ander larvaal stadium, weer te laten groeien en ontwikkelen zoals wildtype dieren.

Hoofdstuk 3 gaat in op een fundamentele vraag in de biologie: hoe komt het dat cellen die zich differentiëren (tot bijvoorbeeld een spiercel) niet meer delen? Wij maken gebruik van de spiercellen van *C. elegans* als een model voor terminaal gedifferentieerde cellen. Eén van de manieren hoe gedifferentieerde cellen zich terugtrekken uit de celcyclus is door de afbraak en inactivatie van Cycline/Cdk complexen tijdens differentiatie. Door deze Cycline/Cdk complexen na de differentiatie weer te activeren, kunnen we kijken hoe belangrijk dit mechanisme is in het behouden van het celcyclus arrest in deze cellen. We ontdekten dat het aanzetten van CYD-1/CDK-4 of CYE-1/CDK-2 , de Cycline/Cdk complexen die de G1/S transitie reguleren, leidt tot reactivering van de celcyclus in deze terminaal gedifferentieerde spiercellen. Voorts bleek dat dit leidde tot het activeren van een gentranscriptie programma met een sterke celcyclus signatuur. De activiteit van genen die belangrijk waren voor spierfunctie veranderden echter niet en de wormen bewogen zich normaal nadat we deze Cycline/Cdk complexen hadden gereactiveerd. Celcyclus reactivering leidt dus niet tot een verandering in spiercel differentiatiestatus. Ook hebben we gezocht naar genen die het aantal extra celdelingen in CYD-1/CDK-4 transgene dieren kon versterken. Twee genen werden gevonden die wellicht een specifieke rol spelen in het beperken van het delingsvermogen van gedifferentieerde cellen. Dit onderzoek laat zien dat het celcyclus arrest in terminaal gedifferentieerde cellen niet zo statisch is als wordt gedacht,. Er werdt ook duidelijk dat er meerdere niveaus van controle in deze cellen zijn die het delingsvermogen beperken. Het identificeren van deze controlestappen kan van groot belang zijn voor kankeronderzoek en het onderzoek naar regeneratie van beschadigde, terminaal gedifferentieerde cellen.

Hoofdstuk 4 laat zien dat deregulatie van de G1/S transitie in de celcyclus kan leiden tot een verandering van het lot van de cel: cel “fate”. De *C. elegans* huid, de hypodermis, wordt mede gevormd door een groep cellen die de seam cellen worden genoemd. Deze seam cellen liggen in een rij aan de buitenkant van de worm, van voor (anterior) tot achter (posterior), en delen zich asymmetrisch tijdens elk van de vier larvale stadia. Na deling fuseert één van de dochtercellen, de anteriore dochtercel, met een cel van de omliggende hypodermis, terwijl de posteriore dochtercel de seam cel fate behoudt en in het volgende stadium wederom asymmetrisch deelt. Dit type asymmetrische deling lijkt in vele aspecten op de deling van stamcelpopulaties in de humane huid en darm. Hoofdstuk 4 toont aan dat deregulatie van de G1/S transitie in deze seamcellen niet alleen leidt tot teveel deling, maar ook tot een verlies van de timing ervan,

alsmede een verandering in de fate van de dochtercellen. Als we CYE-1/CDK-2 specifiek tot expressie brengen in de seam cellen, leidt dit tot G1/S transitie deregulatie, maar de anteriore dochtercellen, die normaal fuseren, veranderen vaak ook in seam cellen, die extra delen tijdens het eerste larvale stadium. Dit toont aan dat deregulatie van de G1/S transitie kan leiden tot onverwachte fenotypes, zoals verandering van cel fate. Hoe G1/S regulatoren het lot van cellen bepalen is nog niet duidelijk.

In **hoofdstuk 5** wordt de functie van CDK-4 in G1/S transitie in meer detail bestudeerd. De belangrijkste functie van het Cycline D/Cdk4 complex is de fosforylering van de belangrijke negatieve G1/S regulator: het Rb eiwit. Naast deze kinase functie wordt er gedacht dat het Cycline D/Cdk4 complex ook de negatieve G1/S regulator p27 (CKI-1 in *C. elegans*) kan wegvangen. Deze rol van het Cycline D/Cdk4 complex zou los staan van de kinase functie. Wij onderzochten of een CDK-4 zonder kinase functie nog een rol speelt in G1/S transitie in *C. elegans*. We plaatsten een wildtype CDK-4 of een CDK-4 zonder kinase functie terug in *cdk-4* mutant wormen en analyseerden of dit de celdelingdefecten van deze mutant kon herstellen. Het wildtype CDK-4 herstelde, zoals verwacht, de celdelingdefecten in *cdk-4* mutant wormen. Het kinase-loze CDK-4 echter, was niet in staat om de celdelingdefecten in *cdk-4* mutanten te herstellen. Dit toont aan dat de kinase functie van cruciaal belang is voor CDK-4 om zijn functie uit te oefenen in de celcyclus in *C. elegans*. We kunnen echter niet uitsluiten dat de kinase functie invloed heeft op een mogelijke binding aan CKI-1. **Hoofdstuk 6** geeft een samenvattende discussie, met aandachtspunten voor toekomstig onderzoek.

