

**Mechanisms of Cell Cycle Remodeling at the MBT  
during the Development of *Xenopus laevis* Embryos**

Matthew Petrus

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**Committee Chair**

Jill Sible

**Committee Members**

John Tyson

Rich Walker

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# Mechanisms of Cell Cycle Remodeling at the MBT during the Development of *Xenopus* Embryos

Matthew Petrus

(Abstract)

During the early development of *Xenopus laevis* embryos, cells divide without checkpoints. At the midblastula transition (MBT), the cell cycle is remodeled as the division time lengthens and checkpoints are acquired. Initiation of the MBT depends upon the degradation of maternally supplied cyclin E, which is the regulatory partner of the cyclin dependent kinase, Cdk2. To study the program that drives cyclin E degradation and cell cycle remodeling at the MBT, embryos were treated with two cell cycle inhibitors, GST- $\Delta$ 34Xic1 and XChk1.

Injection of embryos with GST- $\Delta$ 34Xic1, a stoichiometric inhibitor of cyclin E/Cdk2, delays degradation of cyclin E and onset of the MBT. GST- $\Delta$ 34Xic1 lowers Wee1 level, a kinase that maintains Cdks in an inactivate state. Eventual degradation of cyclin E is preceded by degradation of GST- $\Delta$ 34Xic1. The mathematical modelers, Andrea Ciliberto and John Tyson, incorporated the data into a kinetic model and set of ordinary differential equations. The model accurately described the experimental data and made additional predictions, which were tested experimentally.

Additionally, embryos were injected with mRNA encoding XChk1, a kinase that activates Wee1 and inhibits Cdc25, the phosphatase opposing Wee1. Like GST- $\Delta$ 34Xic1, XChk1 inhibits cyclin E/Cdk2 and delays the degradation of cyclin E. In contrast to GST- $\Delta$ 34Xic1, XChk1 elevates the level of Wee1 at a time when sibling controls begin the MBT, despite cell cycle arrest.

Since XChk1 inhibits both Cdk1 and Cdk2, and GST- $\Delta$ 34Xic1 inhibits only Cdk2, we propose Cdk1 destabilizes Wee1, whereas Cdk2 elevates Wee1 level. Prior to the MBT, when cyclin E/Cdk2 is active, Wee1 is maintained. After cyclin E/Cdk2 is destroyed at the MBT, Wee1 is degraded.

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# Chapter 1

## Introduction

## **The cell cycle: G1, S, G2, M phases**

The typical somatic cell cycle is composed of four phases: G1, S (DNA synthesis), G2 and M (mitosis). A cell must first complete S phase properly before entering M phase, and the cell must divide its DNA at mitosis before it can re-enter S phase. The cell moves between these phases growing, replicating its DNA, ensuring the DNA is replicated completely and not damaged, and then dividing. Entry and exit from the phases is highly regulated, leading to ordered progression through the four phases.

Regulation of a typical somatic cell can be complex and is regulated in a phase-specific manner. In the event of damaged or unreplicated DNA, the cell pauses during the cell cycle, once at G1, just prior to S phase (Moreno and Nurse, 1994; Madine et al., 1995; Zeng et al., 1998), and again at G2, just prior to M phase (Kumagai and Dunphy, 1995; Peng, et al., 1997; Kumagai et al., 1998). The cell pauses again at metaphase prior to anaphase in mitosis if the mitotic spindle is not correctly assembled (Minshull et al., 1994; Elledge, 1996; Arellano and Moreno, 1997; Novak and Tyson, 1998). These pauses in the cell cycle are called checkpoints, and the checkpoints ensure that the cellular components have been properly maintained in the cell (Furnari et al., 1997; O'Connell et al., 1997).

Multiple gene products regulate progression through the cell cycle. These gene products have a high degree of homology across species (Lee and Nurse, 1987; Su et al., 1995; Sanchez et al., 1997). This conservation suggests the importance of cell cycle regulation and supports the use of *Xenopus* embryos in understanding how growth is controlled in most eukaryotic cells.

### **Early cell divisions of *Xenopus laevis* embryos are rapid and unregulated.**

During the early cell divisions of *Xenopus* embryos, checkpoints and gap phases (G1/G2) are not observed (Newport and Dasso, 1989; Frederick and Andrews, 1994). The cell cycles are rapid in the second through twelfth cell divisions and display little regulation. The cell cycle is remodeled five hours post-fertilization, at the midblastula transition (MBT), when the cell cycle is lengthened, zygotic transcription is initiated, cell motility is gained, gap phases and checkpoints are observed (Newport and Kirschner, 1982; Newport and Kirschner, 1989, Frederick and Andrews, 1994). Understanding how cell cycle regulators become active at the MBT, and how the cell regulates the timing of cell cycle remodeling at the MBT can provide insight into how cell cycle regulation is lost in malignantly transformed cells.

*Xenopus laevis* embryos are an excellent model system to investigate the cell cycle and provides an understanding of how growth is controlled in normal eukaryotic cells. During the first five hours of development, embryonic cell divisions proceed unchecked for DNA damage and incompletely replicated DNA. These rapid, unchecked cellular divisions can be compared to the proliferation of cancer cells which often bypass checkpoints. Five hours post fertilization, at the mid-blastula transition (MBT) (Newport and Kirschner, 1982), S phase lengthens and subsequently gap phases appear (Frederick and Andrews, 1994). The transition from the rapidly dividing embryonic cells to the more somatic cell like divisions at the MBT correlates with the initiation of zygotic transcription (Newport and Kirschner, 1982), gain in cell motility (Newport and Kirschner, 1982; Kimelman et al., 1987), and changes in cell cycle regulators (Hartley et al., 1996). Understanding this transition from rapid, unchecked to slower more regulated cell divisions can lend insight into the regulation of the cell cycle.

The cell divisions before the MBT are unique. The first cell cycle progresses over a 90-minute period (Newport and Kirschner, 1982). In the second through twelfth cell cycles, the division time shortens to 30 minutes, as cells move from S phase (DNA synthesis) to M phase (mitosis) without intervening gap phases (G1/G2) (Newport and Kirschner, 1982; Frederick and Andrews, 1994). The rapid cell cycles prior to the MBT

lack full cell cycle checkpoints. Induction of DNA damage by  $\gamma$ -ionizing radiation ( $\gamma$ -IR) before the mid-blastula transition (MBT) does not arrest cell divisions (Anderson et al., 1997; Hensey and Gautier, 1997). Treatment of embryos with aphidicolin to block DNA replication before the MBT (Newport and Dasso, 1989; Rollins and Andrews, 1991) delays but does not arrest cell cycles, suggesting that the DNA replication checkpoint is partially functional in the early cell cycles of the embryo.

### **The cyclins and Cdks**

Progression through the cell cycle is driven by the cyclin-dependent kinases (Cdks) and their respective cyclins (Chen et al., 1995; Luo et al., 1995; Guadagno and Newport, 1996; Arellano and Moreno, 1997; Krude et al., 1997). Displaying a high level of functional homology among eukaryotic organisms, at least eight Cdks have been described thus far, suggesting a highly conserved and regulated family of protein kinases (Lee and Nurse, 1987; Su et al., 1995; Arellano and Moreno, 1997). The Cdks are regulated stoichiometrically by the p21/p27 family of cyclin dependent kinase inhibitors (CKIs) (Luo et al. 1995; Chen et al. 1995; Su et al. 1995; Shou et al. 1996). In addition, phosphorylation positively and negatively regulates Cdk activity. The Cdks must also associate with, and are activated by, the cyclins (Gautier et al., 1990; Solomon et al., 1990; Devault et al., 1992; Coleman et al., 1996; Strausfeld et al., 1996; Baily and Weeks, 1996). The levels of cyclins in the cell are controlled strictly. The presence of a certain cyclin and subsequent activity of its associated Cdk allows passage through a given phase of the cell cycle (Nigg, 1995). In somatic cells, the degradation of cyclins through the ubiquitin-dependent proteasome pathway initiates the progression into mainly into M phase (Arellano and Moreno, 1997).

Each cyclin/Cdk complex drives progression through a specific phase of the cell cycle. The transition in the cell cycle at G1/S, for instance, is associated with a set of Cdks and cyclins separate from those at G2/M. The binding of cyclin D to its respective

Cdks, Cdk4 and Cdk6, occurs in G1 and is highly regulated in somatic cells, but is not extremely relevant in the early embryo. In late G1, Cdk2 associates with cyclin E and plays a role in the initiation of DNA synthesis [(Strausfeld et al.1996; (Heichman and Roberts, 1994; Jackson et al., 1995), see Chapter 3].

### **S phase regulation**

During the transition in the cell cycle at G1/S, cyclin E binds to Cdk2 and in S phase, cyclin A binds to Cdk2. Collectively cyclin A/Cdk2 and then cyclin E/Cdk2 appear to drive the cell through S phase (Chevalier et al., 1996; Strausfeld et al., 1996). The association of these S phase cyclins with Cdk2 initiates DNA synthesis (Heichman and Roberts, 1994; Jackson et al., 1995; Arellano and Moreno, 1997). When cyclin E/Cdk2 is low in frog egg cell-free extracts, M phase entry is delayed (Guadagno and Newport, 1996), but the role for cyclin E/Cdk2 activity in the initiation of DNA synthesis is better characterized.

#### *S phase regulation in somatic cells*

Cyclin E/Cdk2 activity is negatively regulated by the kinases Wee1 and Myt1 on threonine-14 and tyrosine-15 (Gu et al., 1992). The human cyclin dependent kinase inhibitor (CKI) p21<sup>Cip1</sup> (Cip1) inhibits DNA synthesis and cyclin E/Cdk2 activity in somatic cells (Chen, 1995; Luo et al., 1995). The N-terminus of Cip1 has a Cdk-binding domain that primarily inhibits Cdk2, and the C-terminus has a proliferating cell nuclear antigen (PCNA) binding domain (Chen et al., 1995; Luo et al., 1995). Both the N-terminus and the C-terminus of Cip1 inhibit the synthesis of double-stranded DNA (Chen, 1995; Luo et al., 1995; Heichman and Roberts, 1994). This suggested cyclin E/Cdk2 is involved in the initiation of DNA synthesis.

Inactivating phosphorylations of cyclin E/Cdk2 are removed by the phosphatase Cdc25A. Cdc25A has a cyclin binding motif on the N-terminus and is able to bind to and remove the inhibitory threonine-14 and tyrosine-15 phosphate groups from cyclin E/Cdk2 (Hoffman et al., 1994; Saha, 1997). When bound to cyclin E/Cdk2, Cip1 prevents

Cdc25A binding and phosphatase activity, whereas Cdc25A binding inhibits Cip1 binding (Saha et al., 1997).

This competition for cyclin E/Cdk2 binding by Cip1 and Cdc25A regulates the initiation of double-stranded DNA synthesis. The tumor-suppressor, p53, becomes activated in response to damaged DNA and upregulates Cip1, leading to Cip1 binding of cyclin E/Cdk2 over Cdc25A binding to cyclin E/Cdk2. Binding of Cip1 results in a checkpoint at G1/S as the cell pauses to correct the problem before DNA is replicated (el-Deiry et al., 1993; el-Deiry et al., 1994). The human CKI p27<sup>KIP1</sup> (Kip1) is a member of the same family of CKIs as Cip1 but lacks a PCNA binding domain, possessing only a Cdk binding domain. Cip1 inhibits both cyclin E/Cdk2 and cyclin B/Cdc2 (Polyak et al., 1994; Font de Mora, 1997). Unlike Cip1, Kip1 is not upregulated in response to damaged DNA. Instead, Kip1 is upregulated by TGF- $\beta$ , which inhibits growth in many cell types (Font de Mora et al., 1997).

#### *S phase regulation in frog extracts and embryos*

In *Xenopus* embryos, the only S phase Cdk present before the MBT is cyclin E/Cdk2. During the cell cycles before the MBT, although cyclin E level does not vary, cyclin E/Cdk2 activity oscillates twice per cell cycle, once at S phase and once at M phase (Hartley et al., 1996). Thus, cyclin E/Cdk2 may play a role in both the initiation of DNA replication and mitosis (Guadagno and Newport, 1996; Hartley et al., 1996; Hartley et al., 1997). Because cyclin E level is constant before the MBT, level of cyclin E does not regulate cyclin E/Cdk2 activity. CKIs are not present in the early cell divisions (Su et al., 1995; Shou and Dunphy., 1996) and therefore do not regulate cyclin E/Cdk2 activity. Phosphorylation events of cyclin E/Cdk2 have been detected in egg extracts (D'Angiolella et al., 2001) and may account for the oscillations in cyclin E/Cdk2 activity. Currently only one *Xenopus* homologue, Xic1 or Kix1, of the human CKIs, Cip1 and Kip1 (Su et al., 1995; Shou and Dunphy, 1996) has been identified. The amino acid sequence of Xic1 indicates that a CDK-binding domain is present on the N-terminus and

a PCNA-binding domain (Su et al., 1995) and nuclear localization signal (Shou and Dunphy, 1996) are found on the C-terminus, similar to the structure of Cip1. Xic1 is expressed at a low level in oocytes and remains low until gastrulation when the cell cycle becomes more somatic-like, suggesting a role for Xic1 in the lengthening of the cell cycle after the MBT (Shou and Dunphy, 1996).

Recombinant, exogenous CKIs that inhibit cyclin E/Cdk2 have been a powerful tool in studying phase regulation in frogs. The recombinant Xic1 protein, GST- $\Delta$ 34Xic1, is missing the first 34 amino acids on the N-terminus and is highly specific for the inhibition of cyclin E/Cdk2 (Su et al., 1995; Hartley et al., 1997). GST- $\Delta$ 34Xic1 inhibits DNA synthesis *in vitro*, but GST- $\Delta$ 34Xic1 only causes a 25% delay in the cell cycle *in vivo* (Hartley et al., 1997).

The full-length protein, Xic1, and its human homologue, Cip1, function similarly in frog egg extracts. Xic1 inhibits DNA synthesis in a dose-dependent manner (Su et al., 1995; Shou and Dunphy, 1996). The inhibition of DNA synthesis is due to the ability of Xic1 to bind to cyclin E/Cdk2 via the Cdk binding domain. In addition, Xic1 inhibits DNA synthesis when bound to PCNA via the PCNA-binding domain on the C-terminus (Su et al., 1995; Shou and Dunphy, 1996). Cip1 also binds PCNA and cyclin E/Cdk2 in frog egg extracts, effectively inhibiting double stranded DNA synthesis (Heichman and Roberts, 1994; Jackson et al., 1995; Jackson et al., 2000). However, replication of single-stranded DNA was not inhibited by Cip1. This suggests that Cip1 and Xic1 inhibit cyclinE/Cdk2 early in the initiation of DNA synthesis (Jackson et al., 1995; Jackson et al., 2000). The binding of PCNA by Cip1 inhibits DNA replication, but not PCNA-mediated DNA excision and repair. Because Xic1 does not inhibit cyclin B/Cdc2 activity as strongly as cyclin E/Cdk2 activity, Xic1 may primarily function in S phase regulation and secondarily, in M phase regulation (Su et al., 1995; Shou and Dunphy, 1996). Collectively, these data indicate that cyclin E/Cdk2, and PCNA are inhibited by Xic1 and Cip1, this is sufficient for a block in DNA replication in egg extracts, but does not delay DNA replication *in vivo*, and a delay in cell cycle progression in embryos.

If Xic1 inhibits DNA replication *in vivo* as effectively as it does in egg extracts, then Xic1 must be degraded to release cyclin E/Cdk2 from inhibition. The ubiquitin-mediated degradation of G1 regulators by Cdc34 is essential for entry into S phase in *Xenopus* embryos (Yew and Kirschner, 1997), suggesting that perhaps Cdc34 leads to the degradation of Xic1, allowing the necessary cyclin E/Cdk2 activity for entry into S phase. In frog egg extracts sperm nuclei must be present for Xic1 degradation in a cell cycle-dependent manner. As a complex, Xic1-cyclin E/Cdk2 are transported into the nucleus (Swanson et al., 2000; Chuang and Yew, 2001; Furstenthal et al., 2001b). Once in the nucleus, Xic1-cyclin E/Cdk2 bind to Cdc6-ORC and the replication factor Cdt1, which binds MCM to the DNA [(Furstenthal et al., 2001a; Furstenthal et al., 2001b). The chromatin-associated Xic1-cyclin E/Cdk2 complex is required for the recruitment of the ubiquitin-ligase SCF, which ubiquitinates and leads to Xic1 degradation in the nucleus (Furstenthal et al., 2001b). The Xic1-cyclin E/Cdk2 complex is bound to the chromatin at the origin of replication and is essential for both Xic1 degradation and the initiation of DNA synthesis.

Experiments conducted in egg extracts indicate cyclin E/Cdk2 plays a role early in the initiation of DNA synthesis. If cyclin E/Cdk2 is inhibited by Cip1, then double-stranded DNA synthesis is inhibited, but not single-stranded DNA synthesis is not (Jackson et al., 1995; Sherr, 1994). This indicates that cyclin E/Cdk2 initiates DNA synthesis at unwinding. *In vivo*, the inhibition of cyclin E/Cdk2 by a recombinant form of the *Xenopus* CKI, Xic1, only delayed the cell cycle by approximately 25% (Hartley et al., 1997).

### **M phase regulation**

Significant similarities and differences exist in the regulation of S phase and M phases. The transition in the cell cycle at G2/M is associated with Cdk1, also called Cdc2, dimerized with cyclin A or cyclin B (Dunphy et al., 1988; Gautier et al., 1988; Gautier et al., 1990; Weeks et al., 1991; Devault et al., 1992).

During G2, Cdc2 and cyclin B or cyclin A form a dimer called maturation promoting factor (MPF), which is essential for progression into M phase (Peng et al., 1997). The activity of MPF is tightly controlled in normal cell cycles and has just one peak of activity, at M phase (Arellano et al. 1997; Lee, 1987; Hoffman et al., 1993; Kumagai and Dunphy, 1995; Lee and Kirschner, 1996).

#### *M phase regulation in somatic cells*

*Normal cell cycle:* Similarly to cyclin E/Cdk2 binding in G1/S, cyclins A and B bind to Cdc2 in G2, and Cdc2 activity is regulated by phosphorylation, cyclin level and by CKIs. The Cdk activating kinase (CAK) activates MPF by phosphorylating it on Thr-161, and MPF is inhibited by phosphorylation on threonine-14 and tyrosine-15 by Myt1 and Wee1 [(Mueller et al., 1995), Figure 1].

In a positive feedback loop, MPF inactivates Myt1 and Wee1, and activates the phosphatase Cdc25 (Figure 1), which removes inhibitory phosphates (Hoffman et al., 1993), leading to self activation (Strausfeld et al., 1994b). In a negative feedback loop, MPF leads to the degradation of cyclin B by indirectly activating the protease Cdc20/fizzy (Kramer et al.; 2000). Progression through M phase is dependent upon the presence of cyclin B, and to a lesser extent cyclin A. When present in the cell cyclin B ensures the formation of MPF. When cyclin B is degraded, MPF activity is low and the cell exits mitosis.

*Checkpoint at G2/M:* Normal progression through the eukaryotic cell cycle is delayed when DNA is damaged. This delay allows the cell to repair the damage, or, if the damage is great, a program of apoptosis may be initiated (Basu et al., 1998). The kinases Chk1 and Chk2 are activated by the kinase ATR and ATM in response to damaged DNA and phosphorylate Cdc25 on serine-216 (Sanchez et al., 1997; Peng et al., 1997; Nakajo et al., 1999). This leads to Cdc25 binding to 14-3-3 (Figure 1), preventing Cdc25 from entering the nucleus and activating MPF, thereby blocking M phase entry in response to

damaged DNA (Sanchez et al. 1997; Matsuoka et al., 1998; Brown et al., 1999; Sanchez et al., 1999).

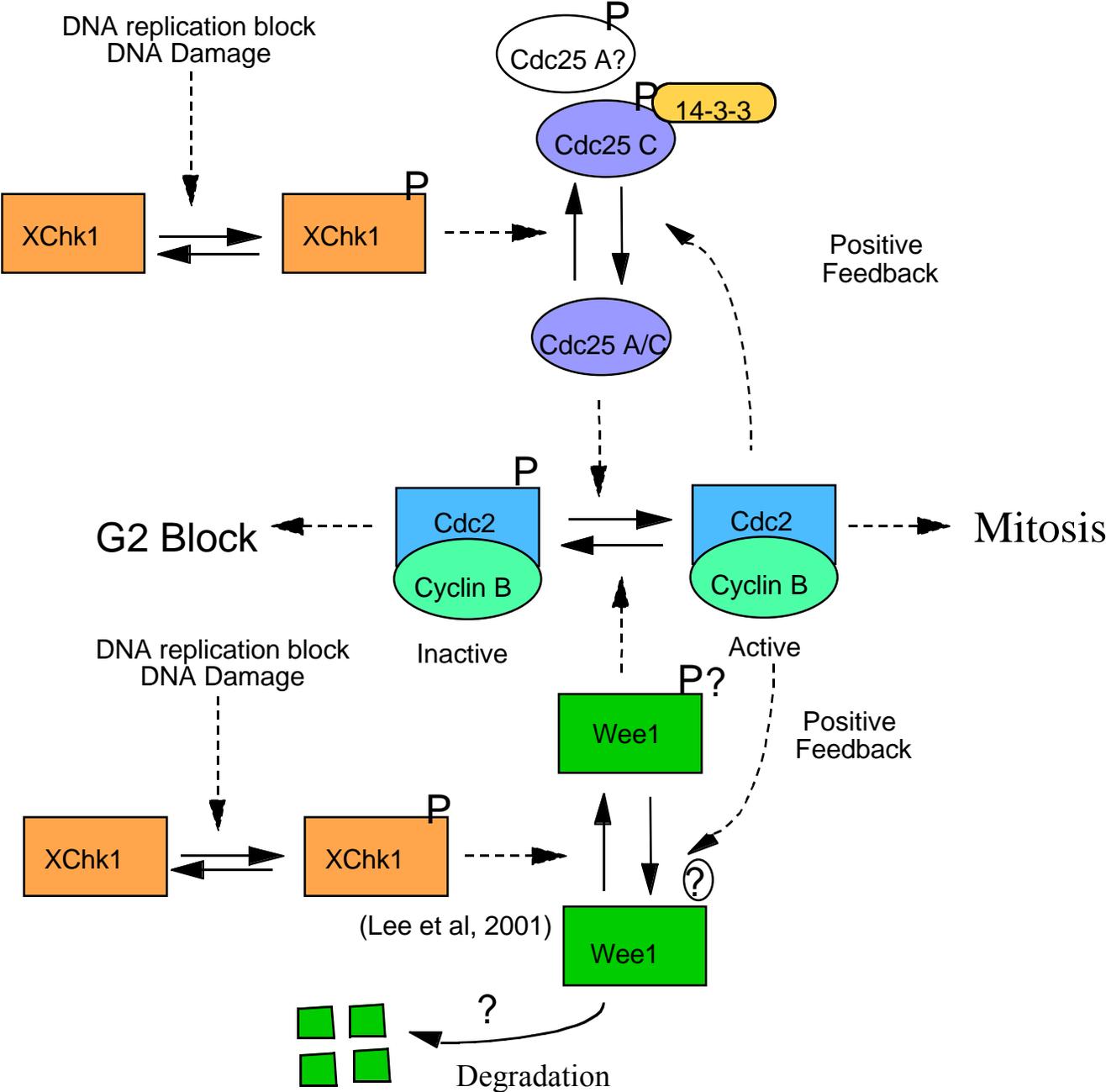
### *M phase regulation in frog egg extracts and embryos*

The regulation of MPF has also been determined through observations in the frog oocyte, embryo and egg extracts. As the oocyte matures, MPF level becomes high (Figure 2), and the cell is blocked in metaphase of meiosis II by cytostatic factor (CSF) (Nigg, 1995; Murakami and Woude, 1998). When the egg is fertilized, cyclins are degraded and MPF drops during the first cell cycle which lasts for 90 minutes. In cycles two-twelve, the cell division time is only 30 minutes long. MPF is found in both activated and inactivated states, as it oscillates with a high degree of synchrony. The level of inactive MPF rises as cyclin B level increases, and the cell progresses towards M phase as a threshold level of cyclin B is reached, and MPF becomes activated. The protein Fizzy, or Cdc20, recognizes cyclin B in frog egg extracts and presents it to the anaphase promoting complex (APC) where it is degraded each cell cycle (Lorca et al., 1998). MPF level drops, and S phase is initiated as cyclin B is degraded (Dunphy et al., 1988; Murray et al., 1989; Gautier et al., 1990).

During pre-MBT cell cycles cyclin B level fluctuates [(Murray et al., 1989; Gautier et al., 1990), Figure 2], and MPF activity oscillates once per cell cycle as in typical somatic cells. When bound to cyclin A or B, Cdc2 is phosphorylated on three regulatory sites, two inactivating: threonine-14, tyrosine-15, and one activating: threonine-161 (Kumagai and Dunphy, 1995). Little phosphorylation of threonine-14 and tyrosine-15 is detected during cycles 2 through 12 (Ferrell et al. 1991; Hartley et al. 1996; Kim et al. 1999). This is supported by mathematical modeling (Novak and Tyson, 1993; Novak and Tyson, 1995.; Borisuk and Tyson; 1998) and suggests that the level of cyclin B, not phosphorylation, causes the oscillation in MPF activity before the MBT (Murray and Kirschner, 1989; Gautier et al., 1990; Solomon et al., 1990).

**Figure 1. The XChk1 signaling pathway regulates entry into mitosis.** XChk1 is activated in response to a DNA replication block, and perhaps to DNA damage (Flaggs, Plug et al., 1997; Furnari, Rhind et al., 1997; O'Connell, Raleigh et al., 1997; Sanchez, Wong et al., 1997; Brondello, Boddy et al., 1999; Chen, Liu et al., 1999). Activated XChk1 phosphorylates Cdc25C (Kumagai and Dunphy, 1992; Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Zeng et al., 1998), Wee1 (Mueller et al., 1995; Lee et al., 2001), and perhaps Cdc25 A. When phosphorylated by XChk1, Cdc25 binds a 14-3-3 protein and becomes sequestered in the cytosol, preventing Cdc25 from entering the nucleus and activating cyclin B/Cdc2 (MPF) (Sanchez et al. 1997; Matsuoka et al., 1998; Brown et al., 1999; Sanchez et al., 1999). XChk1 phosphorylates and activates Wee1 (Lee et al.), which phosphorylates and inhibits MPF. Thus MPF is maintained in its phosphorylated, inactive state, leading to cell cycle arrest at G2/M.

Figure 1



The kinase Wee1, phosphorylates and inactivates MPF (Mueller et al., 1995; Kumagai et al., 1998; Murakami and Woude, 1998; Lee et al., 2001), and the phosphatases Cdc25A and C remove the inhibitory phosphorylations on MPF (Gautier et al., 1991; Kumagai and Dunphy, 1991; Gabrielli et al., 1992; Izumi and Maller, 1993; Kim et al., 1999). Cdc25 C remains in the cell from oocyte to stage 13 (Gautier et al., 1991). Its activity oscillates during the cell cycle, peaking at about the same time active MPF reaches its highest concentration during each cycle (Gautier et al., 1991; Izumi et al., 1992; Kumagai and Dunphy, 1992). Cdc25A, on the other hand, is not found until after the first cycle and is degraded at the MBT [(Kim et al., 1999), Figure 2]. Since there is little tyrosine- phosphorylation on MPF during the early embryonic cell cycles, this suggests MPF is highly activated in response to Cdc25A and Cdc25C, which overcome Wee1 activity (Gautier et al., 1991; Kumagai and Dunphy, 1991; Gabrielli et al., 1997). MPF enhances its own activation by phosphorylating and activating Cdc25C, and inactivating Wee1 in a positive feedback loop [(Izumi et al., 1992; Izumi and Maller, 1993; Mueller et al., 1995), Figure 1].

The kinase XChk1 is activated in response to unreplicated and damaged DNA, and regulates MPF activity by negatively regulating Cdc25C (Furnari, Rhind et al., 1997; Sanchez et al., 1997). When phosphorylated by XChk1, Cdc25 C binds 14-3-3 and is sequestered in the cytosol [(Izumi et al., 1992; Conklin et al., 1995; Peng et al., 1997; Kumagai et al., 1998; Kumagai and Dunphy, 1999), Figure 1] leading to the inactivation of MPF (Peng et al., 1997; Kumagai et al., 1998; Kumagai and Dunphy, 1999). Since Cdc25C functions in the nucleus, 14-3-3 binding prevents MPF activation and the cell cycle is blocked in G2 (Izumi et al., 1992; Izumi and Maller, 1993; Kumagai and Dunphy, 1997; Nakajo et al., 1999). Whether XChk1 is directly responsible for the phosphorylation of Cdc25A in *Xenopus* embryos, or if Cdc25A functions in M phase, is unclear.

XChk1 further regulates the activity of MPF indirectly (Furnari et al., 1997; O'Connell et al., 1997; Kumagai et al., 1998; Nakajo et al., 1999) by phosphorylating and

activating Wee1 [(Lee et al., 2001), Figure 1]. When phosphorylated by XChk1, Wee1 becomes bound to 14-3-3 in the nucleus activated, but not stabilized (Lee et al., 2001). Once activated, Wee1 directly phosphorylates and inactivates MPF, creating a checkpoint arrest.

Although XChk1 is present in the embryo prior to the MBT (O'Connell et al., 1997), XChk1 is not activated by damaged or unreplicated DNA as the cell moves from S to M phase without checkpoints or gap phases. In the absence of cell cycle arrest, the cell is led into a program of apoptosis instead of a checkpoint when DNA is damaged or replication is blocked (Anderson et al., 1997; Hensey and Gautier, 1998; Carter et al. unpublished). After the MBT, unreplicated DNA leads to high concentrations of phosphorylated active XChk1 (Kappas et al.).