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Ten eerste natuurlijk mijn promotor en begeleider Sander. Ik ben je vanuit Boston weer naar Utrecht gevolgd en heb daar nooit spijt van gehad. Samen hebben we het stof soms letterlijk van het lab geklopt en de groep zien groeien. Je hebt altijd vertrouwen in mij en het project getoond wat hopelijk snel zal worden beloond met een mooie publicatie. Ook heb ik veel van je geleerd (o.a. over het nut van controles) en zal onze soms hilarische, soms felle discussies over wetenschap wel missen. Inge, jij was er ook vanaf het prille begin bij. Je bent van groot belang geweest voor dit project. Samen hebben we toch mooi de Horizon binnengehaald en hopelijk zal het project in de toekomst nog lang doorgaan! Vincent: bedankt voor je onmisbare hulp bij kloneren, injecteren, PCR-en, invriezen, mijn muziek collectie, het meezingen van Toto en het verkrijgen van concertkaartjes voor Marduk. Mijn mede AIO-maatjes Christian en Matilde : vele malen hebben we samen een biertje (te veel) gedronken op het lab en daarbuiten en het was altijd even leuk. Zet hem nog effe op: heb er het volste vertrouwen in dat jullie binnenkort ook eens aan het schrijven gaan. Selma: jij staat nog aan het begin van je AIO-schap, maar het gaat je tot nu toe prima af! Veel succes gewenst. Monique: veel succes met jouw promotie de 20e. Marjolein, mijn "plantje"! Wat hebben we gelachen, gediscussieerd over die seamcellen en *cki-1* en gevochten. Ja, het was altijd fijn om weer even onze energie fysiek op elkaar af te reageren. Nog effe dooroefenen en je wint nog wel eens van me met armpje drukken! Mike, het was altijd leuk met jouw droge humor op het lab en daarbuiten en heb ook ontzettend veel van je geleerd, zowel theoretisch als wat betreft "mad computerskillz". Wat kan ik nog aan G1/S regulatie toevoegen na jouw baanbrekend werk? Adri, bedankt voor je kritische blik en het snoepjes jatten. Gelukkig ben ik niet meer zo bang voor je als in mijn 2e jaar en ik kan gerust zeggen dat je onmisbaar bent geweest in het rekruteren van veel van mijn studenten. Ja, mijn studentjes...Sigmarlis, Teije, Tessa, Suzan en Beck: jullie waren mijn oogappeltjes en hebben in grote mate bijgedragen aan de totstandkoming van dit proefschrift. Mijn soms bikkelharde manier van studentbegeleiding heeft tot mijn grote schik tot mooie resultaten geleid. En aangezien jullie allen verder zijn gegaan in de wetenschap, moet het voor jullie toch ook een leerzame, leuke ervaring zijn geweest. Sigmarlis, T-Bob, Tesbob en S-bob: bedankt voor alles, inclusief de gezelligheid buiten het lab. B-Bob: I hope you have enjoyed your time in Holland. Although heat-shock in *C. elegans* isn't all what it's cracked up to be, I enjoyed supervising such a young enthusiastic scientist. I also would like to thank all the other students in the lab for good times, funny lunchtime discussions and buffers I might have "borrowed". Martine en Jana: Ik hoop dat jullie veel plezier hebben in ons lab! Het Holstege lab, in het bijzonder Marian: bedankt voor de prettige samenwerking aan hoofdstuk 3. Door de microarray is dit hoofdstuk echt de kroon op mijn werk geworden. Graag zou ik ook het "Bos-Burgening cluster" bedanken. Boudewijn: al waren Western blots niet mijn ding, jij hebt me toch verder geholpen en heb het altijd gezellig gehad met iedereen op het Stratenum.

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Jerôme

Curriculum Vitae

Jerôme Korzelius werd op 1 januari 1979 geboren in Dordrecht. In deze stad bezocht hij het Johan de Witt Gymnasium, waar hij in 1997 zijn VWO diploma behaalde. In datzelfde jaar begon hij aan zijn studie Biologie aan de Universiteit Utrecht. Tijdens zijn studie liep hij zijn eerste stage bij de leerstoelgroep Plant-Microbe Interactions aan de Universiteit Utrecht onder supervisie van prof.dr. Corné Pieterse, waar hij de 'crosstalk' tussen verschillende plantenafweer-routes bestudeerde. Vervolgens was hij een half jaar werkzaam bij het Hubrecht laboratorium als research analist bij drs. Romke Koch, waar hij meehielp aan het assembleren van het zebravis genoom. Na een half jaar in het Verre Oosten besloot hij zijn 2e stage te lopen bij het Hubrecht laboratorium in de groep van dr. Rik Korswagen onder begeleiding van drs. Damien Coudreuse. Hier werkte hij aan de Wnt signaleringscascade in *C. elegans*. Zijn scriptie, over de invloed van Ras en Wnt signalering op vulva formatie in *C. elegans*, werd tevens in dit lab onder begeleiding van dr. Rik Korswagen geschreven. Het doctoraaldiploma werd behaald in 2003. Na een kortstondige flirt met biochemie en celcultuur in het lab van prof.dr. Boudewijn Burgering in het UMC Utrecht, besloot hij wederom het pad van de worm te volgen. In november 2004 vertrok hij naar Boston, U.S.A. om bij prof.dr. Sander van den Heuvel te gaan werken bij het MGH/Harvard Cancer Center. Vervolgens volgde hij zijn toekomstige begeleider terug naar Utrecht, waar hij in mei 2005 het hierin beschreven promotie-onderzoek begon.

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