### **Cell cycle remodeling at the MBT**

Prior to the MBT, the embryo relies on maternally supplied mRNAs and protein to regulate cell cycle progression (Newport and Kirschner, 1982). As the embryo reaches the proper cell number and nuclear-to-cytoplasmic ratio at the MBT, zygotic transcription is initiated and the embryo moves towards a program of zygotic regulation (Newport and Kirschner, 1982; Krieg and Melton, 1987). Degradation of cyclin E occurs rapidly at the MBT (Howe and Newport, 1996; Hartley et al., 1997), and degradation of cyclin A occurs at gastrulation (Howe et al., 1995). S phase lengthens and gap phases are observed as zygotic transcription is initiated in a nuclear-to-cytoplasmic ratio-dependent manner at the MBT. However, the degradation of cyclin E is independent of nuclear-to-cytoplasmic ratio, cell number, and protein synthesis [(Howe et al., 1995; Rempel et al., 1995; Howe and Newport, 1996; Hartley et al., 1997), Figure 2]. Degradation of cyclin E at the MBT is said to be regulated by a developmental timer (Howe et al., 1995; Howe and Newport, 1996) because it is degraded so consistently at the time of the MBT independent of zygotic transcription.

Oscillations in cyclin E/Cdk2 activity are associated with a developmental timer that regulates the timing of the MBT and are also independent of cell number, protein

synthesis, and nuclear-to-cytoplasmic ratio (Howe and Newport, 1996; Hartley et al., 1997). The mechanisms of "cell cycle remodeling" (Frederick and Andrews, 1994) at the MBT are not well understood. A fundamental control system may exist that links the events of the MBT, like zygotic transcription, to the regulation of cyclin E/Cdk2 oscillations and cyclin E degradation. The oscillations in Cdk2 activity might be generated by a negative feedback similar to the one found in cyclin B/Cdc2 regulation (Figure 1). But since cyclin E level does not change in the second through twelfth cell divisions in the *Xenopus* embryo (Figure 2), the oscillations in Cdk2 activity are probably due to phosphorylation state rather than cyclin level. We hypothesize that cyclin E/Cdk2 activity drives cyclin E degradation at the MBT.

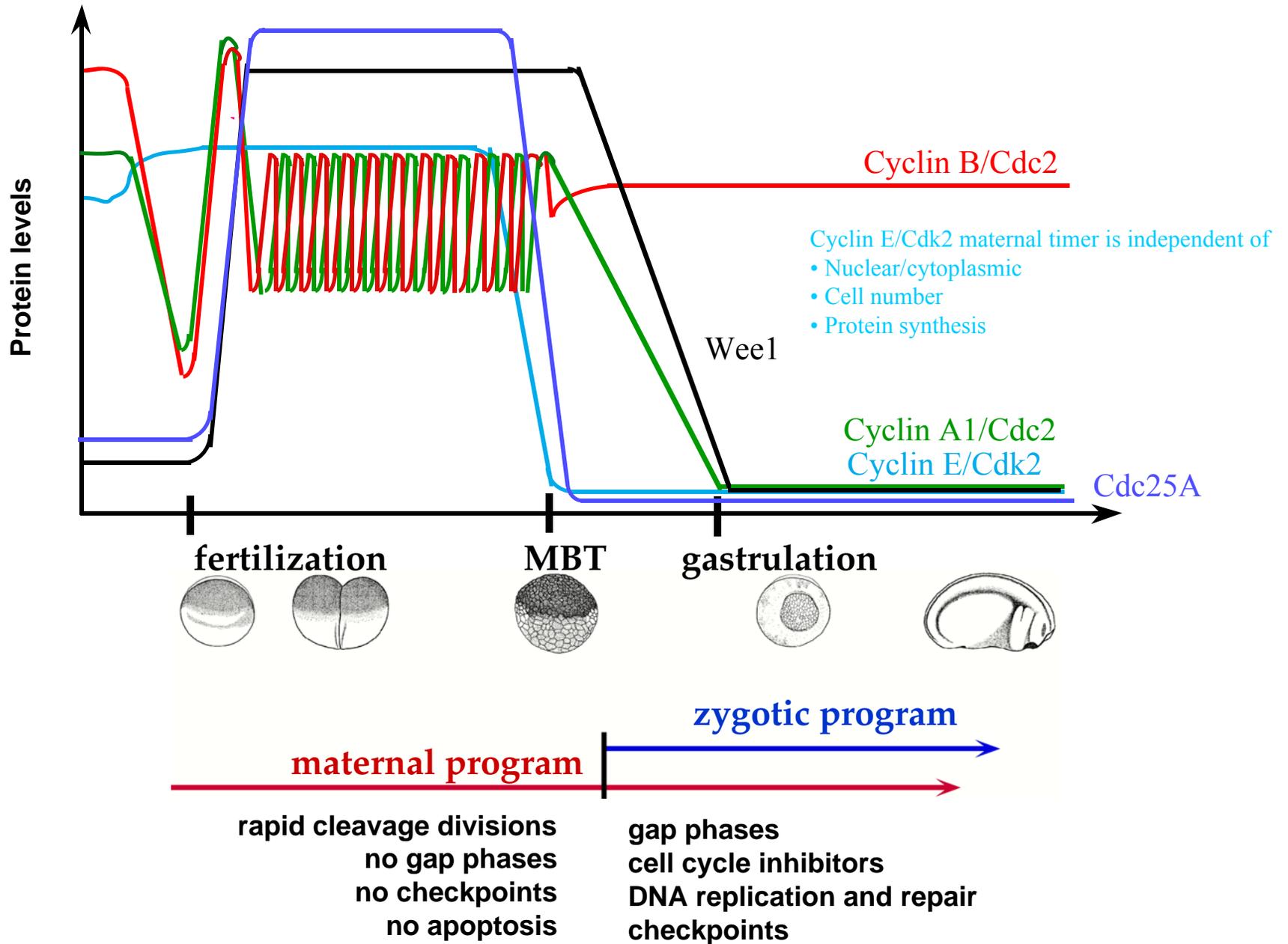
Prior to the MBT, gap phases and checkpoints are not observed. During the early, rapid cell divisions of the embryo, activated Cdc25A (Kim et al., 1999) and Cdc25C (Izumi et al., 1992; Kumagai and Dunphy, 1992; Izumi and Maller, 1993) are found in high concentrations, driving the cell into S and M phase. XChk1 (Nakajo et al., 1999), and Wee1 (Mueller et al., 1995; Murakmai and Woude 1998; Nakajo et al., 1999) exist in the cell before the MBT, yet checkpoints are not observed until five hours post-fertilization when the MBT begins (Newport and Dasso, 1989; Anderson et al., 1997; Hensey and Gautier, 1997). When activated and in high concentrations, Cdc25A and Cdc25C together may provide a signal that is too strong to overcome in the cycles before the MBT as they activate MPF and lead the cell from division to division.

The exact reason why the cell lacks "stop division" signals prior to the MBT is unknown. It would appear that all of the necessary checkpoint machinery is present and still no checkpoints are observed. Exogenous XChk1 does lead the embryo into a checkpoint before the MBT, suggesting the cell cycle checkpoint is functional downstream of XChk1 pre-MBT (Kappas et al. 2000). It might be assumed that the inhibition of checkpoints and the lack of active XChk1 in the early cell divisions before the MBT is due in part to the lack of a threshold concentration of replicated DNA (Dabauvalle et al., 1988; Newport and Dasso, 1989). At fertilization, the nuclear-to-cytoplasmic ratio is low and as the embryo divides and replicates its DNA, without

growth, the ratio increases. When a critical nuclear/cytoplasmic ratio is met at division 12, the MBT is triggered as zygotic transcription begins and checkpoints are observed (Newport and Kirschner, 1982).

**Figure 2. The cell cycle is remodeled at the MBT.** Cell cycles are driven by the activity of cyclin-dependent kinases. Levels of cyclins A and B oscillate rapidly prior to the MBT. Cyclin E is present at a constant level until the MBT, when it is rapidly degraded. The degradation of cyclin E is said to be regulated by a developmental timer which is independent of other events at the MBT. This degradation is independent of the nuclear-to-cytoplasmic ratio, cell number and protein synthesis and is difficult to disrupt. The kinase Wee1, which inhibits Cdks, is present in the cell at a constant level until it is degraded at gastrulation and negatively regulates the cell cycle. The phosphatase Cdc25 A activates Cdks and is present from cycle 2 until the MBT.

Figure 2



## Chapter 2

### Materials and Methods

### ***Maintenance, manipulation and microinjections of Xenopus Embryos***

Eggs from wild type *Xenopus laevis* (*Xenopus Express*) were fertilized *in vitro*, dejellied in 2% cysteine in 0.1 X MMR (0.5 mM HEPES, pH 7.8, 10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO<sub>4</sub>, 0.2 mM CaCl<sub>2</sub>, 0.01 mM EDTA) and maintained in 0.1 X MMR. Embryos were collected at stages spanning the midblastula transition (MBT) and staged according to Nieuwkoop and Farber (1975). XChk1 and luciferase mRNA were dissolved in TE buffer (10mM Tris, pH 8.0, 1 mM EDTA), and 10 ng RNA in approximately 25 nL total volume was microinjected into one-cell embryos. GST-Xic-C, and GST-Δ34-Xic1 proteins were dissolved in Xic1 buffer (20 mM HEPES, pH7.5, 88 mM NaCl, 20 mM βME, 7.5 mM MgCl<sub>2</sub>, 5% glycerol) and microinjected into one-cell stage embryos at 2.5 ng, 5 ng or 10 ng, as indicated, in a 30 nL total volume. GST-Xic-C and GST-Δ34-Xic1 proteins were provided by Dr. James Maller (University of Colorado Health Sciences Center, Denver, CO) (Hartley et al., 1997). For microinjections of GST-Δ34-Xic1 and α-amanitin (Sigma, St. Louis, MO), all reagents were dissolved in water. Five ng of GST-Δ34-Xic1 and 50 ng of α-amanitin were microinjected or co-injected in a 50 nL total volume into one-cell staged embryos. A Medical Systems Corp. microinjector, with RNase-free glass needles, were used for microinjections, and embryos were placed in a 5% Ficoll in 0.1X MMR solution after microinjection to minimize leakage of cytoplasm. Embryos were observed with an Olympus SZX12 stereo microscope and photographed with an Olympus DP10 digital camera.

### ***In vitro transcription of XChk1 and luciferase mRNA***

The *xchk1* gene had been subcloned with a FLAG-tag into the pSP64polyA vector (Promega, Madison, WI) as previously described (Kappas et al., 2000). The plasmid construct was linearized and used as template for the Ambion SP6 mMessage mMachine *in vitro* transcription kit to produce polyadenylated FLAG-tagged XChk1 mRNA. A luciferase cDNA clone (Kappas et al., 2000) was used as template for transcription of FLAG-tagged luciferase mRNA.

### ***Retroelution of cyclin E antibody***

Cyclin E antibody used for Western analysis was purified using cyclin E protein and rabbit immunoserum obtained from Dr. Rebecca Hartley (Department of Anatomy and Cell Biology, College of Medicine, University of Iowa). 100-250 µg purified cyclin E protein was electrophoresed on a modified Anderson gel (separating gel = 10-12% acrylamide, 0.1-0.13% bis-acrylamide, 0.37 M Tris-HCL, pH 8.7, 0.1% SDS, running buffer = 0.025 M Tris pH 8.8, 0.192 M glycine, 0.1% SDS) and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau S (Fisher Scientific, Pittsburgh, PA) and strips containing cyclin E were cut and incubated overnight with 1 mL of anti-cyclin E rabbit immunoserum and 9 mL 10% nonfat dry milk in PBS (80 mM Na<sub>2</sub>HPO<sub>4</sub>•7H<sub>2</sub>O, 20 mM NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O, 100 mM NaCl). Strips were then washed in PBS 0.05% Tween, and the antibody was eluted three times with 12 mL of 100 mM Glycine, pH 2.8, for each elution collected. 1.2mL 1M Tris pH 8.0, 1.5mL 10X PBS, and 3 µL 10% sodium azide was added to each of the three antibody elutions, and the cyclin E antibody elutions were stored at 4°C for future use.

### ***Western analysis of cell cycle proteins***

Embryos were collected at the indicated times, snap frozen on dry ice, and homogenized in EB (Hartley+3p: 80 nM β-glycerophosphate, 20 mM HEPES pH 7.5, 20 nM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM Na Vanadate, 50 mM NaF, 1 mM DTT, 0.05 mM PMSF, 6 µg/mL leupeptin, 1 µM microcystin, 3 µg/mL pepstatin, and 3 µg/mL chymostatin. TR EB: 30 mM HEPES pH 7.5, 20 mM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM Na Vanadate, 1 mM DTT, 1 mM PMSF, 3 µg/mL leupeptin, 1 µM microcystin). Samples blotted for Wee1 were resolved on either modified Anderson (as above) or Laemmli SDS-polyacrylamide gels (separating gel = 12.2% acrylamide, 0.4% bis-acrylamide, 0.3 M Tris, pH 8.8, 0.1% SDS, 10% glycerol, running buffer = 0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS). Western analysis of samples for cyclin E, Xic-1, FLAG, and Cdc25A or Cdc25C were resolved on 10-15% Anderson gels. All samples were transferred to nitrocellulose membranes, and those immunoblotted for Wee1, Cyclin E, Xic-1, and FLAG were

blocked in 10% nonfat dry milk in PBS. Membranes immunoblotted for Cdc25A or Cdc25C were blocked in 5% BSA in TBS (20 mM Tris, pH 7.6, 137 mM NaCl), 0.1% Tween. The Wee1 antibody was obtained from Dr. Monica Murkami (National Cancer Institute, National Institutes of Health) and was diluted in 10% nonfat dry milk in PBS with or without Amersham Pharmacia Biotech Liquid Blocking Reagent diluted 1:20 (Piscataway, NJ.; catalog number EC60152). Cyclin E antibody was retroeluted as described above and diluted in 1% BSA in PBS. Xic-1 and FLAG antibodies were diluted in 10% nonfat dry milk in PBS, and Cdc25 A and Cdc25C antibodies were diluted in 5% BSA in TBS-0.1% Tween. The Xic-1 and Cdc25 A and Cdc25C antibodies were obtained from Dr. James Maller (University of Colorado Health Sciences Center, Denver, CO). Membranes were washed in PBS or TBS-0.1% Tween after incubation in the primary antibody. Immunoreactive proteins were visualized by hybridizing them to a horseradish peroxidase-conjugated secondary antibody diluted 1:10,000 in 10% nonfat dry milk in PBS (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), raised in donkey against appropriate species, and chemiluminescence was detected with the ECL Plus kit (Amersham Pharmacia Biotech, Piscataway, N.J) or SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

### ***Northern analysis of Wee1 and GS17 expression***

Northern analysis of *Wee1* mRNA and *GS17* mRNA expression during *Xenopus* development was performed essentially as described previously (Sible et al., 1997; Hartley et al., 1997). Total RNA was isolated from embryos using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). Ten micrograms of each RNA was resolved by denaturing gel electrophoresis, transferred to a 0.2 $\mu$ m Nytran membrane in 20X SSC (3 M NaCl, 300 mM Na citrate, pH 7.0) with a TurboBlotter apparatus (Schleicher and Scheull, Keene, NH) and then cross-linked to the membrane with a Stratagene UV cross-linker. The *wee1* gene (Accession number, AF035443) inserted into the pBS $\pm$  vector (Stratagene, La Jolla, CA) was provided by Monica Murakami and heat shock transformed into DH5 $\alpha$  competent *E. coli* cells (Invitrogen, Frederick, MD).

The plasmid was isolated using the Qiafilter Maxi Plasmid Kit (Qiagen, Valencia, CA), and the gene was excised from the plasmid by cutting with the restriction enzymes BamHI and DraI (New England BioLabs, Beverly, MA). The *wee1* gene fragment was resolved by agarose electrophoresis and gel-extracted using Qiagen Qiaquick Gel Extraction Kit, heated at 100°C for ten minutes and used as a single stranded template in random priming (Random Priming Kit, Roche Diagnostics Corporation, Indianapolis, IN) to produce an [ $\alpha$ -<sup>32</sup>P] dCTP (Amersham Biosciences, Piscataway, NJ) labeled probe. The GS17 cDNA probe was made in a similar fashion (Krieg and Melton, 1985). Each probe was added to a Bio-Spin Chromatography Column (Bio-Rad Laboratories, Hercules, CA). Incorporation of [ $\alpha$ -<sup>32</sup>P] dCTP into probe was determined by Cerenkov scintillation counting of TCA-precipitable material. The probe was heated with 100  $\mu$ g/mL of sonicated herring sperm DNA at 100°C for ten minutes. The single-stranded cDNA probes were used at  $2 \times 10^6$  cpm counts per mL and hybridized with the RNA blot for 2-4 hours at 65°C in QuickHyb solution (Stratagene, La Jolla, CA). The blots were washed twice for 15 minutes in 2X SSC, 0.1% SDS at room temperature, and once in 0.2X SSC, 0.1% SDS at 60°C for 30 minutes. After washing, the blots were dried and exposed to Kodak BioMax film.

### ***Immunoprecipitation and kinase assays of cyclin-Cdk complexes***

Embryos injected with GST-Xic1-C and GST- $\Delta$ 34-Xic1 were collected at various timepoints and snap-frozen on dry ice. Protein G immobilized on sepharose beads (Sigma, St. Louis, MO) was prepared by washing three times for ten minutes each in 10% BSA in H<sub>2</sub>O and again in EB buffer (80 nM  $\beta$ -glycerophosphate, 20 mM HEPES pH 7.5, 20 nM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM Na Vanadate, 50 mM NaF, 1 mM DTT, 0.05 mM PMSF, 3  $\mu$ g/ml leupeptin, 1  $\mu$ M microcystin). The embryos were homogenized in the same EB buffer and pre-cleared by incubating for 30 minutes at 4°C with protein G beads. Lysates were then centrifuged, and the supernatant was collected. Antiserum

against either cyclin E or cyclin B (cyclin antibodies were kindly supplied by Dr. James L. Maller) was added to the homogenized embryos and incubated on ice overnight at 4°C. The prepared protein G beads were added to precipitate the antibody-Cyclin/Cdk complexes from the embryo lysates. Beads were incubated 1-2 hours at 4°C washed in low-salt buffer (20 mM Tris pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 100 mM NaCl) and high-salt buffer (20 mM Tris pH7.4, 5 mM EDTA, 0.1% Triton X-100, 1 M NaCl) to remove any nonspecifically bound proteins. The protein G-cyclin/Cdk complex was incubated for 20 minutes at 23°C in 1X kinase reaction buffer (20 mM HEPES, pH 7.4, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5 mM DTT), 5 μCi [<sup>32</sup>P] γ-ATP, 100 μM ATP, 1μg histone H1, and H<sub>2</sub>O or 50 mM sodium phosphate. To terminate the reaction, 5X sampling buffer (463 mM Tris, 13.3% glycerol, 0.002% bromophenol blue, 20% SDS, 13.75 mM β-mercaptoethanol (BME), pH 6.8) was added, and the samples were heated at 95°C for 2 minutes and resolved by SDS-PAGE on 12.5% acrylamide gels. The gel was stained with Coomassie blue for about 1 hour and destained overnight in destain solution (45% methanol, 10% glacial acetic acid). The gel was rinsed in water and exposed to Kodak BioMax film for the autoradiography. The histone H1 bands were cut out and counted for radioactivity in 3 mL safe-scintillation fluid, in a Beckman LS 6000SC scintillation counter.

## Chapter 3

Xic1 inhibits cyclin E/Cdk2 and  
lowers the level of Wee1

## Introduction

A typical, somatic cell cycle is divided into four phases: G1, S (synthesis of new DNA), G2, and M (mitosis) (Elledge, 1996). Checkpoints exist to ensure that genetic and cellular components have been properly maintained in the cell at G1 prior to S phase, G2 prior to M phase and at metaphase prior to anaphase (Hartwell and Weinert, 1989). The G1 checkpoint ensures that the cell is the appropriate size for initiation of DNA replication and that any damaged DNA has been repaired (Moreno and Nurse, 1994; Sherr, 1994). The G2 checkpoint ensures that DNA replication is complete and DNA is not damaged before entering mitosis (Newport and Dasso, 1989). The checkpoint at metaphase ensures the chromosomes are properly aligned along the mitotic spindle. In contrast to a typical somatic cell where three checkpoints are observed, the early cell divisions in *Xenopus* embryos appear to bypass these checkpoints entirely (Newport and Kirschner, 1982 a; Murray and Kirschner, 1989). As the embryo moves from these unregulated cell divisions prior to the midblastula transition (MBT) to the more regulated somatic cell divisions after the MBT, checkpoints are acquired (Newport and Kirschner, 1982 a).

In addition to the acquisition of checkpoints, multiple events characterize the onset of the MBT. At the MBT, cell division time lengthens, zygotic transcription initiates, cells become motile, and the expression of cell cycle regulators changes (Newport and Kirschner 1982; Newport and Kirschner 1989). Degradation of cyclin E occurs rapidly at the MBT. Unlike transcription, which is dependent upon the proper nuclear-to-cytoplasmic ratio (Newport and Kirschner 1982a), the degradation of cyclin E is independent of nuclear-to-cytoplasmic ratio, cell number, protein synthesis, and transcription [(Howe et al., 1995; Howe and Newport, 1996), Figure 2]. The timing of the MBT may be regulated by a developmental timer that triggers degradation of cyclin E at approximately six hours post-fertilization (Howe and Newport, 1996). If the degradation of cyclin E is delayed, then all other events of the MBT are delayed,

including transcription and degradation of cyclin A (Howe et al., 1995; Howe and Newport, 1996).

The only known agent to delay the timing of the degradation of cyclin E is the cyclin-dependent kinase inhibitor (CKI) Xic1 (Hartley et al., 1997). Initially described by Su et al. (1995), Xic1 was shown to have an isoform, Kix1, with 90% homology, as described by Shou and Dunphy (1996). Xic1/Kix1 is a member of the mammalian p21<sup>Cip1</sup>/p27<sup>Kip1</sup>/p57<sup>Kip2</sup> CDK inhibitor family (Su et al. 1995, Shou et al. 1996). Xic1 has a Cdk binding-domain on the N-terminus and proliferating cell nuclear antigen (PCNA) binding-domain on the C-terminus (Su et al., 1995; Shou and Dunphy, 1996). The presence of these two domains are similar to the structure of p21<sup>Cip1</sup> (Chen, 1995), whereas p27<sup>Kip1</sup> has only a Cdk binding domain (Polyak et al., 1994; Font de Mora et al., 1997). Presumably, the two functional domains allow Xic1 to inhibit DNA synthesis via Cdk and PCNA binding.

In the cell cycles before the MBT, although cyclin E level does not vary [(Hartley et al., 1996; Howe and Newport, 1996; Hartley et al., 1997), Figure 2], cyclin E/Cdk2 activity oscillates twice per cell cycle (Hartley, Rempel et al., 1996). This suggests that cyclin E/Cdk2 has a role in the initiation of DNA synthesis and mitosis (Guadagno and Newport, 1996; Hartley et al., 1996; Hartley et al., 1997). In higher eukaryotes, the activity of cyclin E/Cdk2 is essential for the replication of DNA at unwinding, and PCNA activity is required for the formation of the initiation complex (Strausfeld et al., 1994; Chen, 1995; Jackson et al., 1995; Luo et al., 1995; Su et al., 1995; Shou and Dunphy, 1996). Therefore, Xic1 may inhibit initiation of replication via cyclin E/Cdk2.

The ability of Xic1 to bind and inhibit cyclin E/Cdk2 and delay the degradation of cyclin E was of interest to our lab. Previously Su et al. (1995) had generated two recombinant Xic1 fusion proteins, 1) GST-Xic1-C, composed of the C-terminal (residues 97-210) half of Xic1 including the PCNA-binding domain (within residues 165-186) and 2) GST- $\Delta$ 34-Xic1, composed of the N-terminal Cdk-binding domain and C-terminal PCNA binding domain with the first 34 amino acids deleted, therefore consisting of amino acids 35-210. The GST-Xic1-C protein is unable to inhibit the activity of

cyclin/Cdk complexes, and the GST- $\Delta$ 34-Xic1 protein is a highly specific inhibitor of cyclin E/Cdk2 but not cyclin A/Cdc2 or cyclin B/Cdc2 (Su et al., 1995; Shou and Dunphy, 1996; Hartley et al., 1997). Microinjections of GST- $\Delta$ 34-Xic1 protein into fertilized eggs to specifically inhibit cyclin E/Cdk2 activity leads to a delay in cyclin E degradation until 10 hours post-fertilization, thus altering the cyclin E/Cdk2 developmental timer. In addition, GST- $\Delta$ 34-Xic1 also delays the cell cycle by approximately 25%, delaying the embryo from reaching the nuclear-to-cytoplasmic ratio required for the MBT until 10 hours post-fertilization, coincident with degradation of cyclin E (Hartley et al., 1997). GST-Xic1-C does not inhibit cyclin E/Cdk2 activity, cell cycle length, or the timing of cyclin E degradation; therefore, GST-Xic1-C can be used as a negative control for GST- $\Delta$ 34-Xic1 (Su et al., 1995; Hartley et al., 1997). These recombinant proteins provide powerful tools by which investigations into the cyclin E/Cdk2 developmental timer and cell cycle regulation can be conducted.

Cell cycle regulation has been extensively studied experimentally in cell-free extracts in *Xenopus* (Murray and Kirschner, 1989; Solomon et al., 1990; Gautier et al., 1991; Kumagai and Dunphy, 1991; Devault et al., 1992; Kumagai and Dunphy, 1992; Smythe and Newport, 1992; Solomon et al., 1992; Izumi and Maller, 1993; Mueller et al., 1995 a; Mueller et al., 1995 b; Lorca et al., 1998). In egg extracts, cyclin B/Cdc2 (MPF) activity promotes the degradation of cyclin B in a negative feedback loop, which causes oscillations in MPF activity that drive the cell cycle (Lorca et al., 1998). MPF is also inhibited via phosphorylation on Thr-14 and Tyr-15 by Myt1 and Wee1 (Mueller et al., 1995 a; Mueller et al., 1995 b). In a positive feedback loop, MPF activates the phosphatase Cdc25, which removes inhibitory phosphorylations on MPF, and inactivates Myt1 and Wee1, leading to self activation (Izumi and Maller, 1993; Mueller et al., 1995 a; Mueller et al., 1995 b; Kumagai and Dunphy, 1997).

Mathematical modeling has also been used to describe and predict how the cell cycle is regulated (Novak and Tyson, 1993; Novak and Tyson, 1995; Borisuk and Tyson, 1998; Marlovits, 1998). Ordinary differential equations (ODEs) are used to predict and model events in the cell cycle. For instance, the role of MPF in promoting the

degradation of cyclin B (negative feedback loop) explains the oscillations in MPF activity. The feedback loops between MPF and Cdc25/Myt1 and Wee1 are not required for the oscillations, but explain the autocatalytic nature of MPF (Hoffman et al., 1993; Izumi and Maller, 1993; Kumagai and Dunphy, 1997; Mueller et al., 1995a; Mueller et al., 1995b).

On the other hand, cyclin E degradation at the MBT (the cyclin E developmental timer) and the observation of two oscillations in cyclin E/Cdk2 activity per cell cycle before the MBT have not been characterized by a mathematical model. A mathematical model may incorporate the similarities and differences in MPF and cyclin E/Cdk2 regulation to create ODEs. The ODEs are then supplied with estimated rate constants and concentrations of any given component in cyclin E/Cdk2 regulation to predict a role for cyclin E/Cdk2 activity in cyclin E degradation.

In an attempt to understand the regulation of the cyclin E/Cdk2 developmental timer and the oscillations in cyclin E/Cdk2 activity, a mathematical model was built in conjunction with the experiments described here (Appendix A). This mathematical model, by Andrea Ciliberto and John Tyson, took into consideration the similarities and differences in Cdk2 and Cdc2 regulation. For instance, Cdk2 is not regulated by the level of cyclin E in pre-MBT cells, since cyclin E level is constant (Hartley et al., 1997). In addition, the cyclin E/Cdk2 inhibitor, Xic1, is present in low a level previous to late-gastrulation, 11-12 hours post-fertilization and therefore, no CKI regulation is likely for Cdk2 as for Cdc2 (Shou and Dunphy, 1996). It has been noted that Cdk2 is regulated by phosphorylation events (Kappas et al., 2000; D'Angiolella et al., 2001). If Cdk2 activity is regulated by a negative feedback loop, similarly to Cdc2, then the oscillations in cyclin E/Cdk2 activity are likely due to a negative feedback loop at the site of inhibitory phosphorylation. Therefore, initial design of a mathematical model and experimentation was based on the assumption that oscillations in cyclin E/Cdk2 activity are due to the phosphorylation state of Cdk2. To provide critical data for the model and to experimentally validate the predictions made in the comprehensive mathematical model, we performed the following sets of experiments.

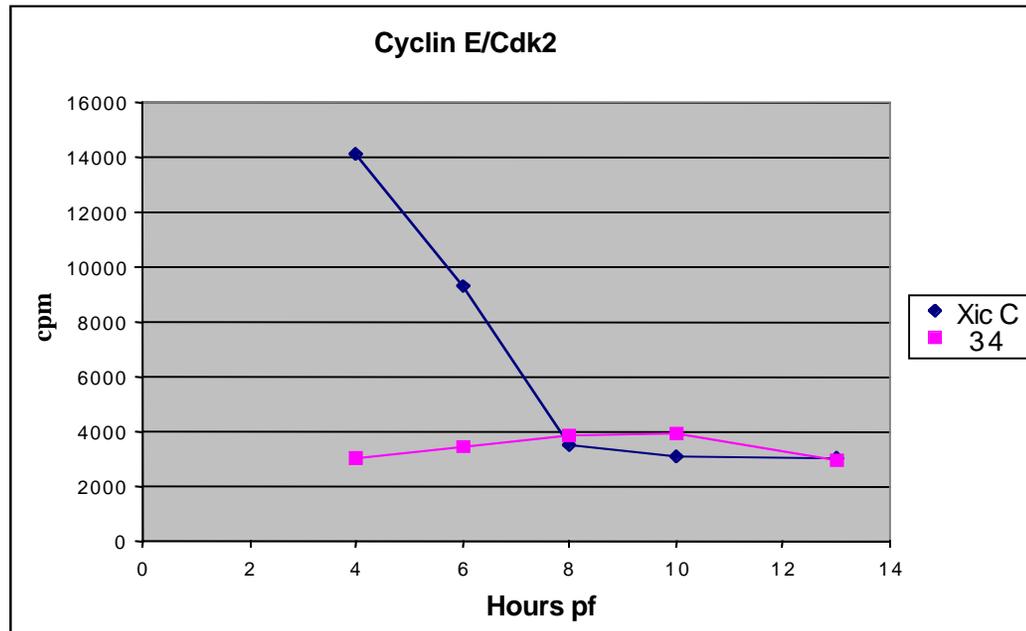
## Results

***The inhibition of cyclin E/Cdk2 by GST-Δ34Xic1 leads to decreased level of Wee1 protein and mRNA in vivo.*** Cyclin E/Cdk2 has two peaks of activity during the cell divisions before the MBT (Hartley et al., 1996). Cyclin E level is constant, and we assume cyclin E/Cdk2 is primarily regulated by phosphorylation. To explore the causes of cyclin E/Cdk2 oscillations, we began by looking for evidence of a negative feedback loop between Cdk2 and its regulatory kinase, Wee1. If changes in the phosphorylation state of Cdk2 cause the observed oscillations in cyclin E/Cdk2 activity, then Wee1 might be activated by cyclin E/Cdk2 in a negative feedback loop. We hypothesized that Wee1 is indeed activated by cyclin E/Cdk2 and performed experiments to test this hypothesis.

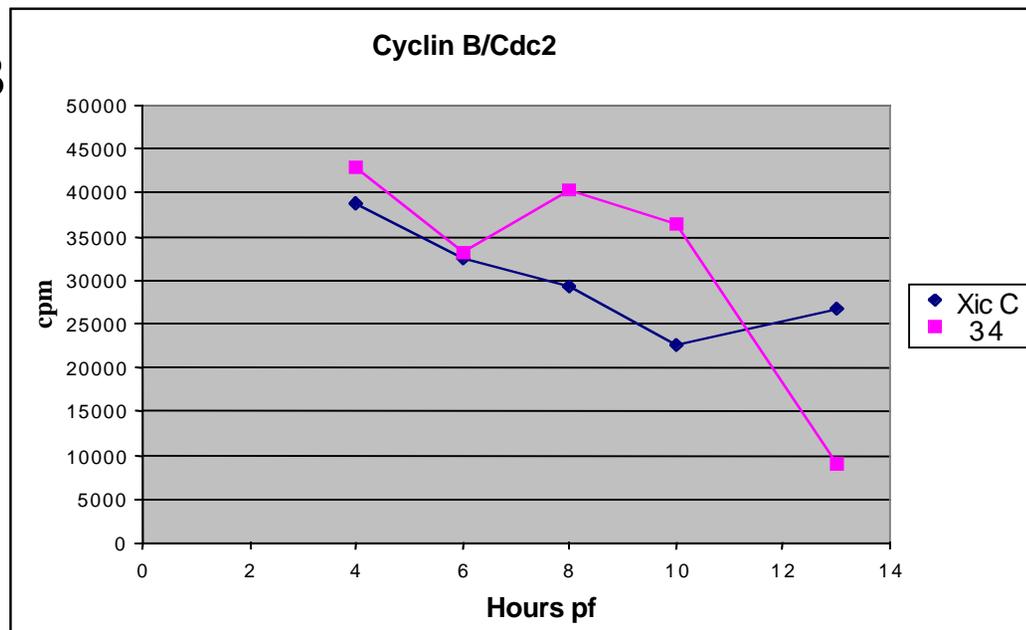
Embryos were microinjected prior to the first cleavage with 5 ng GST-Xic1-C or GST-Δ34-Xic1. Xic1-C lacks the first 96 amino acids and does not bind Cdks; therefore, it serves as a negative control (Su et al., 1995; Hartley et al., 1997). GST-Δ34-Xic1 is missing the first 34 amino acids on the N-terminus and is highly specific for the inhibition of cyclin E/Cdk2 (Su et al., 1995; Hartley et al., 1997). We have confirmed that GST-Δ34-Xic1 significantly inhibits cyclin E/Cdk2 activity (Figure 3 A) without inhibiting cyclin B/Cdc2 activity (Figure 3 B). In addition, cell cycle lengthening of approximately 25% was observed in embryos microinjected with GST-Δ34-Xic1 compared to those microinjected with GST-Xic1-C was observed (Figure 4 A). These results are in agreement with Hartley et al. (1997). As described previously (Hartley et al., 1997), the disappearance of cyclin E in control embryos begins after the MBT, about 7 hours post-fertilization. GST-Δ34-Xic1 delays this disappearance until approximately 10 hours post-fertilization, until after the embryos have approximately reached the MBT number of cells (Figure 4 B).

**Figure 3. GST- $\Delta$ 34-Xic1 specifically inhibits cyclin E/Cdk2.** Embryos were microinjected with 5 ng GST-Xic1-C (Xic C) or GST- $\Delta$ 34Xic1 (34) and collected at the indicated times. Cyclin E/Cdk2 and cyclin B/Cdc2 complexes were immunoprecipitated with antisera against cyclins E and B, respectively. Kinase activity was determined by the incorporation of [ $^{32}$ P] into histone H1 using [ $^{32}$ P]  $\gamma$ -ATP. (A) Cyclin E/Cdk2 activity is inhibited in the GST- $\Delta$ 34Xic1 injected embryos. Cyclin E/Cdk2 activity decreases in GST-Xic1-C injected embryos, coincident with cyclin E degradation at the MBT. (B) Cyclin B/Cdc2 activity is not inhibited in GST- $\Delta$ 34Xic1 injected embryos.

Figure 3 A

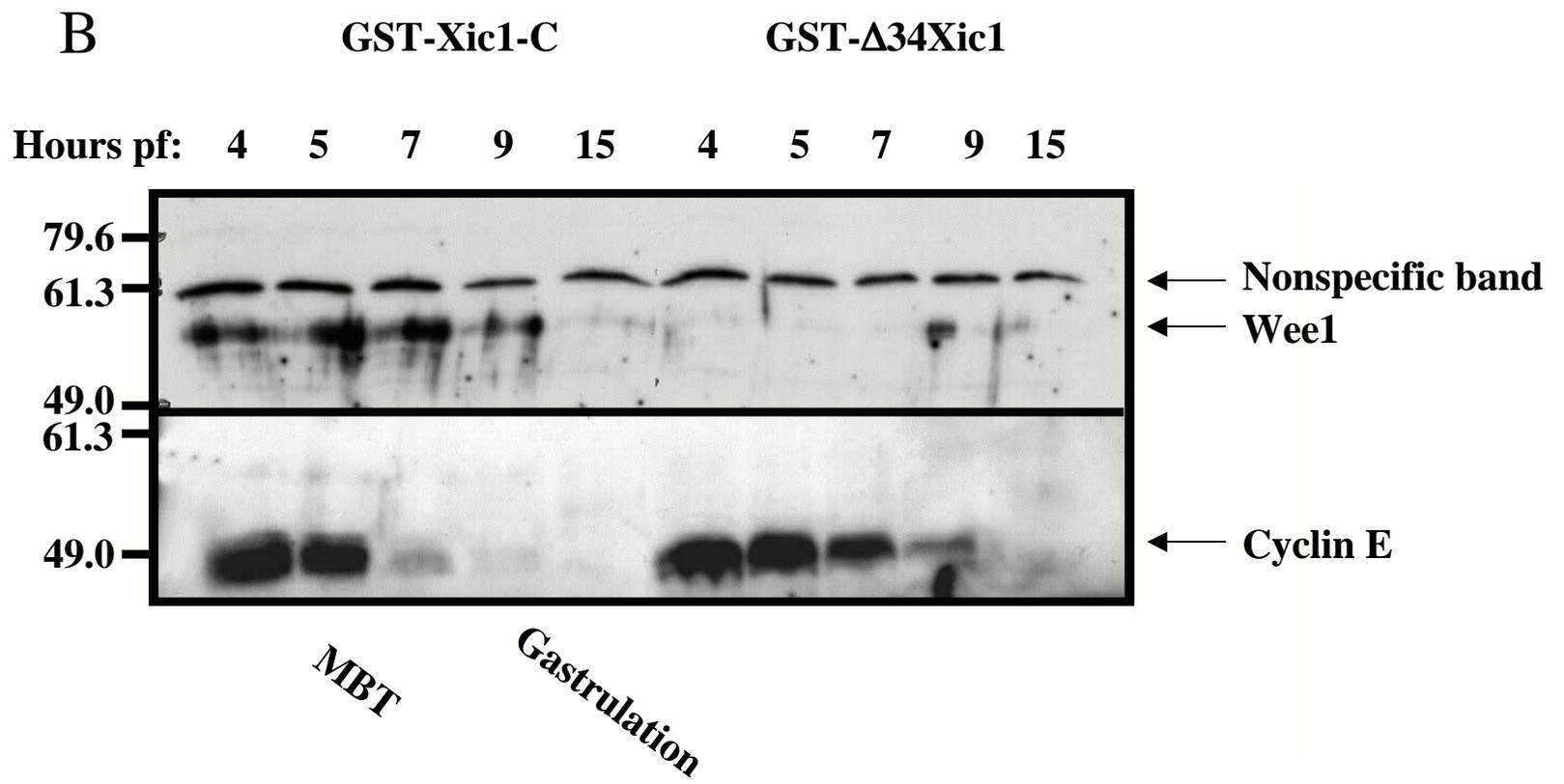
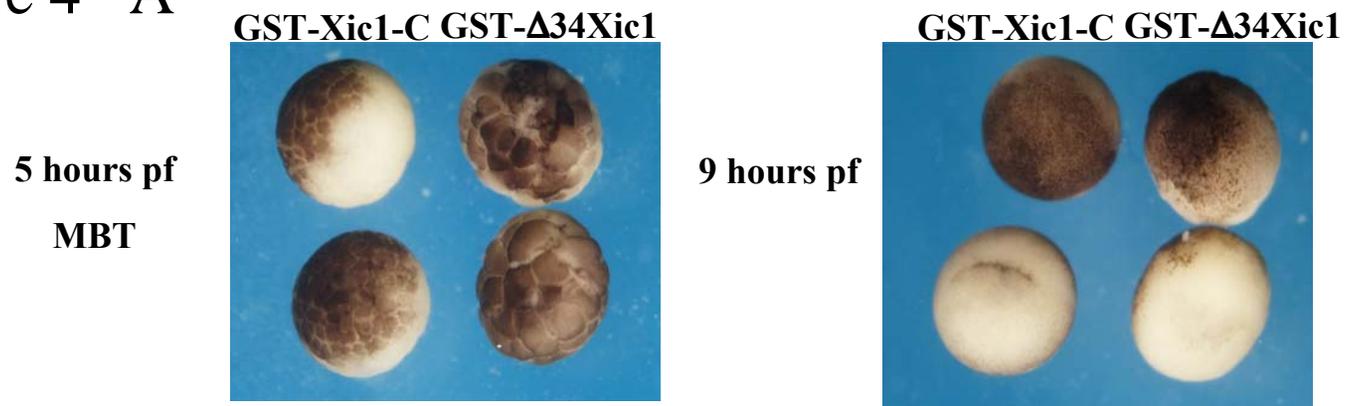


B



**Figure 4. Inhibition of cyclin E/Cdk2 by exogenous GST- $\Delta$ 34Xic1 in early *Xenopus* embryos results in a loss of Wee1 protein.** At the one-cell stage, embryos were microinjected with either 5 ng GST-Xic1-C or GST- $\Delta$ 34Xic1. (A) Embryos were fertilized and photographed at the indicated time post-fertilization (pf). Cell cycles are delayed in GST- $\Delta$ 34Xic1 microinjected embryos. (B) Embryos were collected at the indicated times pf and analyzed by Western analysis for steady state level of Wee1 (Hartley et al. 1996) and cyclin E protein (Rempel et al., 1995; Hartley et al., 1997; Audic et al., 2001). The migration of molecular weight standards in kDa is indicated. The detection of a nonspecific band on Western analysis of Wee1 is consistent with reports of Murakami and Vande Woude (1998).

Figure 4 A



In control embryos, Wee1 level is constant until the late gastrula/early neurula stage (Figure 4 B), when Wee1 level declines [(Mueller et al., 1995; Murakami and Woude, 1998), Figure 4 B]. In the embryos injected with GST- $\Delta$ 34-Xic1, the level of Wee1 is very low, even prior to the MBT (Figure 4 B). These data suggest that cyclin E/Cdk2 activity is required to maintain Wee1 protein level prior to the MBT, evidence for a negative feedback loop between Cdk2 and Wee1.

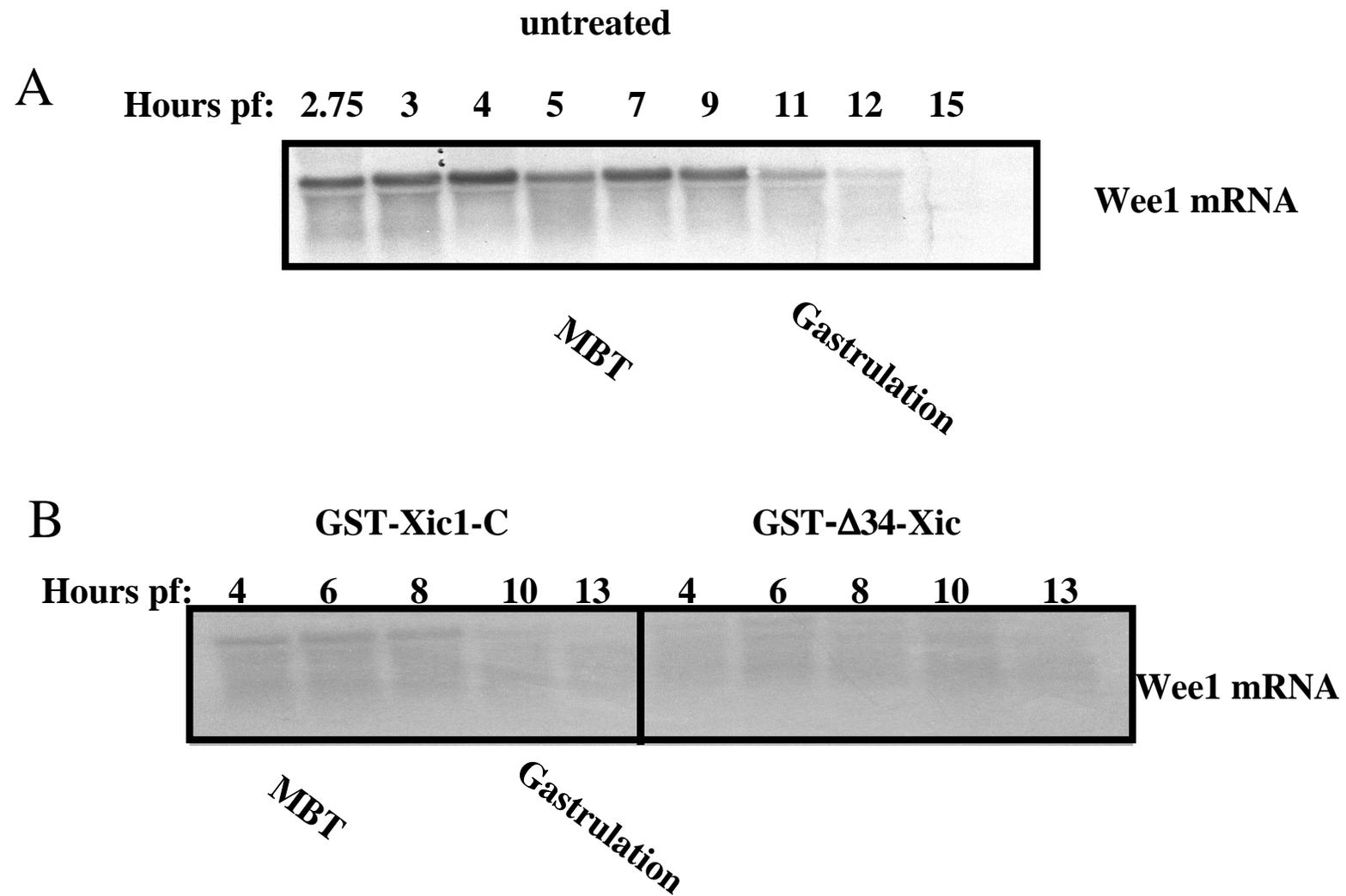
To determine whether the decrease in Wee1 protein level in GST- $\Delta$ 34-Xic1-injected embryos was related to a change in Wee1 mRNA level, we performed Northern analysis. Prior to determining the effect of GST- $\Delta$ 34-Xic1 on Wee1 mRNA, we collected a time course of untreated *Xenopus* embryos. Total RNA was extracted from the embryos and a total of 10  $\mu$ g was resolved on an agarose gel and transferred to a nylon membrane. A copy of the *wee1* gene provided by Dr. Monika Murakami was used as template for synthesis of radiolabeled DNA probe (see Chapter 2). The probe was hybridized with the RNA extracted from the collection of normal embryos. Similar to the degradation of Wee1 protein, Wee1 mRNA in normal embryos is degraded at gastrulation, or approximately 11 hours post-fertilization (Figure 5 A).

Embryos were then microinjected with 5 ng of GST-Xic1-C or GST- $\Delta$ 34-Xic1 for Northern blot analysis and were collected at the indicated times. Injections of GST- $\Delta$ 34-Xic1 led to a decreased level of Wee1 mRNA (Figure 5 B). Therefore, inhibition of cyclin E/Cdk2 by GST- $\Delta$ 34-Xic1 leads to lower a level of Wee1 mRNA and Wee1 protein, indicating that cyclin E/Cdk2 activity may maintain the Wee1 level by stabilizing Wee1 mRNA.

**Figure 5. Microinjection of GST- $\Delta$ 34-Xic1 leads to lower levels of Wee1 mRNA.**

(A) Normal embryos were collected at the indicated times for Northern analysis of steady state level of Wee1 mRNA. (B) Northern blot analysis of embryos injected with either GST-Xic1-C or GST- $\Delta$ 34Xic1 indicated that steady state level of Wee1 mRNA is lower when cyclin E/Cdk2 activity is inhibited by GST- $\Delta$ 34Xic1.

Figure 5

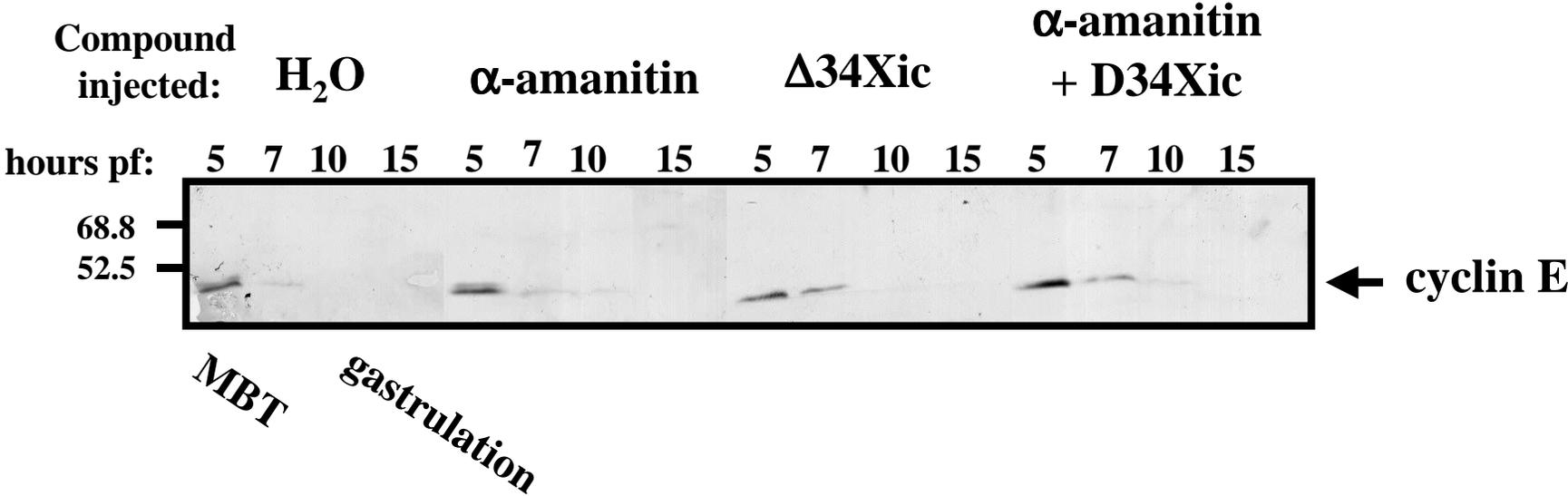


***GST-Δ34Xic1 delays the degradation of cyclin E in a dose-dependent and transcription-independent manner.*** In the initial attempts at constructing a mathematical model, the eventual degradation of cyclin E in embryos injected with GST-Δ34-Xic1 (Hartley et al., 1997) was not explained (Figure 3 B). The model predicted that in the absence of cyclin E/Cdk2 activity, cyclin E persists indefinitely, unless another mechanism to degrade developmental cyclin E exists. Since the degradation of cyclin E in both control and GST-Δ34-Xic1-injected embryos occurs coincident with the onset of zygotic transcription (Hartley et al., 1997), we explored the possibility that a product of zygotic transcription turned on degradation of developmental cyclin E by a second mechanism, independent of cyclin E/Cdk2 activity.

To test this possibility, embryos were injected with either H<sub>2</sub>O; α-amanitin, an inhibitor of transcription; GST-Δ34-Xic1; or a combination of α-amanitin and GST-Δ34-Xic1 (Figure 6). In embryos injected with H<sub>2</sub>O or α-amanitin, cyclin E disappeared between 5 and 7 hours post-fertilization, consistent with previous reports that degradation of developmental cyclin E at the MBT does not depend on transcription (Howe and Newport, 1996; Hartley et al., 1997). As predicted [(Hartley et al., 1997) and Figure 4 B], degradation of cyclin E was delayed in embryos injected with GST-Δ34-Xic1, but was complete by 10 hours post-fertilization. In embryos injected with both α-amanitin and GST-Δ34-Xic1, cyclin E was degraded at the same time as in embryos injected with GST-Δ34-Xic1 alone (Figure 6). These results indicate that transcription at the MBT is not required for the eventual degradation of cyclin E in embryos lacking cyclin E/Cdk2 activity. Therefore, our data is consistent with the hypothesis that cyclin E degradation is dependent upon cyclin E/Cdk2 activity and not another mechanism. As long as GST-Δ34-Xic1 is present in the embryo, cyclin E/Cdk2 activity will be inhibited along with cyclin E degradation. However, there is an eventual degradation of cyclin E in embryos injected with GST-Δ34-Xic1 [(Hartley et al., 1997), Figures 5 B, and 7]. This degradation of cyclin E could be explained if GST-Δ34-Xic1 is eventually degraded and precedes degradation of cyclin E.

**Figure 6. Zygotic transcription is not required for degradation of cyclin E.** Embryos were injected with H<sub>2</sub>O, GST-Δ34Xic1, α-amanitin, or GST-Δ34Xic1 and α-amanitin. Embryos were collected at the indicated times pf for Western analysis of cyclin E. The inhibition of transcription with α-amanitin does not further delay the degradation of the cyclin E in GST-Δ34Xic1 microinjected embryos.

Figure 6



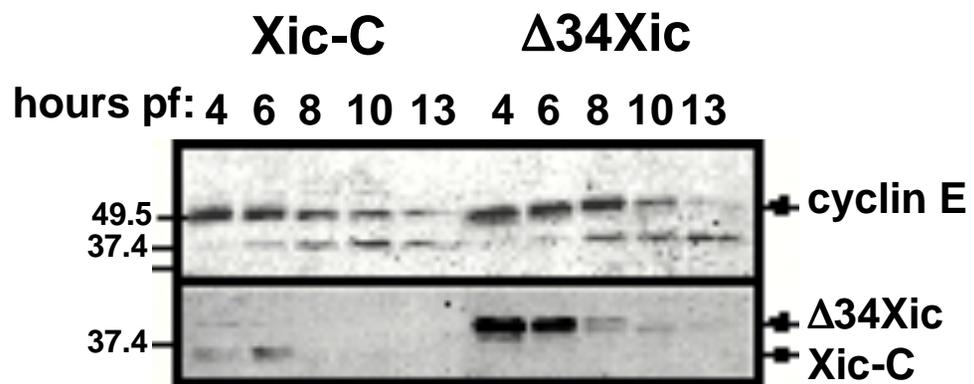
To test for the degradation of GST- $\Delta$ 34-Xic1 in embryos, we injected GST-Xic1-C or GST- $\Delta$ 34-Xic1 into one-cell staged embryos and collected them as in previous experiments. Western analysis with an anti-Xic1 antibody, demonstrated the degradation of GST- $\Delta$ 34-Xic1 and GST-Xic1-C just prior to, or coinciding with, the degradation of cyclin E (Figure 7).

The mathematical model predicted that if the degradation of cyclin E is dependent upon its own activity, then the delay in cyclin E degradation will be prolonged as the total concentration of GST- $\Delta$ 34-Xic1 injected into one-cell stage embryos increases (Appendix A). To test this prediction, varying amounts of GST- $\Delta$ 34-Xic1 were injected into embryos, and the timing of cyclin E degradation was determined by Western analysis. Cyclin E degradation was further delayed in embryos injected with increasing amounts of GST- $\Delta$ 34-Xic1 (Figure 8). These results are consistent with the hypothesis that Xic1 must be degraded prior to cyclin E in the embryo.

Collectively, the mathematical model (Appendix A) and the experimental data suggest that cyclin E/Cdk2 activity leads to the stabilization of Wee1 in a negative feedback loop. The inhibition of cyclin E/Cdk2 by GST- $\Delta$ 34-Xic1 disrupts this stabilization, and Wee1 protein and mRNA levels are decreased. In addition, cyclin E/Cdk2 activity may be essential for the degradation of cyclin E. Consistent with the mathematical model, these data predict that in the absence of cyclin E/Cdk2 activity, cyclin E will persist, and Xic1 degradation precedes cyclin E degradation *in vitro*.

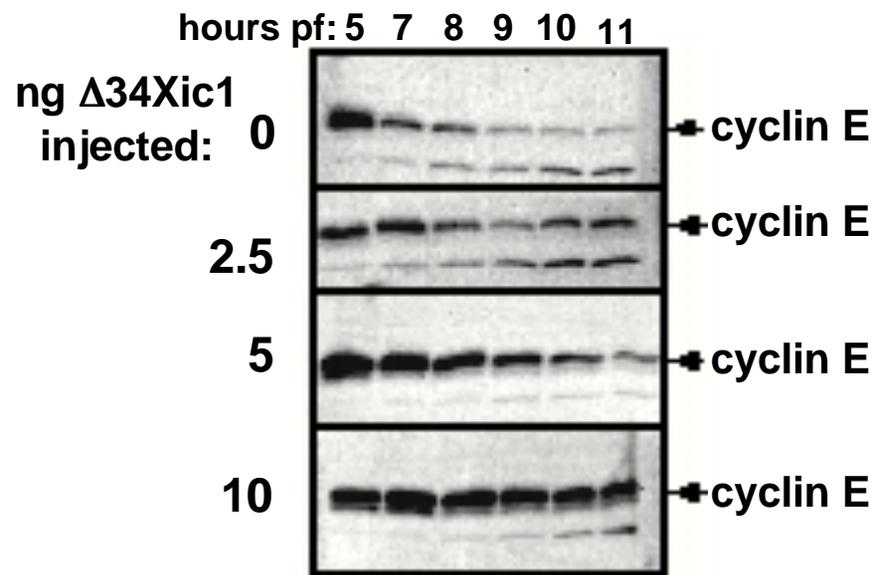
**Figure 7. GST- $\Delta$ 34Xic1 is degraded prior to the degradation of cyclin E.** Embryos were collected at the indicated times for Western blot analysis of cyclin E and Xic1. GST- $\Delta$ 34Xic1 is degraded prior to cyclin E. Migration of molecular weight markers in KDa is indicated.

Figure 7



**Figure 8. Timing of the degradation of cyclin E depends upon the dose of GST- $\Delta$ 34-Xic1.** Embryos were injected with either buffer as a control, 2.5 ng, 5 ng, or 10 ng of GST- $\Delta$ 34-Xic1. In buffer injected embryos, cyclin E is degraded at the MBT. Increasing concentrations of GST- $\Delta$ 34Xic1 lead to a prolonged delay in degradation of cyclin E. Inhibiting cyclin E/Cdk2 activity delays the degradation of cyclin E, suggesting cyclin E degradation is dependent upon the activity of cyclin E/Cdk2.

Figure 8



## Discussion

During the cell cycles before the MBT, although cyclin E level does not vary [(Rempel et al., 1995; Hartley et al., 1996; Howe and Newport, 1996; Hartley et al., 1997), Figure 2], cyclin E-associated kinase activity oscillates twice per cell cycle (Hartley et al., 1996). These oscillations occur independently of nuclear-to-cytoplasmic ratio, cell number, protein translation, and transcription (Hartley et al., 1997; Graves et al., 2000). MPF activity oscillates once per cell cycle in frog embryos and is regulated by cyclin B level (Murray et al., 1989a; [Murray et al., 1989b; Gautier et al., 1990). In contrast, we propose that cyclin E/Cdk2 activity is regulated by phosphorylation in a negative feedback loop where cyclin E/Cdk2 promotes synthesis of Wee1.

In control embryos, Wee1 protein level is constant until the late gastrula/early neurula stage when Wee1 protein level declines (Figure 4 B). In the embryos injected with GST- $\Delta$ 34-Xic1, the level of Wee1 protein is very low, even prior to the MBT (Figure 4 B). The level of Wee1 mRNA is also lower in GST- $\Delta$ 34-Xic1 injected embryos (Figure 5 B). Because GST- $\Delta$ 34-Xic1 specifically inhibits cyclin E/Cdk2, these data suggest that cyclin E/Cdk2 activity is required to maintain Wee1 protein level prior to the MBT. One possibility, incorporated into the mathematical model (Appendix A), is that a negative feedback loop exists between cyclin E/Cdk2 and Wee1, in which cyclin E/Cdk2 phosphorylates and inhibits a kinase that phosphorylates Wee1, targeting it for degradation. In embryos injected with GST-Xic1-C, cyclin E is degraded prior to Wee1 (Figure 4 B), further suggesting a role for cyclin E/Cdk2 in the stabilization of Wee1 via Wee1 mRNA (Figure 5 B).

The inhibition of cyclin E/Cdk2 by Xic1 only leads to a temporary delay in cyclin E degradation. GST- $\Delta$ 34Xic1 inhibits cyclin E/Cdk2 by >90%, and early drafts of the mathematical model predicted that in the absence of cyclin E/Cdk2 activity, cyclin E persists indefinitely, unless another mechanism to degrade developmental cyclin E exists.

Since the degradation of cyclin E in both control and GST- $\Delta$ 34Xic1-injected embryos occurs coincident with the onset of zygotic transcription, we explored the possibility that

a product of zygotic transcription turned on degradation of developmental cyclin E, independent of cyclin E/Cdk2 activity. The co-injection of  $\alpha$ -amanitin and GST- $\Delta$ 34-Xic1 did not prolong the delay in cyclin E degradation (Figure 6). Our data corresponds to Hartley et al., (1997) and suggests that cyclin E degradation is independent of transcription. The prediction that transcription lead to another mechanism of cyclin E degradation was not supported by the data.

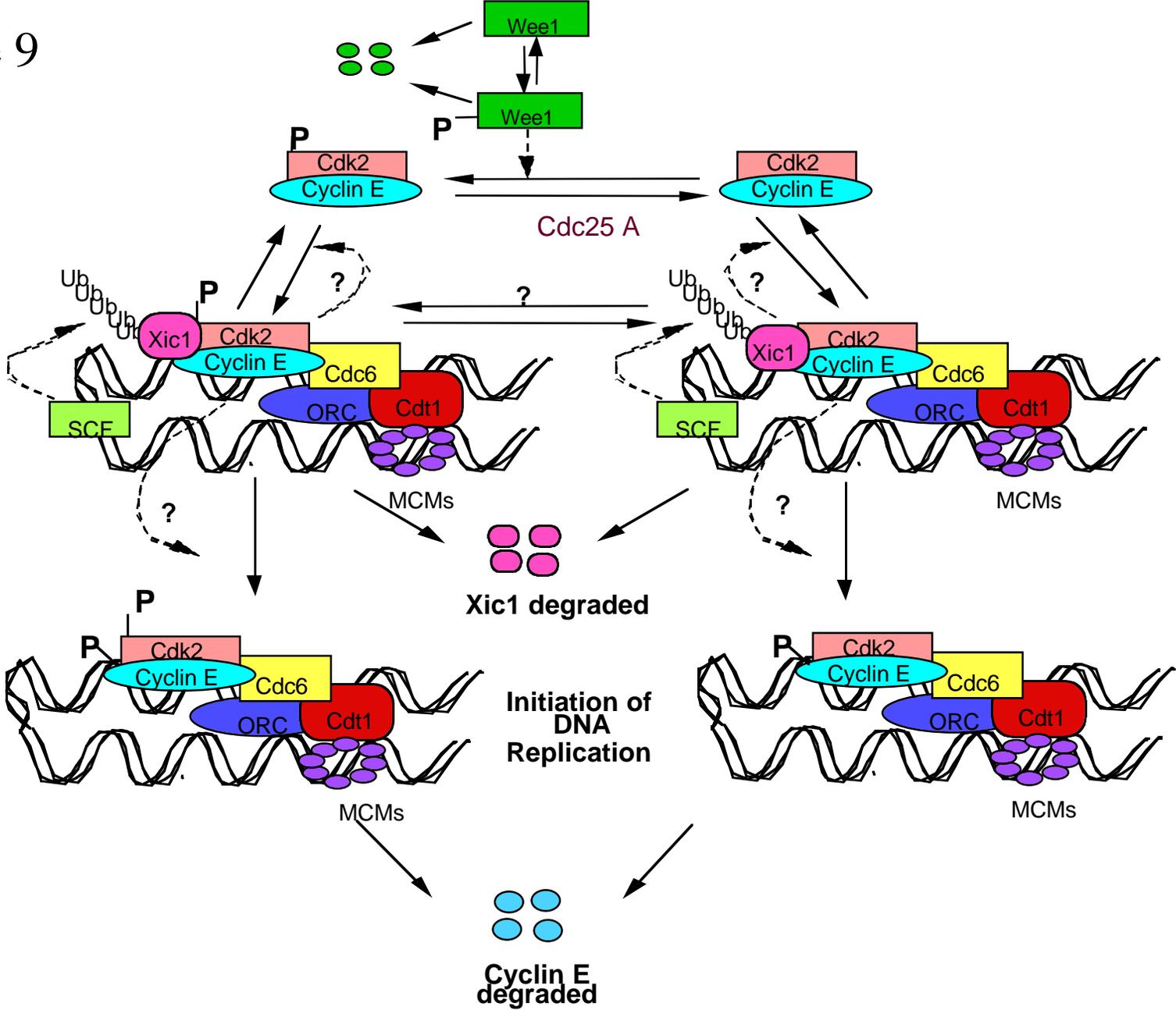
Initial drafts of the mathematical model were unable to explain the eventual degradation of cyclin E in the absence of cyclin E/Cdk2 activity. We hypothesized that if cyclin E/Cdk2 activity is essential for the degradation of cyclin E, then GST- $\Delta$ 34Xic1 eventually must be degraded prior to cyclin E degradation and increasing amounts of GST- $\Delta$ 34Xic1 will further inhibit cyclin E/Cdk2 activity and delay cyclin E degradation. Our data indicates that GST- $\Delta$ 34-Xic1 is, in fact, degraded prior to cyclin E (Figure 7) and higher concentrations of GST- $\Delta$ 34Xic1 do prolong the delay in cyclin E degradation (Figure 8).

The observed degradation of GST- $\Delta$ 34-Xic1 prior to cyclin E is supported by studies in *Xenopus* egg extracts. Cyclin E/Cdk2 binds Xic1 and loads Xic1 onto the chromatin (Figure 9), promoting Xic1 degradation (Swanson et al., 2000; Chuang and Yew, 2001; Furstenthal et al., 2001). Xic1 binds cyclin E/Cdk2 in the cytosol and is transported as a complex into the nucleus [(Swanson et al., 2000; Chuang and Yew, 2001; Furstenthal et al., 2001), Figure 9]. Once in the nucleus, the Xic-cyclin E/Cdk2 complex binds to Cdc6 and ORC and associates with the chromatin (Swanson et al., 2000; Chuang and Yew, 2001; Furstenthal, 2001; Furstenthal et al., 2001). This association is essential for Xic1 ubiquitination and degradation (Chuang and Yew, 2001; Furstenthal et al., 2001). These data and our data together suggest that Xic1 binds cyclin E/Cdk2 and is transported into the nucleus and the initiation of DNA synthesis is inhibited. On the chromatin at origins of replication, Xic1 is degraded, and DNA synthesis is initiated (Figure 9). Once Xic1 is degraded, cyclin E/Cdk2 initiates DNA synthesis and its activity may eventually lead to the degradation of cyclin E (Figure 9, see model in Appendix A).

We suggest cyclin E/Cdk2 is regulated primarily by phosphorylation. Together with the mathematical model (Appendix A), our data suggests cyclin E/Cdk2 is linked to Wee1 in a negative feedback loop, stabilizing Wee1 protein via Wee1 mRNA. This negative feedback loop explains the degradation of cyclin E at the MBT, and Wee1 at gastrulation. The oscillations in cyclin E/Cdk2 activity before the MBT, however, can not be explained by the same negative feedback loop with Wee1. In the absence of zygotic transcription in the pre-MBT cell divisions, the removal of Wee1 mRNA is terminal. To explain the oscillations in cyclin E/Cdk2 activity before the MBT we hypothesize cyclin E/Cdk2 inhibits an inhibitor of Wee1 and the mathematical model (Appendix A) suggests that will lead to the observed oscillations in cyclin E/Cdk2 activity prior to the MBT. In addition, our data suggests that cyclin E/Cdk2 activity appears to play a role in the degradation of cyclin E at the MBT.

**Figure 9. Xic1 binds to and inhibits cyclinE/Cdk2, which loads Xic1 on to the chromatin for degradation.** Cyclin E/Cdk2 is negatively regulated when phosphorylated by the kinase Wee1. The phosphatase Cdc25A removes this inhibitory phosphorylation and sends cyclin E/Cdk2 into the activated state. When Xic1 is present in egg extracts, it binds cyclin E/Cdk2 or cyclin E/Cdk2-P and is transported into the nucleus as a complex (Swanson et al., 2000; Chuang and Yew, 2001; Furstenthal et al., 2001). Once in the nucleus, the complex associates with Cdc6-ORC and is bound to the chromatin in complex with Cdt1 and MCMs, where cyclin E/Cdk2 recruits the ubiquitin-ligase SCF, and Xic1 becomes ubiquitinated and degraded in the nucleus (Fursenthal, 2001; Furstenthal et al., 2001). We hypothesize (question marks) that cyclin E/Cdk2 might move between the inactive and active states once bound to the chromatin. In addition, we hypothesize that cyclin E/Cdk2 actively recruits more cyclin E/Cdk2 to the chromatin. After Xic1 is degraded in somatic cells we hypothesize that the activity of cyclin E/Cdk2 leads to DNA synthesis and the degradation of cyclin E (See Appendix A). Our data suggest that cyclin E/Cdk2 is involved in a negative feedback loop with Wee1. This model became the basis for the mathematical model developed by Andrea Ciliberto and John Tyson (See Chapter 3).

Figure 9



## Chapter 4

XChk1 is linked to the timing of, and cell cycle remodeling at the MBT.

## Introduction

During the cell divisions before the MBT, damaged or unreplicated DNA (Hensey, 1997) does not induce a checkpoint, and the cell cycle proceeds (Anderson et al., 1997; Hensey and Gautier, 1997). After the MBT, on the other hand, damaged DNA will prevent the activation of MPF, allowing cells to correct the damage and survive (Anderson et al., 1997; Sible et al., 1998). The negative cell cycle regulator *Xenopus* Chk1 (XChk1) becomes activated in response to unreplicated DNA, and potentially to damaged DNA (Kappas et al., 2000), and indirectly leads to the inactivation of MPF [(Furnari et al., 1997; O'Connell et al., 1997), Figure 1]. During the rapid early cell divisions of *Xenopus* embryos, activated Cdc25 (Izumi et al., 1992; Kumagai and Dunphy, 1992; Izumi and Maller, 1993) is found in high concentrations and removes phosphorylations on Thr-14 and Tyr-15 of MPF caused by Wee1 and Myt1 (Gautier et al., 1991; Kumagai and Dunphy, 1991; Gabrielli et al., 1992; Mueller et al., 1995a; Mueller et al., 1995b). Cdc25C reaches its highest activity at about the same time active MPF reaches its highest concentration during each cycle, and there is little tyrosine phosphorylation during the early cell divisions (Ferrell et al., 1991; Hartley et al., 1996). This suggests MPF is highly activated in response to Cdc25A and Cdc25C, and this activation signal is stronger than the inactivation signal by Wee1 before the MBT, causing the observed rapid cell divisions.

Although XChk1 and Wee1 are present in the cell before the MBT, checkpoints and gap phases are not observed until five hours post-fertilization when the MBT begins (Newport and Dasso, 1989; Mueller et al., 1995; Anderson et al., 1997; Hensey and Gautier, 1997; Kumagai et al., 1998; Murakami and Woude, 1998; Nakajo et al., 1999). In egg extracts and somatic cells, XChk1 activation in response to unreplicated DNA leads to the inactivation of Cdc25C by phosphorylating it and allowing it to bind 14-3-3, sequestering Cdc25C in the cytosol (Peng et al., 1997; Kumagai et al., 1998; Kumagai, 1999). This leads to the inactivation of MPF and a cell cycle block at G2/M [(Izumi, Walker et al., 1992; Kumagai and Dunphy, 1992; Izumi and Maller, 1993; Nakajo et al.,

1999) Figure 1]. It is currently unclear whether XChk1 phosphorylates Cdc25A (Figure 1). In cell cycle extracts, there is data to suggest XChk1 directly phosphorylates and activates Wee1, but there is no evidence that this activation stabilizes Wee1 (O'Connell et al., 1997; Lee et al., 2001). Wee1 concentrations in the nucleus are seen to be highest when MPF is inactive, and when Wee1 concentrations decrease in the nucleus as a result of proteolysis, the cell enters mitosis, consistent with the role for Wee1 in the inactivation of MPF [(Michael and Newport, 1998) Figure 1]. This suggests XChk1 becomes activated in response to unreplicated DNA and then activates Wee1, leading to MPF inactivation after the MBT.

We have previously demonstrated that in addition to inactivation of MPF, XChk1 inhibits cyclin E/Cdk2 activity indirectly. Earlier (see Chapter 3), we demonstrated that the specific inhibition of cyclin E/Cdk2 activity by the recombinant protein GST- $\Delta 34Xic1$  led to a delay in cyclin E degradation. If the degradation of cyclin E is dependent upon cyclin E/Cdk2 activity, then XChk1 should also block or delay cyclin E degradation. To study and to better understand the role of cyclin E/Cdk2 activity in the degradation of cyclin E, and the role of XChk1 in cell cycle remodeling at, and the timing of, the MBT, we microinjected one-cell staged *Xenopus* embryos with mRNA encoding XChk1 or luciferase as control. Embryos were collected at various time points that span the MBT for Western and Northern blot analysis.

XChk1 microinjected embryos were arrested at the two cell stage (Kappas et al., 2000). This indicates that exogenous XChk1 arrests cell divisions, and leads to a checkpoint before the MBT (Kappas et al., 2000). Because XChk1 microinjected embryos do not divide beyond the eight-cell stage, this indicates that the XChk1 checkpoint pathway is functional downstream of XChk1 in the pre-MBT cell divisions of the embryo.

## Results

***XChk1 alters the timing of the MBT.*** Microinjections of one-cell stage embryos with mRNA encoding XChk1 led to cell cycle arrest and tyrosine phosphorylation of Cdc2 and Cdk2 in embryos (Kappas et al., 2000). The embryos stop dividing at the two-to eight-cell stage (Figure 10 A), suggesting that the XChk1 checkpoint pathway is functional in the cell divisions before the MBT. Firefly luciferase mRNA acts as a good control element in the frog since it is not normally expressed by *Xenopus* and exerts no observable effect on development (Figure 10 A). To determine if the XChk1 and luciferase mRNAs are translated, one-cell stage embryos were microinjected with 15 ng mRNA encoding FLAG-tagged XChk1 or FLAG-tagged luciferase. The embryos were collected at times that span the MBT for Western blot analysis. Western blot analysis of the embryos with an anti-FLAG antibody demonstrated that the FLAG-tagged luciferase protein is present for up to 15 hours post-fertilization, and FLAG-tagged XChk1 protein is present up to 11-15 hours post-fertilization (Figure 10 B).

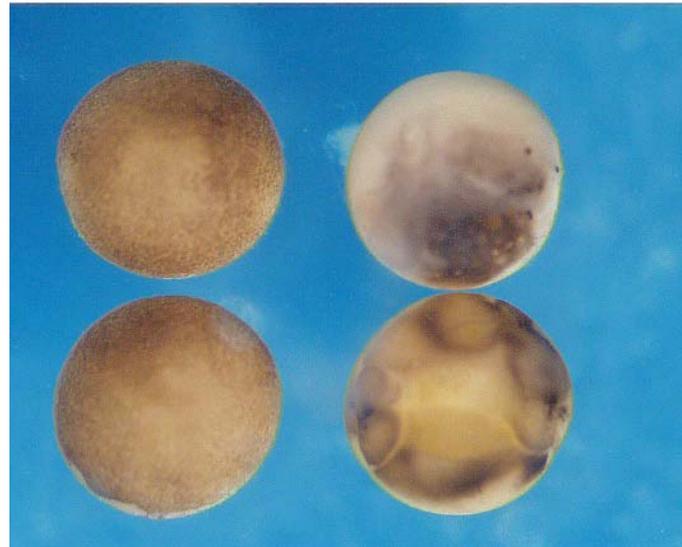
The degradation of cyclin E occurs at seven hours post-fertilization, independent of nuclear-to-cytoplasmic ratio, cell number, and protein synthesis (Howe and Newport, 1996). This consistent degradation of cyclin E at the MBT is linked to a developmental timer (Howe et al., 1995; Howe and Newport, 1996), and is linked to cyclin E/Cdk2 activity (Hartley et al., 1997). The recombinant protein GST- $\Delta$ 34-Xic1 specifically inhibits cyclin E/Cdk2 activity (Hartley et al., 1997) and delays degradation of cyclin E (see Chapter 3). We have also previously demonstrated that exogenous XChk1 inhibits cyclin E/Cdk2 activity (Kappas et al., 2000). Microinjections with mRNA encoding XChk1 were conducted as above. The Western blot analysis demonstrated that XChk1 delays the degradation of cyclin E (Figure 11 A) in *Xenopus* embryos. In addition, exogenous

**Figure 10. Expression of exogenous XChk1 disrupts pre-MBT cell cycles.** Embryos were microinjected at the one-cell stage with 15 ng mRNA encoding FLAG-tagged luciferase or FLAG-tagged XChk1. (A) Embryos were photographed when the controls reached Stage 10 (~10 hours post fertilization). In multiple experiments, the control embryos appeared normal, and the XChk1 embryos stopped dividing at the 2-4 cell stage. (B) Western blots were performed with antibodies against FLAG in lysates from embryos collected at the time indicated. Migration of molecular weight markers (in KDa) is indicated.

Figure 10

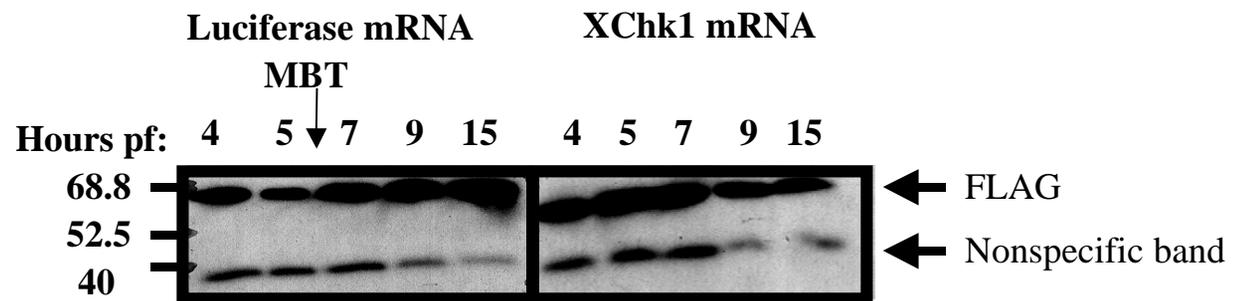
A

Luc mRNA      XChk1 mRNA



5 hours post-fertilization  
(MBT)

B

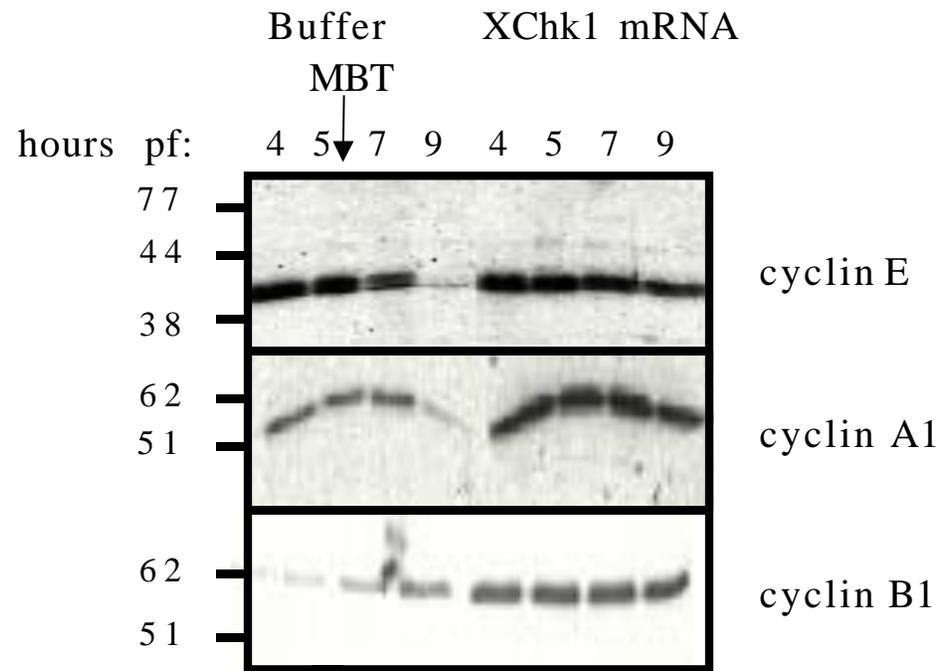


XChk1 led to elevated levels of cyclins A1 and B (Figure 11 A), consistent with cell cycle arrest in mitosis when those cyclins accumulate. Since the disappearance of cyclin E at the MBT and the disappearance of cyclin A1 at gastrulation are delayed in embryos expressing XChk1 (Figure 10), these data demonstrate that like Xic1, the inhibition of Cdk2 by exogenous XChk1 alters the maternal cyclin E/Cdk2 developmental timer.

The timing of the MBT is not only defined by cyclin E degradation but also by cell number, zygotic transcription, nuclear-to-cytoplasmic ratio, and a gain in cell motility. Since both the proper cell number is not reached and degradation of cyclin E is delayed in embryos expressing exogenous XChk1, this suggested the timing of the MBT is altered in embryos microinjected with XChk1. To confirm this, we monitored the initiation of zygotic transcription in control embryos and embryos expressing exogenous XChk1. The gene GS17 is transcribed after the MBT (Krieg and Melton, 1987) at 7 hours post-fertilization (Figure 12 A). Embryos were microinjected at the one-cell stage with mRNA encoding luciferase or XChk1 and collected for Northern blot analysis at the indicated times (Figure 12 B). We demonstrated that zygotic transcription is inhibited past 13-15 hours post-fertilization. These data indicates that exogenous XChk1 not only inhibits cyclin E degradation and stops cell division before the MBT, preventing the embryo from reaching the number of cells required for the MBT, but it also inhibits the initiation of zygotic transcription. Collectively, these data indicates exogenous XChk1 alters the both nuclear-to-cytoplasmic dependent-(zygotic transcription) and independent-(cyclin E degradation) events that characterize the timing of the MBT.

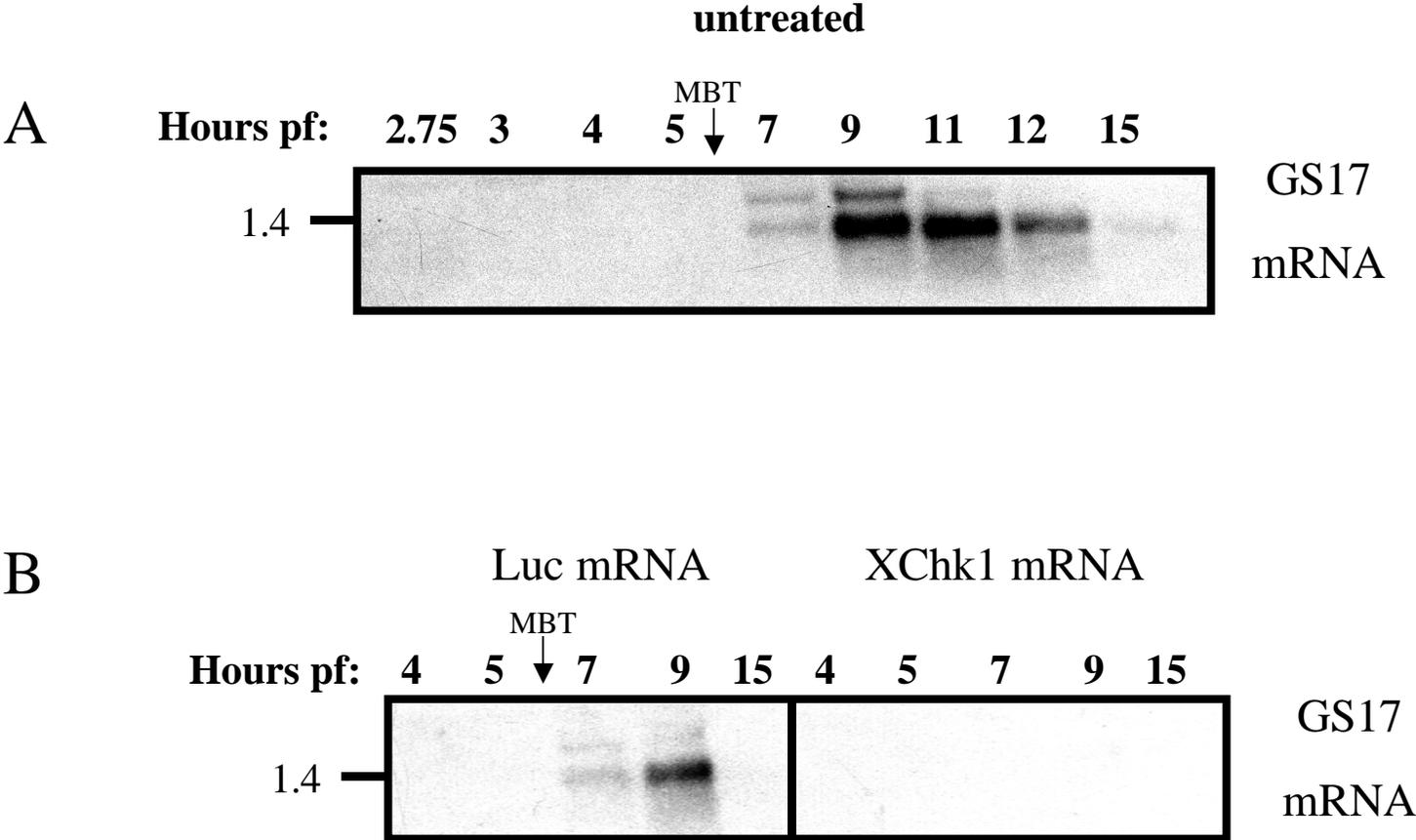
**Figure 11. Expression of exogenous XChk1 delays the degradation of cyclins E and A and elevates level of cyclin B.** Western blots were performed with antibodies against cyclins A, B, and E on lysates from embryos microinjected with 15 ng of control or XChk1 mRNA collected at the times indicated. Cyclin E and A persist, and levels of cyclins B and A are elevated in embryos expressing exogenous XChk1 (performed by Nicholas Kappas).

Figure 11



**Figure 12. Expression of exogenous XChk1 blocks the onset of zygotic transcription.** Total RNA was isolated from embryos collected at the indicated times. Northern blots were performed with a radiolabeled DNA probe against *GS17*, a gene transcribed at the MBT (Krieg and Melton, 1987). (A) Untreated embryos were collected, and the steady state level of *GS17* mRNA was determined. (B) Embryos microinjected with luciferase or XChk1 mRNA were collected and the level of *GS17* mRNA was determined.

Figure 12



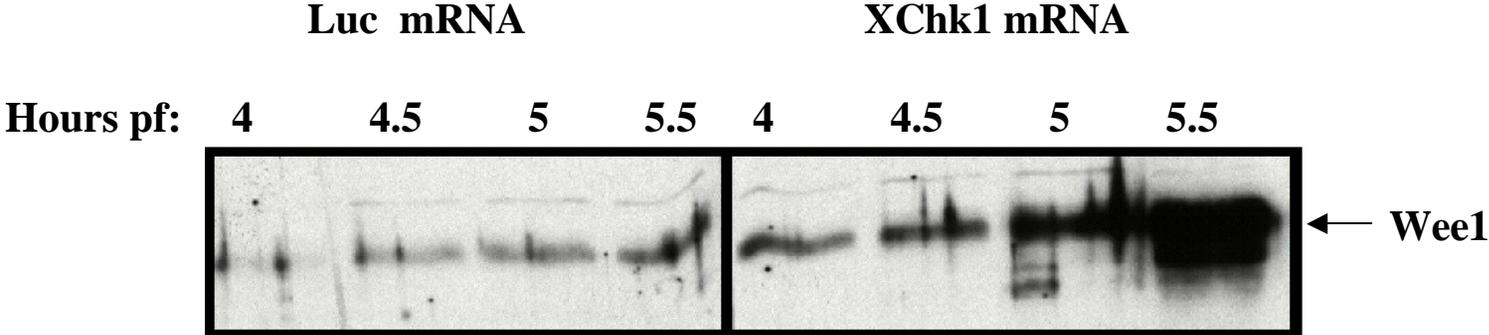
***Expression of exogenous XChk1 leads to an increase in the level of Wee1 and decrease in the level of Cdc25A.*** Both XChk1 and GST- $\Delta$ 34-Xic1 inhibit cyclin E/Cdk2 activity (Su et al., 1995; Hartley et al., 1997). Wee1 is also normally degraded at gastrulation (Mueller et al., 1995), but specific inhibition of cyclin E/Cdk2 by GST- $\Delta$ 34-Xic1 leads to a decrease in the level of Wee1, suggesting a role for cyclin E/Cdk2 activity in the stabilization of Wee1 (Chapter 3, Figure 5 B). In a positive feedback loop, MPF activates itself by inactivating its inhibitor Wee1 (Mueller et al., 1995), and activating its positive regulator Cdc25 (Hoffman et al., 1993). We hypothesized that the inhibition of MPF by XChk1 will interfere with this positive feedback loop, altering Wee1 and Cdc25 protein levels. Furthermore, MPF is known to inhibit Wee1 and based on Chapter 3, we demonstrated cyclin E/Cdk2 stabilizes Wee1. Therefore, the effect of cyclin E/Cdk2 and cyclin B/Cdc2 inhibition by XChk1 is unknown.

To determine the effect of exogenous XChk1 on Wee1 level, embryos microinjected at the one-cell stage with mRNA encoding XChk1 or luciferase. In those XChk1-expressing embryos collected for Western analysis and probed with an anti-Wee1 antibody, the level of Wee1 appeared stable until 5 - 5.5 hours post-fertilization, when the level of Wee1 drastically increased (Figure 13 A). In addition, the positive regulator of MPF, Cdc25A, is present at a constant level until the MBT in embryos injected with mRNA encoding luciferase but exogenous XChk1 leads to lower a level of Cdc25A (Figure 13 B). XChk1 has no observable effect on the level of Cdc25C (Figure 13 B). We hypothesize that MPF targets Wee1 for degradation and stabilizes Cdc25. If this is true, then our data may indicate that the increase in the level of Wee1 and the decrease in Cdc25A is due to the disruption of the MPF positive feedback loop (Figure 1). This increase may also be due, in part, to a direct interaction between exogenous XChk1 and Wee1, or to a stabilization in Wee1 mRNA and increase in protein translation.

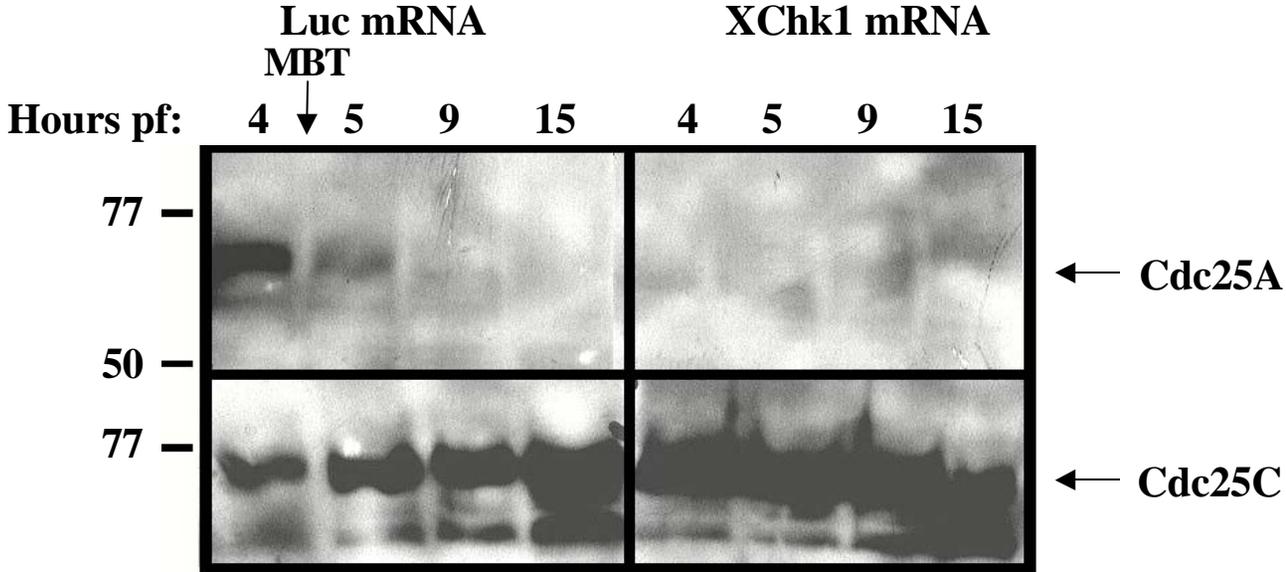
**Figure 13. Expression of exogenous XChk1 induces an increase in level of Wee1 five hours post-fertilization.** Embryos were injected at the one-cell stage with 15 ng of control mRNA or XChk1 mRNA. Western blotting was performed with antibodies against (A) Wee1, (B) Cdc25 A, and Cdc25 C. The migration of molecular weight markers is shown in KDa.

Figure 13

**A**



**B**

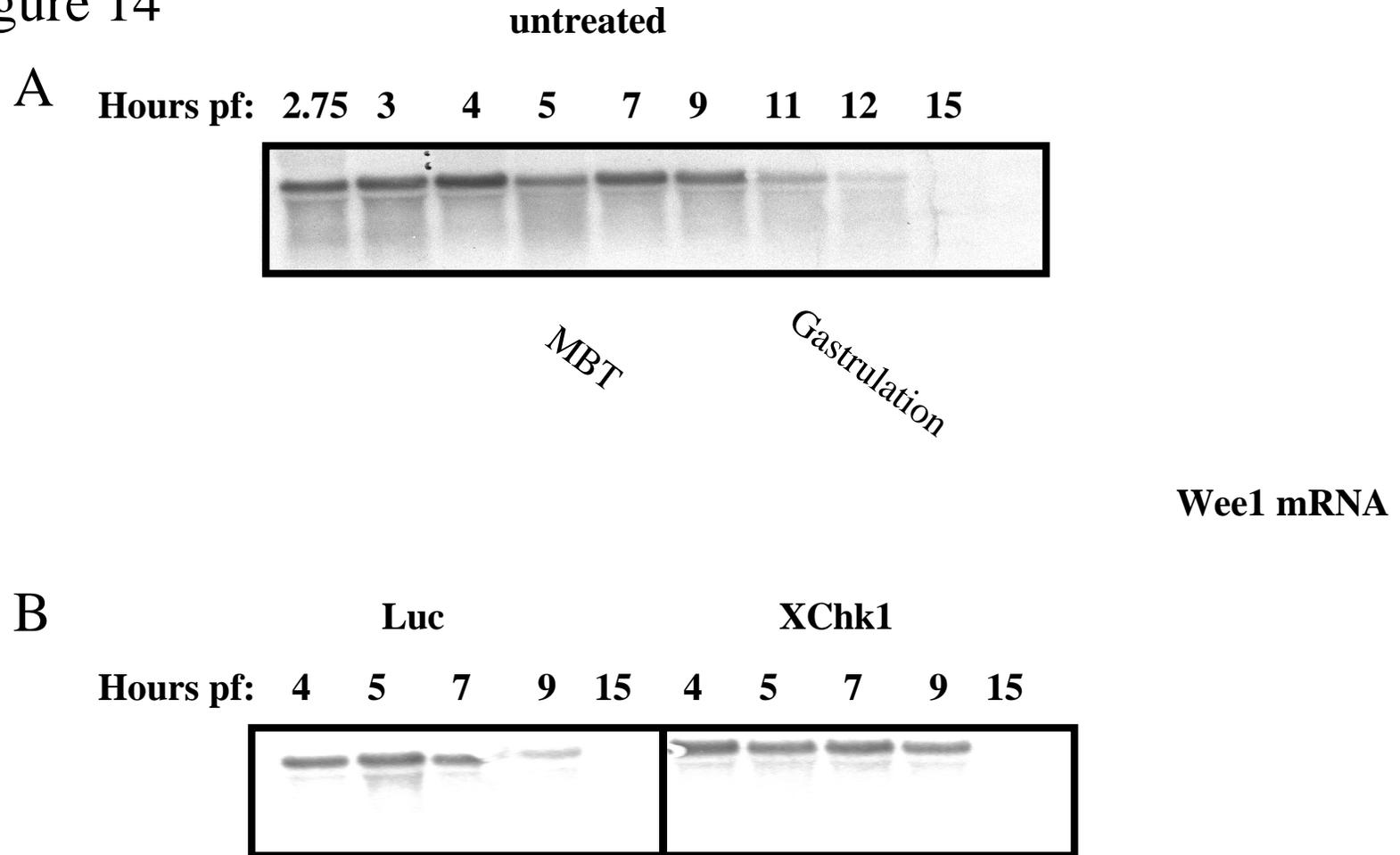


***XChk1 does not alter the level of Wee1 mRNA in Xenopus embryos.*** The specific inhibition of cyclin E/Cdk2 GST- $\Delta$ 34-Xic1 leads to a decrease in the level of Wee1, but the inhibition of cyclin E/Cdk2 and cyclin B/Cdc2 by XChk1 leads to an increase in the level of Wee1. We hypothesized that XChk1 was leading to this increase in Wee1 by altering the level of Wee1 mRNA. To determine the effect of exogenous XChk1 on Wee1 mRNA, we collected a time course of normal *Xenopus* embryos. We used a *wee1* gene provided to us by Monika Murikami (see Chapter 2) as template for our radiolabeled DNA probe. We determined the steady state level of Wee1 mRNA by Northern analysis of total RNA extracted from embryos collected at the times indicated. Similar to the degradation of Wee1 protein, Wee1 mRNA in normal embryos is degraded at gastrulation, or approximately 11 hours post-fertilization (Figure 14 A).

Embryos microinjected with luciferase mRNA, as a control, or XChk1 mRNA were collected in a similar fashion for Northern blot analysis. We determined that the level of Wee1 mRNA is not altered in injected embryos expressing XChk1 (Figure 14 B). Therefore, the stabilization of Wee1 protein is not due to a stabilization of Wee1 mRNA by exogenous XChk1.

**Figure 14. Overexpression of XChk1 does not alter the timing of Wee1 mRNA degradation.** (A) Northern analysis of Wee1 mRNA level in untreated embryos demonstrates Wee1 mRNA is degraded at gastrulation. (B) The steady state level of Wee1 mRNA is not affected by overexpression of XChk1.

Figure 14



## Discussion

In previous experiments, it was shown the negative cell cycle regulator XChk1 inhibited cyclin E/Cdk2 activity [(Kappas et al., 2000), Figure 11] and cyclin E degradation (Figure 11). This suggested a delay in cyclin E degradation is linked to the inhibition of cyclin E/Cdk2. In addition, our data suggests that a negative feedback loop exists between cyclin E/Cdk2 and Wee1 (Figure 4 B). We inhibited cyclin E/Cdk2 with XChk1 to further link cyclin E/Cdk2 activity in cyclin E degradation and in a negative feedback loop with Wee1.

In the presence of unreplicated (Kumagai et al., 1998; Kappas et al., 2000), and perhaps damaged (Kappas et al., 2000) DNA, XChk1 becomes activated. Recently, XChk1 has been shown to phosphorylate Wee1 in egg extracts (Lee et al., 2001). Phosphorylation of Wee1 by XChk1 causes Wee1 to bind to 14-3-3 proteins and become localized in the nucleus where Wee1 can readily phosphorylate MPF (Lee et al., 2001). The phosphorylation of Wee1 by XChk1 thus leads the cell into a checkpoint at G2/M by activating Wee1, and thus indirectly inactivating MPF. To further inactivate MPF and prevent the initiation of mitosis, XChk1 phosphorylates the MPF phosphatase Cdc25 C, allowing it to bind to 14-3-3 in cytosol and prevent it from functioning in the nucleus (Izumi et al., 1992; Kumagai and Dunphy, 1992; Izumi and Maller, 1993; Nakajo et al., 1999).

Our studies indicate it is possible to force a checkpoint in these early embryonic cells. Microinjections of XChk1 mRNA into one-cell staged embryos causes the embryo to stop dividing at the two to four-cell stage (Figure 10 A). Inhibition of Cdc25C function and activation of Wee1 explains the cell cycle arrest observed before the MBT in XChk1-microinjected embryos. This suggests that in the early cell divisions of the embryo, the XChk1 checkpoint pathway is functional downstream of XChk1. It was also shown that exogenous XChk1 inhibits cyclin E/Cdk2 activity and delayed the degradation of cyclin E at the MBT (Figure 11 A). Inhibition of cyclin E/Cdk2 activity

by both GST- $\Delta$ 34-Xic1 and XChk1 demonstrated that cyclin E/Cdk2 activity at least partially regulates the developmental timer.

In embryos microinjected with GST- $\Delta$ 34-Xic1, we showed that the level of Wee1 protein is decreased. This decrease in Wee1 was linked to a negative feedback where cyclin E/Cdk2 activity inhibits Wee1 mRNA degradation. However, when previously described, the phosphorylation of Wee1 by XChk1 did not alter the level of Wee1 protein in egg extracts (Lee et al., 2001). We observed that exogenous XChk1 leads to a dramatic increase in Wee1 protein level at the timing of the MBT (Figure 12 A).

Although Wee1 protein is potentially stabilized by cyclin E/Cdk2 activity and XChk1 inhibits its activity, XChk1 inhibits MPF as well. We hypothesized that the inhibition of MPF by XChk1 leads to a stabilization of Wee1 mRNA, or an increase in translation or a stabilization of Wee1 protein. Perhaps MPF activity leads to the degradation of Wee1, and XChk1 inhibits this, consistent with Michael and Newport's observation that Wee1 is degraded each cell cycle just before mitosis, but not degraded when DNA replication is blocked (Michael and Newport, 1998).

Wee1 mRNA is degraded similarly to the protein at gastrulation, 11 hours post-fertilization (Figure 14 A), and in embryos microinjected with XChk1 mRNA, the level of Wee1 mRNA is not altered (Figure 14 B). This suggests that the Wee1 increase in XChk1 injected embryos is due to an increase in translation or a stabilization of Wee1 protein. We did not, however, look at the effects exogenous XChk1 has on the stabilization or translation of Wee1 protein. To test if the Wee1 increase was due to a stabilization in Wee1, radiolabeled Wee1 protein could be used to determine the fate of Wee1 in embryos expressing exogenous XChk1. In normal embryos, Wee1 is degraded at gastrulation and radiolabeled Wee1 should also be degraded at this time point in embryos. Co-injected with luciferase mRNA or XChk1 mRNA, radiolabeled Wee1 protein can be monitored in embryos. The Wee1 in the control embryos should degrade normally at gastrulation. In the XChk1 injected embryos, Wee1 protein will either degrade normally or will remain in embryos if Wee1 is stabilized by exogenous XChk1. Wee1 translation may increase in XChk1 mRNA injected embryos as

well, leading to the observed increase. During protein translation, ribosomes will be bound to Wee1 mRNA and the amount of ribosomes bound to Wee1 mRNA will increase as the amount of Wee1 translation increases. By isolating the ribosome and mRNA complexes, or polysomes, in the embryo, we can determine if the translation of Wee1 is higher in XChk1 injected embryos by probing the polysomes for Wee1 mRNA.

XChk1 is an inhibitor of MPF, and our data indicates that XChk1 inhibits cyclin E/Cdk2 as well. If Cdc2 were specifically inhibited using antisense technology (Weeks et al., 1991) or morpholinos (Audic et al. 2001), Wee1 protein level could then be monitored. If the specific inhibition of Cdc2 leads to an increase in Wee1 protein level, then we can further demonstrate that XChk1 may lead to a Wee1 increase via the inhibition of Cdc2.

Our data indicate that in embryos, exogenous XChk1 mRNA causes cells to stop dividing in the otherwise rapid early embryonic cell divisions, the protein Wee1 dramatically increases at the time of the MBT, cyclin E protein degradation is delayed and zygotic transcription is delayed. This delay in degradation of cyclin E and zygotic transcription suggests XChk1 is able to alter the timing of the cyclin E/Cdk2 developmental timer, thus altering the onset of the MBT. These data supports the idea that the developmental timer is autonomously regulated by cyclin E/Cdk2 activity.

Indeed, XChk1 does alter the timing of the MBT. Exogenous XChk1 leads to a halt in cell divisions, preventing the proper number of cells normally used to mark the MBT. In addition, exogenous XChk1 prevents the initiation of zygotic transcription, as seen by the lack of transcription of GS17 in embryos microinjected with XChk1 mRNA (Figure 13). These data demonstrates that GST- $\Delta$ 34-Xic1 and XChk1 can perturb to the timing of, and cell cycle remodeling, at the MBT.

# Chapter 5

## Conclusions

The experiments described in the previous chapters were performed coincident with the formulation, by Andrea Ciliberto and John Tyson, of a mathematical model predicting the regulation of cyclin E/Cdk2 in *Xenopus* embryos. The initial predictions made from a mathematical model attempted to explain the oscillations in cyclin E/Cdk2 activity before the MBT and the degradation of cyclin E at the MBT. As experimental data were generated that did not support the model, changes were made in the mathematical model. The predictions made in the altered model were then further tested experimentally. The final predictions and data included the existence of a negative feedback loop between cyclin E/Cdk2 and Wee1, and evidence to support the role of cyclin E/Cdk2 in the degradation of cyclin E (Appendix A).

In the cell divisions before the MBT, two peaks in cyclin E/Cdk2 activity have been observed (Hartley et al., 1997). The causes in the oscillations of cyclin E/Cdk2 activity have not been determined. Wee1 negatively regulates cyclin E/Cdk2 via phosphorylation, and it was hypothesized in the mathematical model that cyclin E/Cdk2 activates Wee1 in a negative feedback loop that creates the observed oscillations in cyclin E/Cdk2 activity. To determine the existence of a negative feedback loop, GST- $\Delta$ 34Xic1 was microinjected into *Xenopus* embryos to specifically inhibit cyclin E/Cdk2 (Figures 4 A and B). The specific inhibition of cyclin E/Cdk2 resulted in a low level of Wee1 protein and mRNA, and delayed the degradation of cyclin E (Figure 4 B). Our data suggested cyclin E/Cdk2 promotes the stabilization of Wee1 in a negative feedback loop (Figure 15) and is linked to cyclin E degradation. Northern analysis of Wee1 mRNA has demonstrated that GST- $\Delta$ 34Xic1 leads to a low level of Wee1 mRNA (Figure 5 B). This suggests cyclin E/Cdk2 promotes Wee1 synthesis by stabilizing Wee1 mRNA and this stabilization accounts for the degradation of cyclin E at the MBT and Wee1 at gastrulation. But in the absence of zygotic transcription in the pre-MBT cell divisions, the removal of Wee1 mRNA is terminal. To explain the oscillations in cyclin E/Cdk2 activity before the MBT we hypothesize cyclin E/Cdk2 inhibits an inhibitor of Wee1 and the mathematical model (Appendix A) suggests this will lead to the observed oscillations in cyclin E/Cdk2 activity prior to the MBT.

The mathematical model had also suggested that as cyclin E/Cdk2 activity is inhibited, degradation of cyclin E will be delayed. The model however did not explain the eventual degradation of cyclin E in GST- $\Delta$ 34Xic1 injected embryos. The degradation of cyclin E is associated with a developmental timer at the MBT (Howe et al., 1995; Howe and Newport, 1996; Hartley et al., 1997), and since cyclin E is degraded with onset of zygotic transcription, we inhibited transcription with  $\alpha$ -amanitin. Our data is consistent with Hartley et al. (1997), where cyclin E degradation is independent of transcription (Figure 6). This further linked cyclin E/Cdk2 to the degradation of cyclin E. To investigate a role for cyclin E/Cdk2 in cyclin E degradation, we injected varied doses of GST- $\Delta$ 34Xic1, extending the delay in cyclin E degradation with increased GST- $\Delta$ 34Xic1 concentrations (Figure 7). Together, our data indicate that cyclin E/Cdk2 activity is associated with cyclin E degradation, regulation of the developmental timer and stabilization of Wee1.

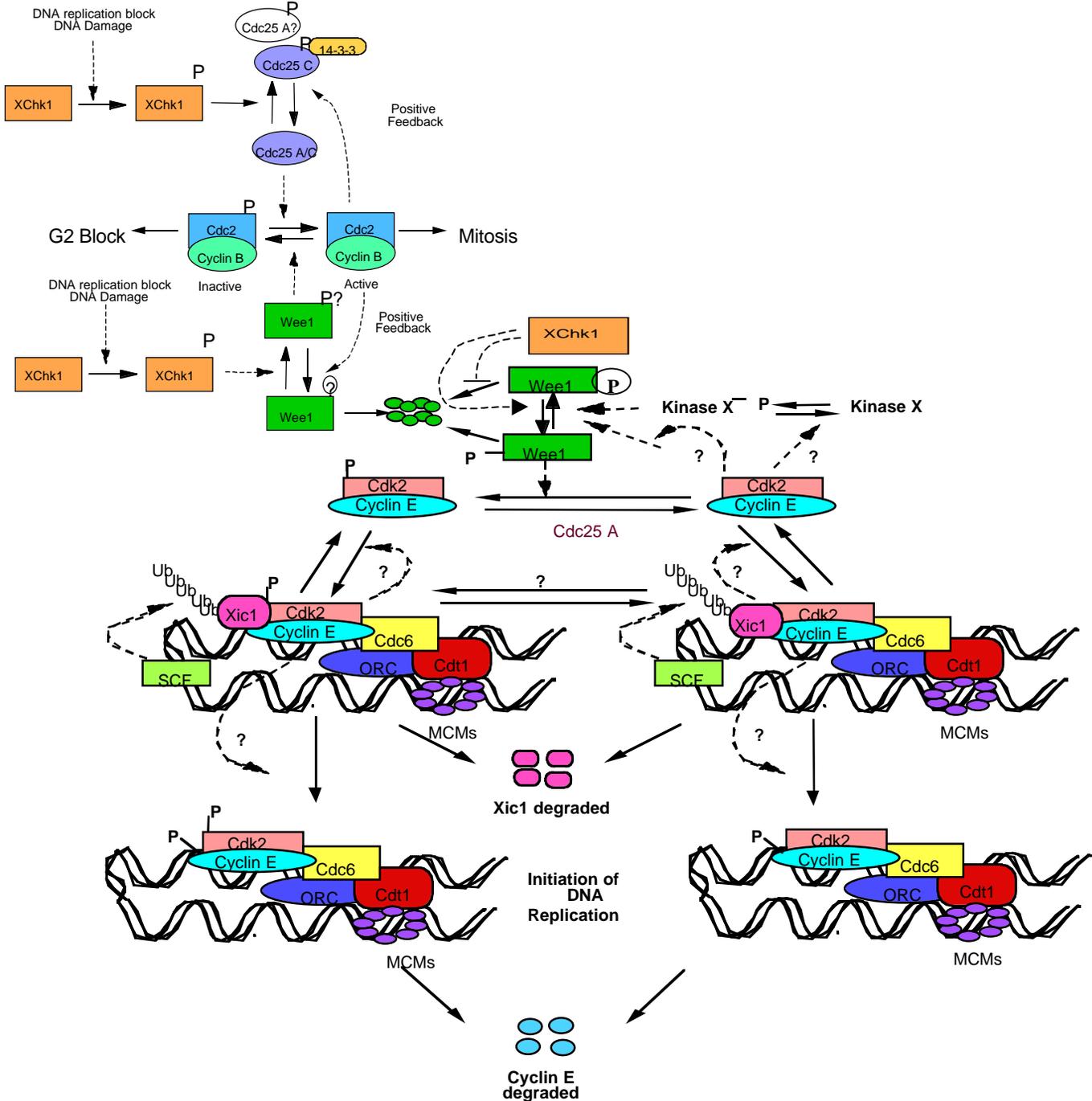
To further link cyclin E/Cdk2 to a negative feedback loop with Wee1 and cyclin E degradation, we inhibited cyclin E/Cdk2 with XChk1. Exogenous XChk1 prevents the initiation of the MBT by preventing the embryo from dividing beyond approximately the four-cell stage (Figure 8), delaying cyclin E degradation (Figure 10) and preventing zygotic transcription (Figure 12 B). Exogenous XChk1 inhibits cyclin B/Cdc2 and cyclin E/Cdk2, and we observed an increase in Wee1 protein at the timing of the MBT (Figure 11) but no effect on Wee1 mRNA (Figure 13). These data support a role for cyclin E/Cdk2 in the degradation of cyclin E and the idea that XChk1 can perturb the timing of, and cell cycle remodeling at, the MBT. Although XChk1 inhibits cyclin E/Cdk2 activity as well, Wee1 protein level is increased. This increase is perhaps due to the inhibition of cyclin B/Cdc2, which may lead to Wee1 degradation in a positive feedback loop (Figure 15). It is unclear how inhibition of cyclin B/Cdc2 by XChk1 leads to the observed increase in Wee1 protein at the timing of the MBT.

GST- $\Delta$ 34Xic1 inhibits cyclin E/Cdk2 (Su et al., 1995; Shou and Dunphy, 1996; Hartley et al., 1997), and XChk1 inhibits cyclin E/Cdk2 and cyclin B/Cdc2 (Kappas et al., 2000). Our data indicates cyclin E/Cdk2 inhibition leads to a delay in cyclin E

degradation and a low level of Wee1 via mRNA. It appears cyclin E/Cdk2 and cyclin B/Cdc2 inhibition delays cyclin E degradation and leads to an increase in Wee1 protein level. These data suggests cyclin E/Cdk2 autonomously regulates the developmental timer and is linked to a negative feedback loop with Wee1, and cyclin B/Cdc2 is linked to the degradation of Wee1 and cell cycle remodeling at the MBT.

**Figure 15. A proposed model for the role of Xic1 and XChk1 in altering the cyclin E/Cdk2 developmental timer.** The inhibition of cyclin E/Cdk2 activity by Xic1 and XChk1 delays cyclin E degradation. Xic1 specifically inhibits cyclin E/Cdk2, preventing the stabilization of Wee1. XChk1 inhibits both cyclin E/Cdk2 and cyclin B/Cdc2 activity, leading to an increase in the level of Wee1 protein.

Figure 15



## References

Anderson, J. A., J. D. Richter, M. E. Chamberlin, D. H. Price, R. J. Britten, D. Smith, and E. H. Davidson (1997). Ionizing radiation induces apoptosis and elevates cyclin A1-Cdk2 activity prior to but not after the midblastula transition in *Xenopus*. *Mol. Biol. Cell* **8**: 1195-1206.

Arellano, M. and S. Moreno (1997). Regulation of CDK/cyclin complexes during the cell cycle. *Int. J. Biochem. Cell Biol* **29**: 559-573.

Audic, Y., B. Boyle, M. Slevin, and R. S. Hartley (2001). Cyclin E morpholino delays embryogenesis in *Xenopus*. *Genesis* **30**: 107-109.

Baily, C. P. and D. L. Weeks (1996). Prevention of cyclin B protein synthesis in early *Xenopus* embryos leads to increased cyclin A levels and cdk activation.

Basu, A. and S. Haldar (1998). The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. *Molecular Human Reproduction*. **4**: 1099-1109.

Borisuk, M. T. and J. J. Tyson (1998). Bifurcation analysis of a model of mitotic control in frog eggs. *J. theor. Biol.* **195**: 69-85.

Brondello, J. M., M.N. Boddy, B. Furnari, and P. Russell (1999). Basis for the checkpoint signal specificity that regulates chk1 and cds1 protein kinases. *Mol. Cell. Biol.* **19**: 4262-4269.

- Brown, A. L., C. H. Lee, J. K. Schwartz, N. Mitiku, H. Piwinica-Worms and J. H. Chuang (1999). A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage. *PNAS*. **96**: 3745-3750.
- Chen, J., Peter K. Jackson, Marc W. Kirschner, and Anindya Dutta (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* **374**: 386-388.
- Chen, L., T.H. Liu and N.C. Walworth (1999). Association of Chk1 with 14-3-3 proteins is stimulated by DNA damage. *Genes. Dev.* **13**: 675-685.
- Chevalier, S., A. Couturier, I. Chartrain, Le Guellec, R., C. Beckhelling, Le Guellec, K., M. Phillippe and C. C. Ford. (1996). Xenopus cyclin E, a nuclear phosphoprotein, accumulates when oocytes gain the ability to initiate DNA replication. *J. Cell Sci.* **109**:1173-1184.
- Chiarugi, V. and M. Ruggiero (1996) Role of three cancer "master genes" p53, bcl2, and c-myc on the apoptotic process. *Tumori.* **82** :205-209.
- Chuang, L.-C. and P. R. Yew (2001). Regulation of nuclear transport and degradation of the *Xenopus* cyclin-dependent kinase inhibitor, p27<sup>Xic1</sup>. *J. Biol. Chem.* **276**: 1610-1617.
- Clark, J. M. and B. G. Gabrielli (1997). Production of a soluble cyclin B/cdc2 substrate for cdc25 phosphatase. *Anal. Biochem.* **254**: 231-235.
- Coleman, T. R., P. B. Carpenter and W. G. Dunphy (1996). The *Xenopus* Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* **87**: 53-63.

- Conklin, D. S., K. Galaktionov and D. Beach (1995). 14-3-3 proteins associate with cdc25 phosphatases. *Proc. Natl. Acad. Sci. USA* **92**: 7892-7896.
- D'Angiolella, V., V. Costanzo, M.E. Gottesman, E.V. Avvedimento, J. Gautier and D. Grieco (2001). Role for cyclin-dependent kinase 2 in mitosis exit. *Curr. Biol.* **11**: 1221-1226.
- Dabauvalle, M. C., M. Doree, R. Bravo and E. Karsenti (1988). Role of nuclear material in the early cell cycle of *Xenopus* embryos. *Cell* **52**: 525-533.
- Dasso, M. and J. W. Newport (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in *Xenopus*. *Cell* **61**: 811-823.
- Devault, A., D. Fesquet, J. C. Cavadore, A. M. Garrigues, J. C. Labbe, T. Lorca, A. Picard, M. Philippe and M. Doree (1992). Cyclin A potentiates maturation-promoting factor activation in the early *Xenopus* embryo via inhibition of the tyrosine kinase that phosphorylates cdc2. *J. Cell Biol.* **118**: 1109-1120.
- Dunphy, W. G., L. Brizuela, D. Beach and J. Newport (1988). The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* **54**: 423-431.
- el-Deiry, W. S., J.W. Harper, P.M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill and Y. Wang (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Research* **54**(5): 1169-74.
- el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**(4): 817-25.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. *Science* **274**: 1664-1671.

Faure, S., S. Vigneron, S. Galas, T. Brassac, C. Delsert and N. Morin (1999). Control of G2/M transition in *Xenopus* by a member of the p21-activated kinase (PAK) family: a link between protein kinase A and PAK signaling pathways? *J. Biol. Chem.* **274**: 3573-3579.

Ferrell, J. E., M. Wu, J.C. Gerhart and G.S. Martin (1991). Cell cycle tyrosine phosphorylation of p34<sup>cdc2</sup> and a microtubule-associated protein kinase homologue in *Xenopus* oocytes and eggs. *Mol. Cell. Biol.* **11**: 1965-1971.

Flaggs, G., A.W. Plug, K.M. Dunks, K.E. Mundt, J.C. Ford, M.R.E. Quiggle, E.M. Taylor, C.H. Westphal, T. Ashley, M.F. Hoekstra and A.M. Carr (1997). Atm-dependent interactions of a mammalian Chk1 homologue with meiotic chromosomes. *Curr. Biol.* **7**: 977-986.

Font de Mora, J., Aykut Urenk, Mohammed Heidarhan and Eugenia Santos (1997). Biological Activity of p27<sup>Kip1</sup> and its amino- and carboxy-terminal domains in G2/M transition of *Xenopus* oocytes. *Oncogene* **15**: 2541-2551.

Frederick, D. L. and M. T. Andrews (1994). Cell cycle remodeling requires cell-cell interactions in developing *Xenopus* embryos. *J. Exp. Zool.* **270**: 410-416.

Furnari, B., N. Rhind and P. Russell (1997). Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase. *Science* **277**: 1495-1497.

Fursenthal, L., Brett K. Kaiser, Craig Swanson, and Peter K. Jackson (2001). Cyclin E Uses Cdc6 as a Chromatin-associated Receptor Required for DNA Replication. *The Journal of cell Biology* **152**(6): 1267-1278.

Furstenthal, L., C. Swanson, B.K. Kaiser, A.G. Eldridge and P.K. Jackson (2001). Triggering ubiquitination of a CDK inhibitor at origins of replication. *Nat. Cell Biol.* **3**: 715-722.

Gabrielli, B. G., J.M. Clark, A.K. McCormack and K.A.O. Ellem (1997). Hyperphosphorylation of the N-terminal domain of Cdc25 regulates activity toward Cyclin B1/Cdc2 but not Cyclin A/Cdk2. *J. Biol. Chem.* **272**: 28607-28614.

Gabrielli, B. G., M.S. Lee, D.H. Walker, H. Piwnica-Worms and J.L. Maller (1992). Cdc25 regulates the phosphorylation and activity of the *Xenopus* cdk2 protein kinase. *J. Biol. Chem.* **26**: 18040-18046.

Gautier, J., J. Minshull, M. Lohka, M. Glotzer, T. Hunt and J. Maller (1990). Cyclin is a component of maturation-promoting factor from *Xenopus*. *Cell* **1990**: 487-494.

Gautier, J., C. Norbury, M. Lohka, P. Nurse and J. Maller (1988). Purified maturation-promoting factor contains the product of a *Xenopus* homologue of the fission yeast cell cycle control gene *cdc2+*. *Cell* **54**: 433-439.

Gautier, J., M.J. Solomon, R.N. Booher, J.F. Bazan and M.W. Kirschner (1991). *cdc25* is a specific tyrosine phosphatase that directly activates  $p34^{cdc2}$ . *Cell* **67**: 197-211.

Graves, P.,L. Yu, J. Schwartz, J. Gale, E. Sausville, P.O'Connor and H. Piwnica-Worms (2000). The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. *J. Biol. Chem.* **275**: 5600.

Gu, Y., J. Rosenblatt and D.O. Morgan (1992). Cell cycle regulation by phosphorylation of Thr-160 and Tyr-15. *EMBO J* **11**: 3995-4005.

Guadagno and Newport (1996). Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity. *Cell* . **84**: 73-82.

Hartley, R. S., R. E. Rempel and James L. Maller (1996). *In vivo* regulation of the early embryonic cell cycles in *Xenopus*. *Devel. Biol.* **173**: 408-419.

Hartley, R. S., J. C. Sible, A. L. Lewellyn and James L. Maller (1997). A role for cyclin E/Cdk2 in the timing of the midblastula transition in *Xenopus* embryos. *Devel. Biol.* **188**.

Hartwell, L. H. and T. A. Weinert (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**: 629-634.

Heichman, K. A. and J. M. Roberts (1994). Rules to Replicate By. *Cell* **79**: 557-562.

Hendrickson, M., M. Madine, S. Dalton and J. Gautier (1996). Phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex. *Proc. Natl. Acad. Sci. USA* **93**: 12223-12228.

Hensey, C. and J. Gautier (1997). A developmental timer that regulates apoptosis at the onset of gastrulation. *Mech. Devel.* **69**: 183-195.

Hoffman, I., P.R. Clarke, M.J. Marcote, E. Karsenti and G. Draetta (1993). Phosphorylation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO J.* **12**: 53-63.

Hoffman, I., G. Draetta and E. Karsenti (1994). Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at the G<sub>1</sub>/S transition. *EMBO J.* **13**: 4302-4310.

Howe, J. A., M. Howell, T. Hunt and J. W. Newport (1995). Identification of a developmental timer regulating the stability of embryonic cyclin A and a new somatic A-type cyclin at gastrulation. *Genes Dev.* **9**: 1164-1176.

Howe, J. A. and J. W. Newport (1996). A developmental timer regulates degradation of cyclin E1 at the midblastula transition during *Xenopus* embryogenesis. *Proc. Natl. Acad. Sci. USA* **93**: 2060-2064.

Izumi, T. and J. L. Maller (1993). Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. *Mol. Biol. Cell* **4**: 1337-1350.

Izumi, T., D.H. Walker and J.L. Maller (1992). Periodic changes in phosphorylation of the *Xenopus* cdc25 phosphatase regulate its activity. *Mol. Biol. Cell* **3**: 927-939.

Jackson, J., A. Gilmartin, C. Imburgia, J. Winkler, L. Marshall and A. Roshak (2000). An indolocarbazole inhibitor of human checkpoint kinase (Chk1) abrogates cell cycle arrest caused by DNA damage. *Cancer Res.* **60**: 566-.

Jackson, P. K., S. Chevalier, M. Philippe and M.W. Kirschner (1995). Early events in DNA replication require cyclin E and are blocked by p21<sup>CIP1</sup>. *J. Cell Biol.* **130**: 755-769.

Kappas, N., P. Savage, K.C. Chen, A.T. Walls and J.C. Sible (2000). Dissection of the XChk1 signaling pathway in *Xenopus laevis* embryos. *Mol. Biol. Cell* **11**: 3101-3108.

Kim, S., C Li and J. L Maller (1999). A maternal form of the phosphatase Cdc25A regulates early embryonic cell cycles in *Xenopus laevis*. *Devel. Biol.* **212**: 381-391.

Kimelman, D., M. Kirschner and T. Scherson (1987). The events of the midblastula transition in *Xenopus* are regulated by changes in the cell cycle. *Cell* **48**: 399-407.

Kramer, E. R., N. Scheuringer, A. V. Podtelejnikov, M. Mann and J. M. Peters (2000). Mitotic regulation of APC activator proteins CDC20 and CDH1. *Mol. Biol. Cell.* **11**: 1555-1559.

Krieg, P. A. and D. A. Melton (1985). Developmental regulation of a gastrula-specific gene injected into fertilized *Xenopus* eggs. *EMBO J.* **4**: 3463-3471.

Krieg, P. A. and D. A. Melton (1987). An enhancer responsible for activating transcription at the midblastula transition in *Xenopus* development. *Proc. Natl. Acad. Sci. USA* **84**: 2331-2335.

Krude, T., M. Jackman, J. Pines and R. A. Laskey (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell* **88**: 109-119.

Kumagai, A. and W. G. Dunphy (1991). The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell* **64**: 903-914.

Kumagai, A. and W. G. Dunphy (1992). Regulation of the cdc25 protein during the cell cycle in *Xenopus* extracts. *Cell* **70**: 139-151.

Kumagai, A. and W. G. Dunphy (1995). Control of the Cdc2/Cyclin B complex in *Xenopus* egg extracts arrested at a G2/M checkpoint with DNA synthesis inhibitors. *Mol. Biol. Cell* **6**: 199-213.

Kumagai, A. and W. G. Dunphy (1997). Regulation of *Xenopus* Cdc25 protein. *Meth. Enzymol.* **283**: 564-571.

Kumagai, A., Z. Guo, K.H. Emami, S.X. Wang and W.G. Dunphy (1998). The *Xenopus* Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts. *J. Cell Biol.* **142**: 1559-1569.

Kumagai, A., P.S. Yakowec and W.G. Dunphy (1998). 14-3-3 proteins act as negative regulators of the mitotic inducer Cdc25 in *Xenopus* egg extracts. *Mol. Biol. Cell* **9**: 345-354.

Kumagai, A., William G. Dunphy (1999). Binding of 14-3-3 proteins and nuclear export control in the intracellular localization of the mitotic inducer Cdc25. *Genes and Development* **13**: 1067-1072.

Lee, J., A. Kumagai and W. G. Dunphy (2001). Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. *Mol. Biol. Cell* **12**: 551-563.

Lee, M. and P. Nurse. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature* **327**.

Lee, T. H. and M. W. Kirschner (1996). An inhibitor of p34<sup>cdc2</sup>/cyclin B that regulates the G<sub>2</sub>/M transition in *Xenopus* extracts. *Proc. Natl. Acad. Sci. USA* **93**: 352-356.

Lorca, T., A. Castro, A.M. Martinez, S. Vigneron, N. Morin, S. Sigrist, C. Lehner, M. Doree and J. C. Labbe (1998). Fizzy is required for activation of the APC/cyclosome in *Xenopus* egg extracts. *EMBO J.* **17**: 3565-3575.

Luo, Y., J. Hurwitz and J. Massague (1995). Cell-cycle inhibition by independent cdk and PCNA binding domains in p21-CIP1. *Nature* **375**: 159-161.

Madine, M. A., C-Y. Khoo, A. D. Mills and R. A. Laskey (1995). MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells. *Nature* **375**: 421-424.

Marlovits, G. (1998). Modeling M-phase control in *Xenopus* oocyte extracts: the surveillance mechanism for unreplicated DNA. *Biophys. Chem.* **72**: 169-184.

Matsuoka S. and S. J. Elledge (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science*. **282**: 1893-1897.

Michael, W. M. and J. Newport (1998). Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of wee1. *Science* **282**: 1886-1889.

Minshull, J., H. Sun, N. K. Tonks and A. W. Murray (1994). A MAP kinase dependent spindle assembly checkpoint in *Xenopus* egg extract. *Cell* **79**: 475-486.

Moreno, S. and P. Nurse (1994). Regulation of progression through the G1 phase of the cell cycle by the rum1+ gene. *Nature* **367**: 236-242.

Mueller, P. R., T. R. Coleman and W. G. Dunphy (1995). Cell cycle regulation of a *Xenopus* Wee1-like kinase. *Mol. Biol. Cell* **6**: 119-134.

Mueller, P. R., T. R. Coleman, A. Kumagai and W. G. Dunphy (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science* **270**: 86-90.

Murakami, M. S. and G. F. V. Woude (1998). Analysis of the early embryonic cell cycles of *Xenopus*; regulation of cell cycle length by Xe-wee1 and Mos. *Development* **125**: 237-248.

Murray, A. W. and M. W. Kirschner (1989a). Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339**: 275-280.

Murray, A. W., M. J. Solomon and M. W. Kirschner (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339** :280-286.

Nakajo, N., T. Oe, K. Uto and N. Sagata (1999). Involvement of Chk1 kinase in prophase I arrest of *Xenopus* embryos. *Devel. Biol.* **207**: 432-444.

Nieuwkoop, P. D. and J. Faber (1975) Normal table of *Xenopus laevis*. Amsterdam. North Holland Publishing Company.

Newport, J. and M. Dasso (1989). On the coupling between DNA replication and mitosis. *J. Cell Sci. Suppl.* **12**: 149-160.

Newport, J. and M. Kirschner (1982). A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**: 675-686.

Newport, J. and M. Kirschner (1982). A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell* **30**: 687-696.

Nigg, E. (1995). Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioessays* **17**: 471-480.

Novak, B. and J. J. Tyson (1993). Numerical analysis of a comprehensive model of M-phase control in *Xenopus* oocyte extracts and intact embryos. *J. Cell Sci.* **106**: 1153-1168.

Novak, B. and J. J. Tyson (1995). Quantitative analysis of a molecular model of M-phase control in *Xenopus* oocyte extracts and intact embryos. *J. Cell Sci.* **106**: 1153-1168.

Novak, B. and J. J. Tyson (1998). Mathematical model of the fission yeast cell cycle with checkpoint controls at the G1/S, G2/M and metaphase/anaphase transitions. *Biophys. Chem.* **72**: 185-200.

O'Connell, M. J., J. M. Raleigh, H. M. Verkade and P. Nurse (1997). Chk1 is a wee kinase in the G<sub>2</sub> DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. *EMBO J.* **16**: 545-554.

Paulovich, A. G., D. P. Toczyski, L. H. Hartwell (1997) When checkpoints fail. *Cell.* **88**: 315-321.

Peng, C.-Y., P. R. Graves, R. S. Thomas, Z. Wu, A. S. Shaw and H. Piwnicka-Worms (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25 on serine-216. *Science* **277**: 1501-1505.

Polyak, K., J. Y. Kato, M. J. Solomon, C. J. Sherr and J. Massague (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes and Development* **8**: 9-22.

Rempel, R. E., S. B. Sleight and J. L. Maller (1995). Maternal *Xenopus* Cdk2-cyclin E complexes function during meiotic and early embryonic cell cycles that lack a G1 phase. *J. Biol. Chem.* **270**: 6843-6855.

Rollins, M. B. and M. T. Andrews (1991). Morphogenesis and regulated gene activity are independent of DNA replication in *Xenopus* embryos. *Development* **112**: 559-569.

Russell, P. and P. Nurse (1987). Negative regulation of mitosis by *wee1*<sup>+</sup>, a gene encoding a protein kinase homologue. *Cell* **49**: 559-567.

Saha, P., Q. Eichbaum, E. D. Silberman, B. J. Mayer, and A. Dutta (1997). p21<sup>Cip1</sup> and Cdc25A: Competition between an inhibitor and an activator of cyclin-dependent kinases. *Mol. Cell Biol.* **17**(8): 4338-4335.

Sanchez, Y., C. Wong, R.S. Thoma, R. Richman, Z. Wu, H. Piwnicka-Worms and S. J. Elledge (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* **277**: 1497-1501.

Sanchez, Y., J. Bachant, H. Wang, F. Hu, D. Liu, M. Tetzlaff and S. J. Elledge, (1999). Control of DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. *Science*. **286**: 1166-1171.

Sherr, C. J. (1994). G1 Phase Progression: Cycling on Cue. *Cell* **79**: 551-555.

Shou, W. and W. G. Dunphy (1996). Cell cycle control by *Xenopus* p28<sup>Kix1</sup>, a developmentally regulated inhibitor of cyclin-dependent kinases. *Mol. Biol. Cell* **7**: 457-469.

Sible, J. C., J. A. Anderson, A. L. Lewellyn and J. L. Maller (1997). Zygotic transcription is required to block a maternal program of apoptosis in *Xenopus* embryos. *Devel. Biol.* **189**: 335-346.

- Sible, J. C., E. Erikson, M. Hendrickson, J. L. Maller and J. Gautier (1998). Developmental regulation of MCM replication factors in *Xenopus laevis*. *Curr. Biol.* **8**: 347-350.
- Smythe, C. and J. W. Newport (1992). Coupling of mitosis to the completion of S phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates p34<sup>cdc2</sup>. *Cell* **68**: 787-797.
- Solomon, M. J., M. Glotzer, T. H. Lee, M. Phillippe and M. W. Kirschner (1990). Cyclin activation of p34<sup>cdc2</sup>. *Cell* **63**: 1013-1024.
- Solomon, M. J., T. H. Lee and M. W. Kirschner. (1992). Role of phosphorylation in p34<sup>cdc2</sup> activation: identification of an activating kinase. *Mol. Cell. Biol.* **3**: 13-27.
- Strausfeld, U. P., Mike Howell, P. Descombes, S. Chevalier, R. E. Rempel, J. Adamczewski, J. Maller, T. Hunt and J. Julian Blow (1996). Both cyclin A and cyclin E have S-phase promoting (SPF) activity in *Xenopus* egg extracts. *J. Cell Sci.* **109**: 1555-1563.
- Strausfeld, U. P., M. Howell, R. Rempel, J. L. Maller, T. Hunt and J. J. Blow (1994). Cip1 blocks the initiation of DNA replication in *Xenopus* extracts by inhibition of cyclin-dependent kinases. *Curr. Biol.* **4**: 876-883.
- Strausfeld, U., A. Fernandez, J. P. Capony, F. Girard, N. Lautredou, J. Derancourt, J. C. Labbe, and N. J. Lamb (1994). Activation of p34<sup>cdc2</sup> protein kinase by microinjection of human cdc25C into mammalian cells. Requirement for prior phosphorylation of cdc25C by p34<sup>cdc2</sup> on sites phosphorylated at mitosis. *J. Biol Chem* **269**(8):5989-6000.

Su, J.-Y., R.E. Rempel, E. Erikson and J. L. Maller (1995). Cloning and characterization of the *Xenopus* cyclin-dependent kinase inhibitor p27<sup>XIC1</sup>. *Proc. Natl. Acad. Sci. USA* **92**: 10187-10191.

Swanson, C., J. Ross and P. K. Jackson (2000). Nuclear accumulation of cyclin E/Cdk2 triggers a concentration-dependent switch for the destruction of p27<sup>Xic1</sup>. *Proc. Natl. Acad. Sci. USA* **97**: 7796-7801.

Weeks, D. L., J.A. Walder and J. M. Dagle (1991). Cyclin B mRNA depletion only transiently inhibits the *Xenopus* embryonic cell cycle. *Development* **111**: 1173-1178.

Yew, P. R., and Marc W. Kirschner (1997). Proteolysis and DNA Replication: Cdc34 Requirement in the *Xenopus* Egg Cell Cycle. *Science* **277**: 1672-1676.

Zeng, Y., K. C. Forbes, Z. Wu, S. Moreno, H. Piwnica-Worms and T. Enoch (1998). Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. *Nature* **395**: 507-510.

## Appendix A

# **A Kinetic Model of the Cyclin E/Cdk2 Developmental Timer in *Xenopus laevis* Embryos**

**Andrea Ciliberto, Matthew J. Petrus, John J. Tyson and Jill C. Sible**

Biology Department, Virginia Polytechnic Institute and State University, Blacksburg, VA

E-mails: [acilibe@vt.edu](mailto:acilibe@vt.edu), [mapetrus@vt.edu](mailto:mapetrus@vt.edu), [tyson@vt.edu](mailto:tyson@vt.edu), [siblej@vt.edu](mailto:siblej@vt.edu)

Corresponding author: J.C. Sible

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Abbreviations: Cdk, cyclin-dependent kinase; MPF, M-phase promoting factor; MBT, midblastula transition; ODE, ordinary differential equation; pf, post fertilization

## ABSTRACT

Early cell cycles of *Xenopus laevis* embryos are characterized by rapid oscillations in the activity of two cyclin-dependent kinases. Cdk1 activity peaks at mitosis, driven by periodic degradation of cyclins A1 and B. In contrast, Cdk2 activity oscillates twice per cell cycle, despite constant levels of its partner, cyclin E. Cyclin E degrades at a fixed time after fertilization, normally corresponding to the midblastula transition. Based on published data and new experiments, we assumed that (1) oscillations in Cdk2 activity depend upon changes in phosphorylation, (2) Cdk2 participates in a negative feedback loop with the inhibitory kinase Wee1, (3) cyclin E binds cooperatively to chromatin, and (4) chromatin-bound cyclin E is degraded by a pathway activated by cyclin E/Cdk2 itself. A mathematical model of these interactions was constructed and compared with relevant data. The model made predictions about Xic1-injected embryos—(1) an inverse relationship between Xic1 concentration and timing of cyclin E degradation and (2) degradation of Xic1 prior to degradation of cyclin E—that were validated experimentally. Therefore, we propose cyclin E/Cdk2 activity patterns during early development are generated by two feedback loops: negative feedback creating oscillations, and positive feedback causing irreversible cyclin E binding and degradation on chromosomes.

## INTRODUCTION

The chromosome replication-division cycle of cell-free extracts derived from *Xenopus laevis* eggs is well characterized by rigorous experimentation (Murray and Kirschner, 1989; Solomon et al., 1990; Gautier et al., 1991; Kumagai and Dunphy, 1991; Devault et al., 1992; Kumagai and Dunphy, 1992; Smythe and Newport, 1992; Solomon et al., 1992; Izumi and Maller, 1993; Mueller et al., 1995a; Mueller et al., 1995b; Lorca et al., 1998) and mathematical modeling (Novak and Tyson, 1993; Novak and Tyson, 1995; Borisuk and Tyson, 1998; Marlovits et al., 1998). In the egg extract system, the chromosome cycle is driven by oscillations in the activity of M-phase promoting factor (MPF), a dimer of a catalytic subunit, Cdk1, and a regulatory subunit, cyclin A1 or B (Murray and Kirschner, 1989). Oscillations in MPF activity depend upon a negative feedback loop in which active MPF promotes degradation of mitotic cyclins (Lorca et al., 1998). MPF activity is also modulated by inhibitory phosphorylation on threonine 14 and tyrosine 15. These sites are phosphorylated by Wee1 and Mik1 (Mueller et al., 1995a; Mueller et al., 1995b), and dephosphorylated by Cdc25 (Gautier et al., 1991; Kumagai and Dunphy, 1991). The autocatalytic nature of MPF activation (Masui and Markert, 1971; Gerhart et al., 1984; Cyert and Kirschner, 1988) depends upon positive feedback loops, whereby MPF activates Cdc25 (Izumi and Maller, 1993; Kumagai and Dunphy, 1997) and inhibits Wee1 (Mueller et al., 1995a; 1995b).

Cell cycle controls in intact, developing *Xenopus* embryos present additional experimental and theoretical challenges. After fertilization, nuclear fusion and the first mitotic division, embryonic cell cycles 2-12 are rapid, synchronous oscillations between DNA replication and mitosis, lacking intervening gap phases, cell growth, and cell cycle checkpoints. After the twelfth division, the *Xenopus* embryo undergoes the midblastula transition (MBT) when cells become motile, embryonic transcription begins, and the cell cycle acquires the gap phases and checkpoints of a typical somatic cell (Newport and Kirschner, 1982a; 1982b).

The mechanisms of “cell cycle remodeling” (Frederick and Andrews, 1994) are not fully understood, but recent evidence suggests that remodeling depends in part on a maternal developmental timer driven by oscillations in the activity of cyclin E/Cdk2 (Hartley et al., 1997). During cleavage cycles 2-12 in the frog, cyclin E level is constant, while cyclin E-associated

kinase activity (cyclin E/Cdk2) oscillates twice per cell cycle (Hartley et al., 1996; Hartley et al., 1997). Cyclin E/Cdk2 activity promotes DNA replication (Strausfeld et al., 1994; Jackson et al., 1995) and is essential for centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999). At the MBT, maternally supplied cyclin E is degraded (Rempel et al., 1995; Hartley et al., 1996; Howe and Newport, 1996; Hartley et al., 1997), and Cdk2 activity declines (Hartley et al., 1997), coincident with remodeling of the cell cycle. Maternal cyclin E mRNA disappears some hours later (Howe and Newport, 1996).

Injection of embryos with  $\Delta 34$ Xic1, (a recombinant, truncated form of the CKI, Xic1), specifically inhibits cyclin E/Cdk2, causes a 25% increase in interdivision time, and delays the MBT (zygotic transcription) and the degradation of cyclin E until approximately the correct nucleocytoplasmic ratio (Hartley et al., 1997). This observation suggests that cyclin E degradation is causally related to the MBT. However, the two events can be dissociated. Cycloheximide treatment (which inhibits protein synthesis) blocks cell division and the MBT, but does not prevent cyclin E degradation. Nor does  $\alpha$ -amanitin treatment (which blocks zygotic transcription at the MBT) prevent cyclin E degradation (Hartley et al., 1997). Furthermore, oscillations in cyclin E/Cdk2 also occur independently of protein synthesis, the nucleocytoplasmic ratio, and embryonic transcription (Howe and Newport, 1996; Hartley et al., 1997).

We have combined experimental and computational approaches to build a model of the cyclin E/Cdk2 developmental timer that describes both the oscillations in Cdk2 activity and the abrupt destruction of cyclin E at the MBT. The model was constructed along the same lines as the Novak-Tyson model of MPF oscillations in frog egg extracts (Novak and Tyson, 1993), taking into account the similarities and differences between cyclin B/Cdk1 regulation and cyclin E/Cdk2 regulation. For both Cdk1 and Cdk2, there are no abundant Cdk-inhibitors expressed during this time (Shou and Dunphy, 1996); hence, oscillations are due either to periodic synthesis and degradation of cyclin partners or periodic phosphorylation and dephosphorylation of kinase subunits (or both). Pre-MBT oscillations of Cdk1 activity are driven by periodic cyclin B degradation (Murray and Kirschner, 1989); however, pre-MBT oscillations of Cdk2 activity are not (Hartley et al., 1997). Although early reports indicated no periodic tyrosine phosphorylation of either Cdk1 or Cdk2 until the MBT (Ferrell et al., 1991; Hartley et al., 1996),

subsequent studies with a sensitive antibody have detected periodic tyrosine phosphorylation of a Cdk in pre-MBT *Xenopus* embryos (Kim et al., 1999). Furthermore, Cdk2 activity is regulated in cell-free egg extracts by such phosphorylation events (D'Angiolella et al., 2001). Therefore, if oscillations in Cdk2 activity are generated by a negative feedback loop (as for Cdk1), the inhibitory phosphorylation state of Cdk2 (rather than cyclin level) is the likely position of the negative feedback.

A second important distinction between the regulation of Cdk1 and Cdk2 activity is the rapid destruction of cyclin E at the MBT. Because the only manipulation known to delay the degradation of cyclin E is to inhibit Cdk2 activity directly (Hartley et al., 1997), we propose that degradation of cyclin E is intrinsically dependent on Cdk2 activity. Furthermore, we propose that cyclin E degradation is turned on by a chromosome-bound form of cyclin E/Cdk2.

## MATERIALS AND METHODS

### **Maintenance and Manipulation of Embryos**

Eggs from wild type *Xenopus laevis* (*Xenopus* Express) were fertilized *in vitro*, dejellied in 2% cysteine in 0.1X MMR (0.5 mM HEPES, pH 7.8, 10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO<sub>4</sub>, 0.2 mM CaCl<sub>2</sub>, 0.01 mM EDTA) and maintained in 0.1X MMR. Embryos were collected at stages spanning the midblastula transition (MBT) and staged according to Nieuwkoop and Faber (1975). In some experiments, fertilized eggs were injected with indicated amounts of GST-tagged Xic1-C or GST-tagged  $\Delta$ 34Xic1 protein dissolved in Xic1 buffer (20 mM HEPES, pH 7.5, 88 mM NaCl, 20 mM  $\beta$ -mercaptoethanol, 7.5 mM MgCl<sub>2</sub>, 5% glycerol) at a concentration of 0.08 – 0.33 mg/ml. As described by Su et al. (1995), Xic1-C contains amino acids 97-210 and  $\Delta$ 34Xic1 contains amino acids 25-210 of the Xic1 protein. The Xic1 proteins were gifts of James Maller (Howard Hughes Medical Institute, University of Colorado Health Sciences Center). Microinjected embryos were maintained in 5% Ficoll in 0.1X MMR, and snap-frozen at indicated time points. Some embryos were also injected with 50 ng  $\alpha$ -amanitin dissolved in H<sub>2</sub>O. In some experiments, fertilized eggs were injected with buffer or 15 ng mRNA encoding luciferase or XChk1 (transcribed *in vitro* using the mMessage mMachine kit from Ambion), as described in Kappas et al. (2000).

### **Immunoblotting**

Embryos were collected at the indicated times, snap-frozen on dry ice, and homogenized in EB buffer (20-30 mM HEPES, pH 7.5, 15 mM MgCl<sub>2</sub>, 20 mM EGTA, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM microcystin, 1 mM PMSF, and 3  $\mu$ g/ml each leupeptin, pepstatin and chymostatin, with or without 80 mM  $\beta$ -glycerophosphate and 50 mM sodium fluoride). Embryo lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and hybridized with antibodies, as described previously (Sible et al., 1997; Kappas et al., 2000). The cyclin E antibody was purified from serum provided by Rebecca Hartley (University of Iowa). The Wee1 antibodies and Xic1 antibodies were generously provided by Monica Murakami (National Cancer Institute, NIH) and James Maller (Howard Hughes Medical Institute, University of Colorado Health Sciences Center), respectively. To visualize immunoreactive proteins, a

horseradish peroxidase-conjugated secondary antibody was hybridized, and chemiluminescence from the secondary antibody was detected with the ECL Plus (Amersham) or West Pico (Pierce) kits.

### **Northern analysis of Wee1 Expression**

Northern analysis of *Wee1* mRNA expression during *Xenopus* development was performed essentially as described previously (Sible et al., 1997). Total RNA was isolated from embryos using TriReagent (Molecular Research Center). Ten micrograms of each RNA was resolved by denaturing gel electrophoresis, transferred to a 0.2 $\mu$ m Nytran membrane with a TurboBlotter apparatus (Schleicher and Schuell), and then cross-linked to the membrane with a Stratagene UV cross-linker. *Wee1* cDNA, isolated from a plasmid provided by Dr. Monica Murakami (National Cancer Institute, NIH), was used as template to generate <sup>32</sup>P-labeled probe in a random priming reaction (Roche) containing [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham). The probe was hybridized with the RNA blot in QuickHyb solution (Stratagene). After washing, the blot was exposed to Kodak BioMax film.

### **Immunoprecipitation and kinase assays of cyclin /Cdk complexes**

Embryos injected at the one-cell stage with 5 ng Xic-C or  $\Delta$ 34Xic1 were collected at the time indicated, and antibodies against cyclin B or cyclin E were used to immunoprecipitate cyclin B/Cdk1 or cyclin E/Cdk2 complexes, respectively, as described in Kappas et al. (2000). Cyclin antibodies were provided by Dr James L. Maller (Howard Hughes Institute, University of Colorado Health Sciences Center).

### **Mathematical modeling and computational simulation**

Mathematical models of hypothetical molecular mechanisms for regulation of cyclin E/Cdk2 activity were constructed by translating a mechanism into a set of ordinary differential equations (ODEs) by standard principles of biochemical kinetics (Tyson et al., 1996). The ODEs were then provided as input to XPP-AUT, a simulation and analysis software system, freely downloadable from <http://www.math.pitt.edu/~bard/xpp/xpp.html>. XPP runs on the Unix operating system.

Parameter values were chosen by trial-and-error, to fit the basic phenomenological properties of the control system (rapid fluctuations in cyclin E/Cdk2 activity followed by abrupt degradation of cyclin E).

## RESULTS

Based on the experimental evidence summarized in the INTRODUCTION (“phase one”), a preliminary model of the cyclin E/Cdk2 control system is first presented (Figure 1). The model is sufficient to guide a new round of experiments, but not sufficiently well defined to build a mathematical model. After phase-two experiments are described, a fully specified model is presented, with simulations demonstrating that the model is consistent with phase-one and phase-two data. The model makes clear predictions that are tested in phase-three experiments.

### **Preliminary model**

Pathways by which cyclin E/Cdk2 activity might be controlled in the early embryo are sketched in Figure 1. A maternal store of cyclin E combines with free Cdk2 monomers to form active cyclin E/Cdk2 dimers. These dimers can be inactivated by phosphorylation of tyrosine and threonine residues of Cdk2 by Wee1, a reaction that is reversed by the phosphatase Cdc25. It is predicted that cyclin E/Cdk2 somehow activates Wee1 or inhibits Cdc25, creating a negative feedback loop with the capacity to oscillate autonomously under the right conditions. We propose to associate oscillations in Cdk2 phosphorylation state with the rapid, twice-per-mitotic-cycle fluctuations in Cdk2 activity observed before the MBT (Hartley et al., 1997).

To model the abrupt degradation of cyclin E at approximately 7 h post fertilization (pf), it is proposed that cyclin E/Cdk2 is removed from the oscillatory subsystem in a cooperative fashion through a positive feedback loop. Subsequently, the removed-form of cyclin E is degraded, probably by the SCF/proteasome pathway. “Removed” cyclin E/Cdk2 could correspond to a change in phosphorylation state, localization, or some other property of cyclin E. Qualitatively, it is predicted that, during the first 7 h of embryonic development, most cyclin E will cycle between active (unphosphorylated) and inactive (Cdk2-phosphorylated) forms. During these oscillations, an increasing fraction of cyclin E/Cdk2 dimers is removed from the oscillating forms, eventually stimulating a rapid, cooperative (autocatalytic) removal of cyclin E/Cdk2, which quenches the oscillation.

## Phase-two experiments: refinement of the model

*Inhibition of cyclin E/Cdk2 in early Xenopus embryos results in a loss of Wee1 protein.* To explore possible mechanisms for the oscillatory behavior of Cdk2 activity in pre-MBT embryos, we looked for experimental evidence of a negative feedback loop between cyclin E/Cdk2 and the enzymes that regulate Cdk phosphorylation. Prior to the first cleavage, embryos were microinjected with 5 ng Xic1-C or  $\Delta 34$ Xic1. (Xic1-C lacks the first 96 amino acids and does not bind Cdks (Su et al., 1995); therefore, it serves as a negative control. Five ng  $\Delta 34$ Xic1 specifically inhibits cyclin E/Cdk2 in *Xenopus* embryos (Su et al., 1995; Hartley et al., 1997).) Embryos were collected at the times indicated, and immunoblotting of cyclin E and the inhibitory kinase Wee1 was performed (Figure 2A). As described previously (Hartley et al., 1997), the disappearance of cyclin E in control embryos began after the MBT, about 6-7 h pf, and  $\Delta 34$ Xic1 delayed this disappearance until approximately 10 h pf, when embryos reached approximately the MBT number of cells. In control embryos, Wee1 level was constant until the late gastrula/early neurula stage when Wee1 level declined, as previously described (Murakami and Woude, 1998). In the embryos injected with  $\Delta 34$ Xic1, the level of Wee1 was very low, even prior to the MBT. These data suggest that cyclin E/Cdk2 activity is required to maintain Wee1 protein level prior to the MBT.

Embryos injected with  $\Delta 34$ Xic1 demonstrated no difference in the level of Cdc25A compared to control embryos (data not shown). The level of Cdc25C was not determined.

*Wee1 mRNA level correlates with cyclin E/Cdk2 activity.* Since Wee1 protein level depends on the activity of cyclin E/Cdk2, the abundance of Wee1 mRNA during early embryogenesis was determined. Embryos were collected at the indicated times post-fertilization, and total RNA was isolated for Northern analysis of Wee1 mRNA levels. Like Wee1 protein ((Murakami and Woude, 1998) and Figure 2A), Wee1 mRNA level is constant during early development through the MBT, and then levels decline during gastrulation (Figure 2B). Wee1 mRNA levels are lower in  $\Delta 34$ Xic1-injected embryos (Figure 2C), suggesting that cyclin E/Cdk2 maintains the stability of Wee1 mRNA.

*Reagents that inhibit Cdk2 delay the degradation of cyclin E.* A fundamental assumption of our working model is that degradation of cyclin E at the MBT results from a cooperative transition in cyclin E/Cdk2 forms. This assumption is based upon published studies demonstrating that cyclin E is degraded at a fixed time post-fertilization, independent of protein synthesis (hence, independent of Cdk1 activity which requires continued synthesis of mitotic cyclins A1 and B), cell number, the nucleocytoplasmic ratio, and zygotic transcription (Howe and Newport, 1996; Hartley et al., 1997; Sible et al., 1997). Furthermore, the only manipulation known to alter the timing of cyclin E degradation is microinjection of  $\Delta 34Xic1$ , a protein that specifically inhibits Cdk2 but not Cdk1 activity (Hartley et al., 1997). Because  $\Delta 34Xic1$  delays the degradation of cyclin E, it seemed likely that the timing mechanism for cyclin E degradation depends upon cyclin E/Cdk2 activity itself.

If this assumption were correct, then other reagents that interfere with cyclin E/Cdk2 activity should also block or delay the degradation of cyclin E. Expression of exogenous XChk1 in *Xenopus* embryos inhibits both Cdk1 and Cdk2 activity (Kappas et al., 2000); therefore, expression of XChk1 should also block or delay the degradation of cyclin E. Embryos were injected with 15 ng mRNA encoding FLAG-tagged XChk1. Negative control embryos were injected with buffer or 15 ng mRNA encoding FLAG-tagged luciferase. Embryos were collected at the times indicated and analyzed for cyclin E content by immunoblotting (Figure 2D). Analogous to embryos injected with  $\Delta 34Xic1$ , cyclin E degradation was delayed in embryos overexpressing XChk1 compared to both buffer (Figure 2D) and luciferase controls (not shown), supporting the hypothesis that timing of cyclin E degradation depends directly on Cdk2 activity.

*Cyclin E degradation in Xic1-treated embryos does not depend upon transient cyclin E/Cdk2 activity.* When embryos are microinjected with 5 ng  $\Delta 34Xic1$ , cyclin E/Cdk2 activity is inhibited by more than 90% without inhibition of Cdk1 activity (Hartley et al., 1997). Intuitively, one would predict that cyclin E degradation would be blocked indefinitely (or until embryos died by necrosis) in  $\Delta 34Xic1$ -injected embryos. However, the degradation of cyclin E does occur, only several hours delayed, when embryos have reached the nucleocytoplasmic ratio of the MBT (Hartley et al., 1997). This observation challenges the hypothesis that cyclin E degradation is directly linked to cyclin E/Cdk2 activity.

One possible explanation for the eventual degradation of cyclin E in  $\Delta 34Xic1$ -injected embryos is that cyclin E/Cdk2 activity resumes transiently, prior to the degradation of cyclin E, at approximately 9 h pf. This scenario played a central role in a preliminary mathematical model that simulated the delay in degradation of cyclin E in  $\Delta 34Xic1$ -injected embryos. However, kinase assays performed on embryos injected with  $\Delta 34Xic1$  detected no transient peak of Cdk2 activity (Figure 2E), even when samples were collected at 30 min intervals (not shown), ruling out this particular model.

*Cyclin E degradation in Xic1-treated embryos does not depend upon zygotic transcription.* It could be that degradation of cyclin E in embryos injected with  $\Delta 34Xic1$  is initiated by zygotic transcription at the MBT, since transcription initiates coincident with cyclin E degradation in  $\Delta 34Xic1$ -injected embryos (Hartley et al., 1997). To determine whether a product of zygotic transcription turned on degradation of maternal cyclin E, independent of cyclin E/Cdk2 activity, embryos were injected with H<sub>2</sub>O,  $\alpha$ -amanitin (an inhibitor of transcription),  $\Delta 34Xic1$ , or a combination of  $\alpha$ -amanitin and  $\Delta 34Xic1$  (Figure 2F). In embryos injected with H<sub>2</sub>O or  $\alpha$ -amanitin, cyclin E disappeared between 5 and 7 h pf, consistent with previous reports that degradation of maternal cyclin E at the MBT does not depend on transcription (Howe and Newport, 1996; Hartley et al., 1997). As predicted (Hartley et al., 1997), degradation of cyclin E was delayed in embryos injected with  $\Delta 34Xic1$ , and was complete by 10 h pf. In embryos injected with both  $\alpha$ -amanitin and  $\Delta 34Xic1$ , cyclin E was degraded at the same time as in embryos injected with  $\Delta 34Xic1$  alone. These results indicate that transcription at the MBT is not required for the eventual degradation of cyclin E in embryos lacking cyclin E/Cdk2 activity.

### **Kinetic model of the cyclin E/Cdk2 developmental timer**

Based on the information summarized in the INTRODUCTION and the new data presented in Figure 2, a full, working model of the cyclin E/Cdk2 control system was developed (Figure 3). The model is composed of three modules: a negative feedback oscillator, a positive feedback switch, and an irreversibly activated pathway for cyclin E degradation.

*Negative feedback oscillator.* Cyclin E/Cdk2 dimers can be transformed between an inactive,

phosphorylated form (cyclin E/Cdk2P) and an active, non-phosphorylated form (cyclin E/Cdk2) by the action of a kinase, Wee1, and a phosphatase, Cdc25A. We assume that Cdc25A is present at constant activity in the early embryo, and that Wee1 is periodically activated by cyclin E/Cdk2, indirectly through the action of a hypothetical kinase, Kin. (Cdc25C was not included in the model because dominant-negative Cdc25C does not affect cell cycle length in embryos (Kim et al., 1999).) In the model, Kin phosphorylates and inactivates Wee1, and cyclin E/Cdk2 phosphorylates and inactivates Kin. These interactions create a delayed negative feedback loop, consisting of three inactivating phosphorylation steps in sequence (Cdk2 --| Kin --| Wee1 --| Cdk2). The parameters in the model are adjusted to create limit cycle oscillations with a period of about 15 min (two peaks per mitotic cycle). Because a two-component negative feedback loop (Cdk2 --| Wee1 --| Cdk2) cannot oscillate (Griffith, 1968), we are compelled to introduce the hypothetical kinase (as the simplest case of some process that provides a delay between the actions of Cdk2 and Wee1). Furthermore, because Wee1 mRNA levels are lower in  $\Delta 34Xic1$ -injected embryos and disappear after the MBT, when cyclin E is degraded (Figure 2B, C), we assume that the synthesis rate for Wee1 is an increasing function of cyclin E-dependent kinase activity. This assumption is consistent with the observation that nuclear Wee1 is turned over during each cell cycle (Michael and Newport, 1998).

*Positive feedback switch.* Cyclin E/Cdk2 binds to chromatin (Furstenthal et al., 2001a). We assume that the binding step is cooperative (autocatalytic), and that bound dimers activate the cyclin E degradation machinery (presumably SCF). If the binding step is cooperative, then there will be at first little cyclin E bound to chromatin. After several hours, enough cyclin E is chromatin-bound to rapidly accelerate the loading step and bring the entire cyclin E pool onto chromosomes, consistent with the observations of Furstenthal et al (2001a). Naturally, this extinguishes the cyclin E/Cdk2 oscillations among the unbound dimers.

*Cyclin E degradation.* After a further delay, the cyclin E-degradation machinery (“Deg”) activates, and the whole pool of cyclin E is degraded from the chromosomes. We suppose that Deg is activated by the active form of cyclin E/Cdk2 bound to chromatin. Furthermore, the concentration of cyclin E/Cdk2/chromatin must exceed a certain threshold ( $\theta$  in Eq. 8) before it

can activate Deg. As cyclin E is destroyed, we presume that Deg remains active. In mammals, cyclin E is degraded by the SCF after cyclin E is phosphorylated (Clurman et al., 1996; Won and Reed, 1996), so Deg may correspond to a kinase.

*Effect of  $\Delta 34Xic1$ .* We assume that (1) Xic1 binds strongly to all forms of cyclin E/Cdk2 and blocks any catalytic activity they may have; nonetheless, (2) Xic1 binding does not interfere with cyclin E/Cdk2 association to chromatin. Because Xic1 bound to the chromatin-associated forms of cyclin E is slowly degraded (Swanson et al., 2000; Chuang and Yew, 2001; Furstenthal et al., 2001b), the active form of cyclin E/Cdk2 will eventually appear on chromatin and subsequently activate cyclin E degradation. Under these assumptions, Xic1 injections are expected to quench oscillations of cyclin E/Cdk2 activity and to delay (but not eliminate) cyclin E degradation.

*Further assumptions.* (1) The chromatin-bound forms of cyclin E/Cdk2 (1) are not subject to phosphorylation and dephosphorylation by Wee1 and Cdc25. (2) The chromatin-bound form of cyclin E/Cdk2 does not phosphorylate Kin. (3) The chromatin-bound form of cyclin E/Cdk2 does not stabilize Wee1 mRNA. These assumptions make the model a little easier to understand, but they are not necessary. Models in which these assumptions are not made behave very similarly to the model simulated here (not shown).

*The model.* A mathematical model (the differential equations in Table 1) can be derived from the molecular wiring diagram (Figure 3) by using standard rate laws of biochemical kinetics. Basal parameter values are proposed in Table 2. These values were chosen to reproduce the basic quantitative characteristics of the cyclin E/Cdk2 timer in normal frog embryos. We assume that, at  $t=0$  (fertilization, or shortly thereafter), the cyclin E oscillator is in full swing and no cyclin E is chromatin-bound.

## Numerical simulations and phase-three experiments: testing the model

*The model reproduces the fundamental features of the cyclin E/Cdk2 timer.*

Figure 4 presents a numerical solution of the kinetic equations (Table 1) using the basal parameter values proposed for unperturbed embryos (Table 2). The model reproduces the fundamental observations driving this study, that (1) cyclin E is a stable protein at first (Howe and Newport, 1996; Hartley et al., 1997) and then rapidly degraded at the MBT, and (2) cyclin E/Cdk2 activity oscillates twice per cell cycle before the MBT (Hartley et al., 1997) (Figure 4A). We did not attempt to model the distinct cycle 1, which is longer than cycles 2 – 12 and does demonstrate cyclin E degradation (Hartley et al. 1996). An appreciation for the regulatory mechanisms behind the oscillations in kinase activity and the abrupt degradation of cyclin E unfolds from graphing the concentrations of the four forms of cyclin E/Cdk2 over time (Figure 4B). Oscillations in cyclin E/Cdk2 activity prior to the MBT derive from the periodic phosphorylation and dephosphorylation of unbound cyclin E/Cdk2, as a consequence of the negative feedback loop with Wee1. Loss of cyclin E results as chromatin-bound dimers accumulate. Changes in steady state levels of Wee1 over time (Figure 4C) agree with published experimental data (Murakami and Woude, 1998), in which Wee1 is degraded during the late gastrula/early neurula stage, after the disappearance of cyclin E.

*The cyclin E/Cdk2 timer functions independently of protein synthesis.*

Oscillations in cyclin E/Cdk2 activity (Hartley et al., 1997) and disappearance of cyclin E at a fixed time post-fertilization (Howe and Newport, 1996; Hartley et al., 1997) occur even when embryos are treated with cycloheximide to block protein synthesis. The only step in the model that depends upon protein synthesis is the production of Wee1. Therefore, to simulate the effect of cycloheximide,  $k_{\text{wsyn}}$  is set to 0. In the simulations, cyclin E/Cdk2 activity still oscillates 22 times (Figure 5A), is removed from the system by cooperative binding to chromatin (Figure 5A) and is degraded beginning approximately 6 h pf (Figure 5B) despite a dramatic loss of Wee1 within the first 3 h pf (Figure 5B). Therefore, the model faithfully describes a timer that does not

depend upon protein synthesis, a critical constraint from the experimental data (Hartley et al., 1997).

*Exogenous  $\Delta 34Xic1$  is degraded prior to the disappearance of cyclin E.*

A major challenge in developing the model was the experimental result that embryos microinjected with  $\Delta 34Xic1$  eventually degrade cyclin E, with a delay of only several hours (Hartley et al., 1997). If cyclin E degradation were intrinsically linked to cyclin E/Cdk2 activity as we assume, and if  $\Delta 34Xic1$  inhibits cyclin E/Cdk2 activity by more than 90% (Hartley et al., 1997), then cyclin E levels might persist indefinitely in  $\Delta 34Xic1$ -injected embryos. That this is not the case suggests that Xic1 is eventually lost from the treated embryos. In the model, we assume that chromatin-bound Xic1 is subject to steady degradation. Simulations show that this reasonable assumption is sufficient to account for Xic1- and cyclin E fates in treated embryos (Figure 6). To simulate the effect of injecting  $\Delta 34Xic1$ , the initial concentration of "Xic1" in the model was set to 3. (In simulations of unperturbed embryos (Figure 4), [total Xic1] = 0, based on the data of (Shou and Dunphy, 1996).) Simulations indicate that most cyclin E/Cdk2 associates with Xic1, and this complex is gradually loaded onto chromatin, peaking approximately 7 hrs pf (Figure 6A). Once bound to chromatin, Xic1 is degraded there, consistent with experiments (Swanson et al., 2000; Furstenthal et al., 2001b). As long as free Xic1 is present in the cell, chromatin-bound cyclin E/Cdk2 complexes that lose their Xic1 partner will quickly pick up another. Between hours 5-9, a steady drop in concentration of free Xic1 is observed with only a modest rise in chromatin-bound cyclin E/Cdk2 (Figure 6B). Once the supply of Xic1 is exhausted, the active form of chromatin-bound cyclin E/Cdk2 can turn on Deg (Figure 6B), and cyclin E is degraded (Figure 6C). We assume that chromatin bound cyclin E/Cdk2 is not immunoprecipitated and detected in H1 kinase assays because we do not detect a transient peak in cyclin E/Cdk2 activity between the degradation of microinjected  $\Delta 34Xic1$  and the degradation of cyclin E (Figure 2E and data not shown).

Plotting levels of  $\Delta 34Xic1$  and cyclin E over time (Figure 6C) indicates that (1) cyclin E is degraded abruptly at approximately 10 h pf, consistent with observations (Hartley et al., 1997), and (2)  $\Delta 34Xic1$  is degraded a few hours before cyclin E.

Since the content of  $\Delta 34Xic1$  over time had not been monitored in previous studies of

$\Delta 34$ Xic1-injected embryos, the theoretical prediction that  $\Delta 34$ Xic1 would be degraded just prior to the degradation of cyclin E was tested experimentally. Embryos were injected with 5 ng Xic1-C or  $\Delta 34$ Xic1 at the one-cell stage, collected at multiple time points, and analyzed for content of cyclin E and Xic1 (Figure 6D). The level of  $\Delta 34$ Xic1 decreased dramatically between 6 and 8 h pf, prior to the degradation of cyclin E between 8 and 10 h pf. Therefore, the prediction of the model was confirmed. Interestingly, in the Xic1-C-injected embryos, cyclin E degradation began between 6 and 8 h pf, and exogenous Xic1-C was also degraded at this time, indicating that the truncated Xic1-C protein contains the necessary elements to be targeted for degradation.

*Cyclin E degradation is progressively delayed by increasing amounts of  $\Delta 34$ Xic1.*

As a final test of the model, the dose of  $\Delta 34$ Xic1 was altered in simulations to determine the effect on cyclin E degradation. Simulations (Figure 7A) indicated that the timing of cyclin E degradation should be inversely correlated to the quantity of  $\Delta 34$ Xic1 injected over at least a 4-fold range. To test this prediction experimentally, embryos were injected with 0, 2.5, 5 or 10 ng  $\Delta 34$ Xic1. The total injection volume was kept constant. Embryos were analyzed for the degradation of cyclin E at multiple time points (Figure 7B). In embryos lacking  $\Delta 34$ Xic1, most cyclin E degradation occurred between 5 and 7 h pf. In embryos injected with 2.5 or 5 ng  $\Delta 34$ Xic1, cyclin E degradation occurred 7-8 h and 9-10 h pf, respectively. Little degradation of cyclin E was detected in embryos injected with 10 ng  $\Delta 34$ Xic1, even as late as 11 h pf. The simulations and experimental data correlate well, further supporting the theoretical basis of the model.

The good fit between simulations and experiments allows us to estimate the “arbitrary units” of Xic1 concentration in the model. [Total Xic1] = 1 AU corresponds to 1.67 ng  $\Delta 34$ Xic1 per embryo (1.5 AU simulates activity of 2.5 ng  $\Delta 34$ Xic1; 3 AU simulates 5 ng  $\Delta 34$ Xic1, etc.), which is a concentration of about 33 nM (assuming 1 embryo = 1.0  $\mu$ L and MW  $\Delta 34$ Xic1 = 50,000 g/mol). Since Xic1 and cyclin E/Cdk2 bind in 1:1 stoichiometry, this means that [total cyclin E] = 1 AU also corresponds to about 33 nM.

## DISCUSSION

By combining computational and experimental approaches, we have built a model of the cyclin E/Cdk2 developmental timer in early *Xenopus* embryos. Development of the model was constrained by experimental evidence obtained from intact *Xenopus* embryos: (1) oscillations in E/Cdk2 occur twice per cell cycle prior to the MBT (Hartley et al. 1997), (2) the level of cyclin E remains constant during this time (Howe and Newport, 1996; Hartley et al., 1997), and (3) cyclin E is degraded at a fixed time post-fertilization independent of cell number, protein synthesis, nucleocytoplasmic ratio, and zygotic transcription (Howe and Newport, 1996; Hartley et al., 1997). The model faithfully reproduces 22 oscillations in cyclin E/Cdk2 activity and degradation of cyclin E at approximately 6-7 h pf, independent of protein synthesis. Experimental evidence that the degradation of cyclin E is delayed in embryos microinjected with 5 ng  $\Delta 34$ Xic1 (Hartley et al., 1997) also agrees with the simulations.

The model is composed of three “modules”: an oscillatory module, based on a delayed negative feedback loop, a switching module, based on cooperative loading of cyclin E/Cdk2 onto chromatin, and a proteolysis module, based on irreversible activation of the cyclin E degradation pathway.

### **Oscillatory module**

Oscillations in cyclinE/Cdk2 activity are not dependent on cyclin E synthesis or degradation, because total cyclin E level is quite constant throughout the early stages of frog egg embryogenesis (Hartley et al., 1997). Nor are these oscillations attributable to periodic fluctuations in a stoichiometric inhibitor, Xic1, because Xic1 is not normally present in the early embryo (Shou and Dunphy, 1996). That leaves reversible inhibitory phosphorylation of Cdk2 as the prime suspect for generating oscillations in cyclin E/Cdk2 activity. To our knowledge, oscillations in the phosphorylation state of Cdk2 have not been measured in intact embryos and may be difficult to observe because the oscillations are rapid and may be localized within nuclei. On the other hand, Cdk2 activity is regulated by reversible phosphorylation in extracts (D'Angiolella et al., 2001), and embryos microinjected with XChk1, which promotes tyrosine

phosphorylation of Cdks, have low cyclin E/Cdk2 activity (Kappas et al., 2000). If cyclin E/Cdk2 oscillations derive from reversible phosphorylation alone, then the components must be involved in a negative feedback loop, cyclin E/Cdk2 either promoting Wee1 activity or inhibiting Cdc25. This supposition is in sharp contrast to the case of cyclin B/Cdk1, which inhibits Wee1 and activates Cdc25. In that case, oscillations depend on Cdk1 turning on cyclin B degradation by activating Fizzy/APC. Therefore, Cdk2 oscillations are fundamentally different from Cdk1 oscillations.

Inhibition of cyclin E/Cdk2 with  $\Delta 34$ Xic1 results in decreased levels of Wee1 mRNA and protein, evidence for a negative feedback loop in which cyclin E/Cdk2 stabilizes Wee1 mRNA. These data provide a satisfying explanation for the disappearance of Wee1 mRNA (Figure 2D) and protein after the MBT (Murakami and Woude, 1998). However, regulation of mRNA stability is not likely to account for the rapid oscillations in cyclin E/Cdk2 activity prior to the MBT. Because there is no transcription before the MBT (Newport and Kirschner, 1982a), Wee1 mRNA content can only remain steady or decrease until the MBT. For these reasons, we suggest that the negative feedback loop works on Wee1 activity rather than protein level.

At this point, the oscillatory module in Figure 3 is purely hypothetical and awaits experimental validation. One should also keep in mind that a negative feedback loop, whereby cyclin E/Cdk2 indirectly inhibits Cdc25, might be an alternative basis for oscillations in Cdk2 activity.

### **Switching module**

The second major assumption in the model is that the timing of cyclin E degradation depends upon cyclin E/Cdk2 activity. In support of this assumption, degradation of cyclin E is delayed when Cdk2 activity is inhibited by either  $\Delta 34$ Xic1 (Hartley et al., 1997 and Figures 2A, F) or XChk1 (Figure 2B). The model assumes cooperative binding of cyclin E/Cdk2 to chromatin, consistent with the two-step association of cyclin E/Cdk2 to chromatin observed by Furstenthal et al (2001a) in egg extracts. However, in  $\Delta 34$ Xic1-injected embryos, cyclin E is eventually degraded. The model incorporates the idea that cyclin E/Cdk2 promotes chromatin association and subsequent degradation of Xic1, as suggested by experiments of (Furstenthal et al., 2001b). In simulations of  $\Delta 34$ Xic1-injected embryos, cyclin E is degraded some hours after degradation

of  $\Delta 34\text{Xic1}$  (Figure 6A). This prediction was validated experimentally (Figure 6B).

### **Degradation module**

We have not attempted to model the molecular machinery of cyclin E degradation, because it does not feedback, as far as we know, on the dynamics of the oscillatory and switching modules. We use a mathematical trick (the “Heaviside function”) to switch on cyclin E degradation in an irreversible fashion, after a sufficient amount of active cyclin E/Cdk2 has accumulated on chromatin. As new experimental data is generated, a more realistic mechanism for cyclin E degradation can be inserted in place of this module.

### **Bifurcation analysis of the mathematical model**

To better understand the fundamental behavior of the cyclin E/Cdk2 developmental timer, bifurcation analysis was applied to a simplified version of the model (a primer on bifurcation analysis is available in Borisuk and Tyson, 1998). Because our aim is to uncover the dynamics of the oscillatory and switching modules, we have eliminated Xic1 binding and cyclin E degradation from the network (see Table 3). A numerical simulation of these equations is presented in Figure 8A. Chromatin-bound cyclin E/Cdk2 (dashed line) increases abruptly around 6 h pf, indicative of cooperative loading, as postulated by the model and supported by Furstenthal et al. (2001b). Oscillations in active, unbound cyclin E/Cdk2 (solid line) are created by the negative feedback loop involving Wee1 and quenched by the cooperative binding of cyclin E/Cdk2 to chromatin.

In Figure 8B, we consider the behavior of the negative feedback loop as a function of the fraction of cyclin E bound to chromatin. That is, we fix  $[\text{Cdk2:CycE:Chr}] + [\text{PCdk2:CycE:Chr}] = H = \text{constant}$ , and study the behavior of the negative feedback loop as a function of the value of  $H$ . For  $0 < H < 0.416$ , the steady state solution of the negative feedback loop (dashed line) is unstable, and the network executes sustained oscillations (the black circles indicate the maximum and minimum values of active cyclin E/Cdk2 over the course of an oscillation) with period close to 14 min (open triangles). For  $0.416 < H < 1$ , the negative feedback loop has a stable steady state (solid line) and no sustained oscillations. At  $H = 0.416$ , the negative feedback loop is said to undergo a Hopf bifurcation. Now, comparing Figures 8A and B, we see that, as  $H$

increases from 0 to 0.416 (during the first 5 h pf), the negative feedback loop oscillates with a period of about 14 min. But, as soon as  $H$  exceeds the Hopf-bifurcation point, the oscillations are quickly lost.

To see why  $H$  increases as in Figure 8A, we plot the rate of change of  $H$  ( $dH/dt$ ) as a function of  $H$  in Figure 8C. When  $dH/dt$  is positive,  $H$  will increase; when  $dH/dt$  is negative,  $H$  will decrease. Steady states exist wherever  $dH/dt = 0$ . For the parameter set used here (solid line), a single stable steady state exists at  $H$  close to 1. Hence, starting with  $H=0$  (no chromatin-bound cyclin E/Cdk2),  $H$  must increase until it reaches the steady state, where most cyclin E/Cdk2 is bound to chromatin. Notice, however, that the rate of increase of  $H$  is, at first, very small ( $dH/dt \approx 0.0008$ ) and then accelerates rapidly by nearly ten-fold. This acceleration is a reflection of the cooperative binding of cyclin E to chromatin in the model.

Cooperative binding is a form of positive feedback (autocatalysis) that can easily create bistable behavior. If the dissociation rate constant,  $k_{\text{off}}$ , is increased from 0.0001 to 0.01 (perhaps by a mutation in Cdc6 that affects cyclin E/Cdk2 binding to chromatin), then  $dH/dt$  as a function of  $H$  is modified (dashed line) so that there now exist two stable steady states: one with  $H \approx 0.1$  (little cyclin E bound to chromatin), and another with  $H \approx 0.7$  (most cyclin E bound to chromatin). At the lower steady state, the negative feedback loop will be in its oscillatory regime, and at the upper steady state, the negative feedback loop will be quiescent. This behavior of the control system is entirely different from what is observed. We have chosen our parameter set (Table 2) to bring the control system close to but not within its region of bistability. With this choice,  $H$  increases slowly at first, pulling the oscillatory module past its Hopf bifurcation, and then  $H$  increases rapidly, as most of the cyclin E loads cooperatively onto chromatin.

Finally, we have mentioned several times that oscillations in the negative feedback loop require an intermediate,  $K_{\text{in}}$ , between cyclin E/Cdk2 and Wee1.  $K_{\text{in}}$  introduces a time delay between activation of cyclin E/Cdk2 and subsequent increase in Wee1 activity. This time delay depends on the characteristic time,  $\tau$ , required for cyclin E/Cdk2 to inactivate  $K_{\text{in}}$  ( $\tau = 1/k_{\text{inact}}$ ). As this characteristic time gets small, the time delay becomes negligible, and the oscillations

disappear. This effect is illustrated in Figure 8D, where we show that oscillations of the negative feedback disappear as  $\tau$  gets small. (To keep the relative activity of Kin fixed, we steadily increase both  $k_{\text{iact}}$  and  $k_{\text{iinact}}$ , keeping their ratio constant at  $k_{\text{iact}}/k_{\text{iinact}} = 0.25$ .) As  $\tau$  gets small, oscillations are lost by a Hopf bifurcation (at  $\tau = 5 \times 10^{-4}$  in Figure 8D). The amplitude of the negative feedback oscillations is getting quite small even for  $\tau < 5 \times 10^{-4}$ . In other words, oscillations in the negative feedback loop require an intermediate that introduces a minimal time lag between cyclin E/Cdk2 and Wee1.

### **Future developments**

The model presented here will be refined as new experimental and computational information becomes available. Creation of egg extracts that reproduce oscillations in cyclin E/Cdk2 activity would facilitate future experimentation. Although these oscillations have not been observed in egg extracts (Rebecca Hartley, personal communication), it should be possible to use the model to predict which parameters should be varied in egg extracts to promote cyclin E/Cdk2 oscillations. An in-depth investigation of the underlying dynamics of the mathematical model (Table 1) is underway. Additional modeling should be done to study the interaction between Cdk1 and Cdk2 oscillators in determining the timing of cell divisions in the early embryo.

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## REFERENCES

- Audic, Y., Boyle, B., Slevin, M., and Hartley, R. (2001). Cyclin E morpholino delays embryogenesis in *Xenopus*. *Genesis*. *30*, 107-109.
- Borisuk, M.T., and Tyson, J.J. (1998). Bifurcation analysis of a model of mitotic control in frog eggs. *J. theor. Biol.* *195*, 69-85.
- Chuang, L.-C., and Yew, P.R. (2001). Regulation of nuclear transport and degradation of the *Xenopus* cyclin-dependent kinase inhibitor, p27<sup>Xic1</sup>. *J. Biol. Chem.* *276*, 1610-1617.
- Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M. and Roberts, J.M. (1996) Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by Cdk2 binding and cyclin phosphorylation. *Genes Dev.* *10*, 1979-1990.
- Cyert, M., and Kirschner, M. (1988). Regulation of MPF activity *in vitro*. *Cell.* *53*, 185-195.
- D'Angiolella, V., Costanzo, V., Gottesman, M.E., Avvedimento, E.V., Gautier, J. , and Grieco, D. (2001). Role for cyclin-dependent kinase 2 in mitosis exit. *Curr. Biol.* *11*, 1221-1226.
- Devault, A., Fesquet, D., Cavadore, J.-C., Garrigues, A.-M., Labbe, J.-C., Lorca, T., Picard, A., Philippe, M. ,and Doree, M. (1992). Cyclin A potentiates maturation-promoting factor activation in the early *Xenopus* embryo via inhibition of the tyrosine kinase that phosphorylates cdc2. *J. Cell Biol.* *118*, 1109-1120.
- Ferrell, J.E., Wu, M., Gerhart, J.C., and Martin, G.S. (1991). Cell cycle tyrosine phosphorylation of p34<sup>cdc2</sup> and a microtubule-associated protein kinase homolog in *Xenopus* oocytes and eggs. *Mol. Cell. Biol.* *11*, 1965-1971.

Frederick, D.L., and Andrews, M.T. (1994). Cell cycle remodeling requires cell-cell interactions in developing *Xenopus* embryos. *J. Exp. Zool.* 270, 410-416.

Furstenthal, L., Kaiser, B., Swanson, C., and Jackson, P. (2001a). Cyclin E uses Cdc6 as a chromatin-associated receptor required for DNA replication. *J. Cell Biol.* 152, 1267-1278.

Furstenthal, L., Swanson, C., Kaiser, B.K., Eldridge, A.G., and Jackson, P.K. (2001b). Triggering ubiquitination of a CDK inhibitor at origins of replication. *Nat. Cell Biol.* 3, 715-722.

Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F., and Kirschner, M.W. (1991). Cdc25 is a specific tyrosine phosphatase that directly activates p34<sup>cdc2</sup>. *Cell.* 67, 197-211.

Gerhart, J., Wu, M., and Kirschner, M. (1984). Cell cycle dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. *J. Cell. Biol.* 98, 1247-1255.

Goldbeter, A. and Koshland, D.E., Jr. (1981) An amplified sensitivity arising from covalent modification in biological systems. *Proc. Natl. Acad. Sci. USA* 78, 6840-6844.

Griffith, J.S. (1968) Mathematics of cellular control processes. I. Negative feedback to one gene. *J. Theor. Biol.* 20, 202-208.

Hartley, R.S., Rempel, R.E., and Maller, J.L. (1996). *In vivo* regulation of the early embryonic cell cycles in *Xenopus*. *Devel. Biol.* 173, 408-419.

Hartley, R.S., Sible, J.C., Lewellyn, A.L., and Maller, J.L. (1997). A role for cyclin E/Cdk2 in the timing of the midblastula transition in *Xenopus* embryos. *Devel. Biol.* 188,

Hinchcliffe, E.H., Li, C., Thompson, E.A., Maller, J.L., and Sluder, G. (1999). Requirements of Cdk2-cyclin E activity of repeated centrosome reproduction in *Xenopus* egg extracts. *Science.* 283, 851-854.

- Howe, J.A., and Newport, J.W. (1996). A developmental timer regulates degradation of cyclin E1 at the midblastula transition during *Xenopus* embryogenesis. *Proc. Natl. Acad. Sci. USA.* *93*, 2060-2064.
- Izumi, T., and Maller, J.L. (1993). Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. *Mol. Biol. Cell.* *4*, 1337-1350.
- Jackson, P.K., Chevalier, S., Philippe, M., and Kirschner, M.W. (1995). Early events in DNA replication require cyclin E and are blocked by p21<sup>CIP1</sup>. *J. Cell Biol.* *130*, 755-769.
- Kappas, N., Savage, P., Chen, K.C., Walls, A.T., and Sible, J.C. (2000). Dissection of the XChk1 signaling pathway in *Xenopus laevis* embryos. *Mol. Biol. Cell.* *11*, 3101-3108.
- Kim, S., Li, C., and Maller, J. (1999). A maternal form of the phosphatase Cdc25A regulates early embryonic cell cycles in *Xenopus laevis*. *Devel. Biol.* *212*, 381-391.
- Kumagai, A., and Dunphy, W.G. (1991). The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell.* *64*, 903-914.
- Kumagai, A., and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in *Xenopus* extracts. *Cell.* *70*, 139-151.
- Kumagai, A., and Dunphy, W.G. (1997). Regulation of *Xenopus* Cdc25 protein. *Meth. Enzymol.* *283*, 564-571.
- Lacey, K.R., Jackson, P.K., and Stearns, T. (1999). Cyclin-dependent kinase control of centrosome duplication. *Proc. Natl. Acad. Sci. USA.* *96*, 2817-2822.
- Lorca, T., Castro, A., Martinez, A.M., Vigneron, S., Morin, N., Sigrist, S., Lehner, C., Doree, M., and Labbe, J.C. (1998). Fizzy is required for activation of the APC/cyclosome in *Xenopus* egg extracts. *EMBO J.* *17*, 3565-3575.

Marlovits, G., Tyson, C.J., Novak, B., and Tyson, J.J. (1998). Modeling M-phase control in *Xenopus* oocyte extracts: the surveillance mechanism for unreplicated DNA. *Biophys. Chem.* 72, 169-184.

Masui, Y., and Markert, C. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* 177, 129-146.

Michael, W.M., and Newport, J. (1998). Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of wee1. *Science.* 282, 1886-1889.

Mueller, P.R., Coleman, T.R., and Dunphy, W.G. (1995a). Cell cycle regulation of a *Xenopus* Wee1-like kinase. *Mol. Biol. Cell.* 6, 119-134.

Mueller, P.R., Coleman, T.R., Kumagai, A., and Dunphy, W.G. (1995b). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science.* 270, 86-90.

Murakami, M.S., and Woude, G.F.V. (1998). Analysis of the early embryonic cell cycles of *Xenopus*; regulation of cell cycle length by Xe-wee1 and Mos. *Development.* 125, 237-248.

Murray, A.W., and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. *Nature.* 339, 275-280.

Newport, J., and Kirschner, M. (1982a). A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell.* 30, 675-686.

Newport, J., and Kirschner, M. (1982b). A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell.* 30, 687-696.

- Nieuwkoop, P.D., and Faber, J. (1975). Normal Table of *Xenopus laevis*. North Holland Publishing Company, Amsterdam.
- Novak, B., and Tyson, J.J. (1993). Numerical analysis of a comprehensive model of M-phase control in *Xenopus* oocyte extracts and intact embryos. *J. Cell Sci.* *106*, 1153-1168.
- Novak, B., and Tyson, J.J. (1995). Quantitative analysis of a molecular model of M-phase control in *Xenopus* oocyte extracts and intact embryos. *J. Theor. Biol.* *173*, 283-305.
- Rempel, R.E., Sleight, S.B., and Maller, J.L. (1995). Maternal *Xenopus* Cdk2-cyclin E complexes function during meiotic and early embryonic cell cycles that lack a G1 phase. *J. Biol. Chem.* *270*, 6843-6855.
- Shou, W., and Dunphy, W.G. (1996). Cell cycle control by *Xenopus* p28<sup>Kix1</sup>, a developmentally regulated inhibitor of cyclin-dependent kinases. *Mol. Biol. Cell.* *7*, 457-469.
- Sible, J.C., Anderson, J.A., Lewellyn, A.L., and Maller, J.L. (1997). Zygotic transcription is required to block a maternal program of apoptosis in *Xenopus* embryos. *Devel. Biol.* *189*, 335-346.
- Smythe, C., and Newport, J.W. (1992). Coupling of mitosis to the completion of S phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates p34<sup>cdc2</sup>. *Cell.* *68*, 787-797.
- Solomon, M.J., Glotzer, M., Lee, T.H., Phillippe, M., and Kirschner, M.W. (1990). Cyclin activation of p34<sup>cdc2</sup>. *Cell.* *63*, 1013-1024.
- Solomon, M.J., Lee, T.H., and Kirschner, M.W. (1992). Role of phosphorylation in p34<sup>cdc2</sup> activation: identification of an activating kinase. *Mol. Cell. Biol.* *3*, 13-27.

Strausfield, U.P., Howell, M., Rempel, R., Maller, J.L., Hunt, T., and Blow, J.J. (1994). Cip1 blocks the initiation of DNA replication in *Xenopus* extracts by inhibition of cyclin-dependent kinases. *Curr. Biol.* *4*, 876-883.

Su, J.-Y., Rempel, R.E., Erikson, E., and Maller, J.L. (1995). Cloning and characterization of the *Xenopus* cyclin-dependent kinase inhibitor p27<sup>Xic1</sup>. *Proc. Natl. Acad. Sci. USA.* *92*, 10187-10191.

Swanson, C., Ross, J., and Jackson, P.K. (2000). Nuclear accumulation of cyclin E/Cdk2 triggers a concentration-dependent switch for the destruction of p27<sup>Xic1</sup>. *Proc. Natl. Acad. Sci. USA.* *97*, 7796-7801.

Tyson, J.J., Novak, B., Odell, G.M., Chen, K., and Thron, C.D. (1996). Chemical kinetic theory: understanding cell-cycle regulation. *Trends Biochem. Sci.* *21*, 89-96.

Won, K.A., and Reed, S.I. (1996) Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J.* *15*,4182-4193.

**Table 1.** Differential equations describing the model in Figure 3. All concentration variables are expressed in “arbitrary units” (i.e., they are dimensionless numbers). Hence, all rate constants ( $k$ 's) have units  $\text{time}^{-1}$ . In the text, 1 AU of Xic and CycE concentration is estimated to be approximately 33 nM.

$$1. \frac{d}{dt} [\text{Cdk2:CycE}] = -k_{\text{wee}}[\text{Wee1}^*][\text{Cdk2:CycE}] + k_{25A}[\text{PCdk2:CycE}] - k_{\text{on}}\phi [\text{Cdk2:CycE}] \\ + k_{\text{off}}[\text{Cdk2:CycE:Chr}] - k_{\text{assoc}}[\text{Xic}][\text{Cdk2:CycE}] + k_{\text{dissoc}}[\text{Xic:Cdk2:CycE}]$$

$$2. \frac{d}{dt} [\text{PCdk2:CycE}] = +k_{\text{wee}}[\text{Wee1}^*][\text{Cdk2:CycE}] - k_{25A}[\text{PCdk2:CycE}] - k_{\text{on}}\phi [\text{PCdk2:CycE}] \\ + k_{\text{off}}[\text{PCdk2:CycE:Chr}] - k_{\text{assoc}}[\text{Xic}][\text{PCdk2:CycE}] + k_{\text{dissoc}}[\text{Xic:PCdk2:CycE}]$$

$$3. \frac{d}{dt} [\text{Wee1}^*] = \frac{k_{\text{on}}\phi [\text{Cdk2:CycE}] + k_{\text{off}}[\text{Cdk2:CycE:Chr}] + k_{\text{dissoc}}[\text{Xic:Cdk2:CycE}]}{k_{\text{deg}} + k_{\text{wee}}[\text{Wee1}^*]} - k_{\text{wdeg}}[\text{Wee1}^*]$$

$$4. \frac{d}{dt} [\text{Wee1}_{\text{total}}] = \frac{k_{\text{on}}\phi [\text{Cdk2:CycE}] + k_{\text{off}}[\text{Cdk2:CycE:Chr}] + k_{\text{dissoc}}[\text{Xic:Cdk2:CycE}]}{k_{\text{deg}} + k_{\text{wee}}[\text{Wee1}^*]} - k_{\text{wdeg}}[\text{Wee1}_{\text{total}}]$$

$$5. \frac{d}{dt} [\text{Kin}^*] = \frac{k_{\text{on}}\phi [\text{Cdk2:CycE}] + k_{\text{off}}[\text{Cdk2:CycE:Chr}] + k_{\text{dissoc}}[\text{Xic:Cdk2:CycE}]}{k_{\text{deg}} + k_{\text{wee}}[\text{Wee1}^*]} - k_{\text{wdeg}}[\text{Wee1}^*]$$

$$6. \frac{d}{dt} [\text{Cdk2:CycE:Chr}] = +k_{\text{on}}\phi [\text{Cdk2:CycE}] - k_{\text{off}}[\text{Cdk2:CycE:Chr}] \\ - k_{\text{cdeg}}[\text{Deg}][\text{Cdk2:CycE:Chr}] + k_{\text{xddeg}}[\text{Xic:Cdk2:CycE:Chr}] \\ - k_{\text{assoc}}[\text{Xic}][\text{Cdk2:CycE:Chr}] + k_{\text{dissoc}}[\text{Xic:Cdk2:CycE:Chr}]$$

7. 
$$\begin{aligned} \frac{d}{dt} [\text{PCdk2:CycE:Chr}] = & + k_{\text{on}} \phi [\text{PCdk2:CycE}] - k_{\text{off}}[\text{PCdk2:CycE:Chr}] \\ & - k_{\text{edeg}}[\text{Deg}][\text{PCdk2:CycE:Chr}] + k_{\text{xdeg}}[\text{Xic:PCdk2:CycE:Chr}] \\ & - k_{\text{assoc}}[\text{Xic}][\text{PCdk2:CycE:Chr}] + k_{\text{dissoc}}[\text{Xic:PCdk2:CycE:Chr}] \end{aligned}$$
8. 
$$\frac{d}{dt} [\text{Deg}^*] = k_{\text{dact}} \text{Heav}([\text{Cdk2:CycE:Chr}] - \theta)$$
9. 
$$\begin{aligned} \frac{d}{dt} [\text{Xic}] = & - k_{\text{assoc}} [\text{Xic}] ( [\text{Cdk2:CycE}] + [\text{PCdk2:CycE}] + [\text{Cdk2:CycE:Chr}] + [\text{PCdk2:CycE:Chr}] ) \\ & + k_{\text{dissoc}} ( [\text{Xic:Cdk2:CycE}] + [\text{Xic:PCdk2:CycE}] + [\text{Xic:Cdk2:CycE:Chr}] + [\text{Xic:PCdk2:CycE:Chr}] ) \end{aligned}$$
10. 
$$\begin{aligned} \frac{d}{dt} [\text{Xic:Cdk2:CycE}] = & + k_{\text{assoc}}[\text{Xic}][\text{Cdk2:CycE}] - k_{\text{dissoc}}[\text{Xic:Cdk2:CycE}] \\ & - k_{\text{on}} \phi [\text{Xic:Cdk2:CycE}] + k_{\text{off}}[\text{Xic:Cdk2:CycE:Chr}] \\ & - k_{\text{wee}}[\text{Wee1}^*][\text{Xic:Cdk2:CycE}] + k_{25\Lambda}[\text{Xic:PCdk2:CycE}] \end{aligned}$$
11. 
$$\begin{aligned} \frac{d}{dt} [\text{Xic:PCdk2:CycE}] = & + k_{\text{assoc}}[\text{Xic}][\text{PCdk2:CycE}] - k_{\text{dissoc}}[\text{Xic:PCdk2:CycE}] \\ & - k_{\text{on}} \phi [\text{Xic:PCdk2:CycE}] + k_{\text{off}}[\text{Xic:PCdk2:CycE:Chr}] \\ & + k_{\text{wee}}[\text{Wee1}^*][\text{Xic:Cdk2:CycE}] - k_{25\Lambda}[\text{Xic:PCdk2:CycE}] \end{aligned}$$
12. 
$$\begin{aligned} \frac{d}{dt} [\text{Xic:Cdk2:CycE:Chr}] = & + k_{\text{assoc}}[\text{Xic}][\text{Cdk2:CycE:Chr}] - k_{\text{dissoc}}[\text{Xic:Cdk2:CycE:Chr}] \\ & + k_{\text{on}} \phi [\text{Xic:Cdk2:CycE}] - k_{\text{off}}[\text{Xic:Cdk2:CycE:Chr}] \\ & - k_{\text{edeg}}[\text{Deg}][\text{Xic:Cdk2:CycE:Chr}] - k_{\text{xdeg}}[\text{Xic:Cdk2:CycE:Chr}] \end{aligned}$$

$$13. \frac{d}{dt} [\text{Xic:PCdk2:CycE:Chr}] = + k_{\text{assoc}}[\text{Xic}][\text{PCdk2:CycE:Chr}] - k_{\text{dissoc}}[\text{Xic:PCdk2:CycE:Chr}] \\ + k_{\text{on}} \phi [\text{Xic:PCdk2:CycE}] - k_{\text{off}}[\text{Xic:PCdk2:CycE:Chr}] \\ - k_{\text{edeg}}[\text{Deg}][\text{Xic:PCdk2:CycE:Chr}] - k_{\text{xdeg}}[\text{Xic:PCdk2:CycE:Chr}]$$

$$14. \frac{d}{dt} [\text{Xic:Chr}] = + k_{\text{edeg}}[\text{Deg}][\text{Xic:Cdk2:CycE:Chr}] + k_{\text{edeg}}[\text{Deg}][\text{Xic:PCdk2:CycE:Chr}] \\ - k_{\text{xdeg}}[\text{Xic:Chr}]$$

$$\phi = \frac{\varepsilon + [\text{Pool}]^n}{L^n + [\text{Pool}]^n}, \quad \text{Heav}(x) = \begin{cases} 0, & \text{if } x < \theta \\ 1, & \text{if } x \geq \theta \end{cases}$$

$$[\text{Pool}] = [\text{Cdk2:CycE:Chr}] + [\text{PCdk2:CycE:Chr}] + [\text{Xic:Cdk2:CycE:Chr}] + [\text{Xic:PCdk2:CycE:Chr}]$$

**Notes:**

- (i)  $\phi$  is a Hill function describing the cooperativity of CycE:Cdk2 binding to chromatin. It depends on  $[\text{Pool}]$ , the “total pool” of chromatin-bound cyclin E.
- (ii)  $\text{Heav}(x)$  is the Heaviside function, a common mathematical expression for a switch.  $\theta$  is the threshold Cdk2-activity for turning on the switch.
- (iii) Because cyclin E-dependent kinase activity seems to stabilize Wee1 mRNA, we assume that the rate of Wee1 synthesis increases hyperbolically with Cdk2-activity.
- (iv) The activation kinetics of Wee1 and Kin are assumed to be zero-order ultrasensitive switches (Goldbeter and Koshland, 1981).

**Table 2.** Basal parameter values for the differential equations in Table 1.

Rate constants ( min<sup>-1</sup> )

$$k_{wee} = 1.5, k_{25A} = 0.1, k_{on} = 0.02, k_{off} = 0.0001, k_{assoc} = 0.1, k_{dissoc} = 0.001, k_{wsyn} = 0.1, k_{wdeg} = 0.01, \\ k_{wact} = 0.75, k_{winact} = 1.5, k_{iact} = 0.15, k_{iinact} = 0.6, k_{edeg} = 0.017, k_{xdeg} = 0.01, k_{dact} = 0.023$$

Other constants ( dimensionless )

$$J_{wsyn} = 0.1, J_{wact} = 0.01, J_{winact} = 0.01, J_{iact} = 0.01, J_{iinact} = 0.01, \theta = 0.3, \varepsilon = 0.001, L = 0.4, m = 4, \\ n = 4$$

Initial concentrations (arbitrary units)

$$[Cdk2:CycE] = 0.08, [PCdk2:CycE] = 0.92, [Wee1^*] = 0.78, [Kin] = 0.61, [Wee1_{total}] = 8.06$$

**Table 3.** Differential equations used for bifurcation analysis in Figure 8. In the simplified model, cyclin E degradation and Xic1 binding are ignored. These equations are carried over from Table 1, with the understanding that  $H = [\text{cyclin E bound to chromatin}]$ , and  $E_{\text{tot}} = [\text{cyclin E in all forms}] = [\text{Cdk2:CycE}] + [\text{PCdk2:CycE}] + H$ .

$$1. \frac{d}{dt} [\text{Cdk2:CycE}] = -k_{\text{wee}}[\text{Wee1}^*][\text{Cdk2:CycE}] + k_{25A}(E_{\text{tot}} - H - [\text{Cdk2:CycE}])$$

$$3. \frac{d}{dt} [\text{Wee1}^*] = \frac{k_{\text{kin}}[\text{Kin}^*][\text{Wee1}] + k_{\text{on}}[\text{Cdk2:CycE}][\text{Wee1}]}{K_{\text{M}} + [\text{Wee1}]} - k_{\text{wdeg}}[\text{Wee1}^*]$$

$$4. \frac{d}{dt} [\text{Wee1}_{\text{total}}] = \frac{k_{\text{kin}}[\text{Kin}^*][\text{Wee1}] + k_{\text{on}}[\text{Cdk2:CycE}][\text{Wee1}]}{K_{\text{M}} + [\text{Wee1}]} - k_{\text{wdeg}}[\text{Wee1}_{\text{total}}]$$

$$5. \frac{d}{dt} [\text{Kin}^*] = \frac{k_{\text{on}}[\text{Cdk2:CycE}][\text{Kin}]}{K_{\text{M}} + [\text{Kin}]} - k_{\text{deg}}[\text{Kin}^*]$$

$$6. \frac{d}{dt} H = +k_{\text{on}} \frac{[\text{Cdk2:CycE}][\text{H}]}{K_{\text{M}} + [\text{H}]} (E_{\text{tot}} - H) - k_{\text{off}} H$$

## LEGENDS

Figure 1. **Positive and negative feedback loops are thought to underlie the cyclin E/Cdk2 developmental timer.** The hatched box represents a mechanism for removing cyclin E/Cdk2 from oscillatory subsystem (the negative feedback loops through Wee1 and, possibly, Cdc25). “Deg” represents the cyclin E degradation system, which we assume is activated by the “removed” form of cyclin E/Cdk2. Notice that the removal step is cooperative, i.e., the more cyclin E/Cdk2 is bound to the black box, the faster is the association reaction.

Figure 2. **Experimental basis for a mathematical model of the cyclin E/Cdk2 developmental timer.**

**(A) Inhibition of cyclin E/Cdk2 in early *Xenopus* embryos results in a loss of Wee1 protein.**

At the one-cell stage, embryos were microinjected with 5 ng Xic1-C or  $\Delta$ 34-Xic1 (Su et al., 1995). Embryos were collected at the indicated times post-fertilization (pf) and analyzed by immunoblotting for steady state levels of Wee1 (Murakami and Woude, 1998) and cyclin E protein (Rempel et al., 1995; Audic et al., 2001). The migration of molecular weight standards in kDa is indicated. The detection of a nonspecific protein on the Wee1 immunoblot is consistent with reports of Murakami and Vande Woude (1998).

**(B) Wee1 mRNA is degraded during gastrulation.** Embryos were collected at the indicated times, and RNA was isolated for Northern analysis of Wee1 mRNA. The migration of molecular weight standards (in kb) is shown on the left.

**(C) Wee1 mRNA level is decreased in embryos injected with  $\Delta$ 34Xic1.** Embryos were injected at the one cell stage with 5 ng Xic1-C or  $\Delta$ 34Xic1, collected at the time indicated, and processed for Northern analysis.

**(D) Expression of XChk1 delays the degradation of cyclin E in *Xenopus* embryos.** Embryos were injected with buffer or 15 ng mRNA encoding XChk1. Embryos were collected at the time points indicated and analyzed for cyclin E protein content. The migration of molecular weight standards in kDa is indicated. In other experiments, control embryos were injected with mRNA encoding luciferase, and the delay in cyclin E degradation was shown to be a specific effect of XChk1 (data not shown).

**(E) Cyclin E degradation in  $\Delta 34$ -Xic1-treated embryos does not depend upon transient cyclin E/Cdk2 activity.** Embryos microinjected with 5 ng Xic1-C or  $\Delta 34$ -Xic1 were collected at the times indicated, cyclin E/Cdk2 was immunoprecipitated, and immunoprecipitates were analyzed for histone H1 kinase activity. cpm = counts per minute  $^{32}\text{P}$  incorporated into histone H1. Values plotted represents raw cpm – background cpm on gel (1476 cpm). Cyclin B/Cdk1 immunoprecipitates indicated no inhibition of Cdk1 by  $\Delta 34$ -Xic1 (not shown).

**(F) Zygotic transcription is not required for degradation of cyclin E.** Embryos were injected at the one-cell stage with  $\text{H}_2\text{O}$ , 50 ng  $\alpha$ -amanitin, 5 ng  $\Delta 34$ -Xic1, or 50 ng  $\alpha$ -amanitin + 5 ng  $\Delta 34$ -Xic1. Embryos were collected at the indicated times pf and analyzed by immunoblotting for steady state level of cyclin E protein as described in Figure 2A. The migration of molecular weight standards in kDa is indicated.

Figure 3. **A detailed molecular mechanism of the cyclin E/Cdk2 developmental timer is necessary for the construction of a mathematical model.** An extension of Figure 1. We introduce a kinase (“Kin”) between cyclin E/Cdk1, to introduce a time lag in the negative feedback loop. We propose that the removal reaction corresponds to chromatin association. “Pool” corresponds to the sum of all forms of cyclin E/Cdk2 bound to chromatin. We also allow Xic1 to bind to and inhibit all forms of cyclin E/Cdk2. Xic1 associated to chromatin, in any form, is degraded with a constant half-life.

Figure 4. **The mathematical model is consistent with the observed dynamics of cyclin E/Cdk2 in normal frog embryos.**

**(A) Numerical simulation of the equations in Table 1.** Given the parameter values in Table 2, the control system exhibits rapid oscillations in Cdk2 activity (period = 15 min) followed by abrupt degradation of cyclin E about 6 h pf.

**(B) The four forms of cyclin E/Cdk2.**

**(C) Total cyclin E, activity of the cyclin E-degradation machinery, Wee1 activity.** We plot  $[\text{Wee1}_{\text{total}}]$  relative to its initial value, 8.06.

Figure 5. **The cyclin E/Cdk2 timer runs independently of protein synthesis.** The only step in the model (Figure 3) that depends on protein synthesis is Wee1 production. With  $k_{\text{wsyn}} = 0$ ,  $[\text{Wee1}_{\text{total}}]$  drops to zero over the course of 3-4 h, with little effect on the dynamics of cyclin E/Cdk2.

**(A) The four forms of cyclin E/Cdk2.**

**(B) Total cyclin E, activity of the cyclin E-degradation machinery (Deg), Wee1 activity.** We plot  $[\text{Wee1}_{\text{total}}]$  relative to its initial value, 8.06.

Figure 6. **Exogenous  $\Delta 34\text{Xic1}$  is degraded prior to the destruction of cyclin E.**

**(A, B, C) Simulation.**  $[\text{Xic1}]_{\text{initial}} = 3$ . In panel (C) we plot  $[\text{Xic1}_{\text{total}}] / [\text{Xic1}]_{\text{initial}}$ .

**(D) Experiments.** Embryos were injected with 5 ng Xic1-C or  $\Delta 34\text{Xic1}$ , collected at the indicated time points and analyzed by immunoblotting for the content of cyclin E and Xic1. The migration of molecular weight standards is indicated on the left. Note: Xic1-C reacts weakly with the Xic1 antibody but is detected and is degraded at the same time as  $\Delta 34\text{Xic1}$ .

Figure 7. **Timing of cyclin E degradation is dependent upon the dose of  $\Delta 34\text{Xic1}$ .**

**(A) Simulation.** Total cyclin E for different amounts of Xic1 added to the system. As in Figure 6, Xic1 is degraded prior to cyclin E. The times when one-half the original Xic1 has been degraded are 6.4, 7.6 and 10 h for  $[\text{Xic1}]_{\text{initial}} = 1.5, 3, 6$ , respectively. The times when one-half of the original cyclin E has been degraded are 7, 7.7, 10.3, 15.4 h, for  $[\text{Xic1}]_{\text{initial}} = 0, 1.5, 3, 6$ , respectively.

**(B) Experiment.** Embryos were injected with 0, 2.5, 5 or 10 ng  $\Delta 34\text{Xic1}$ , collected at the time points indicated, and analyzed by immunoblotting for content of cyclin E. Arrows denote the position of cyclin E.

Figure 8. **Bifurcation analysis of the cyclin E/Cdk2 developmental timer.**

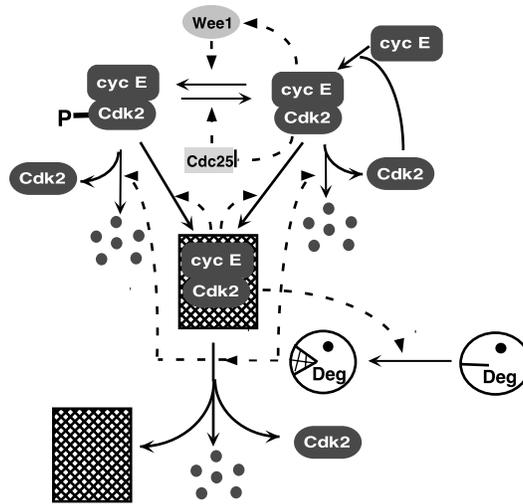
**(A) Simulation of the equations in Table 3.** Chromatin-bound cyclin E/Cdk2 (dashed line;  $H = [\text{Cdk2:CycE:Chr}] + [\text{P Cdk2:CycE:Chr}]$ ) and active, unbound cyclin E/Cdk2 (solid line) as functions of time.

**(B) Dependence of negative feedback oscillations on  $H$ .** The ordinate is [Cdk2:CycE], except for the open diamonds, which indicate the period of oscillation (scale on right). Solid line: stable steady state; dashed line: unstable steady state; black circles: maximum and minimum values of [Cdk2:CycE] during the course of an oscillation at fixed  $H$ . The control system undergoes a Hopf bifurcation at  $H = 0.41$ .

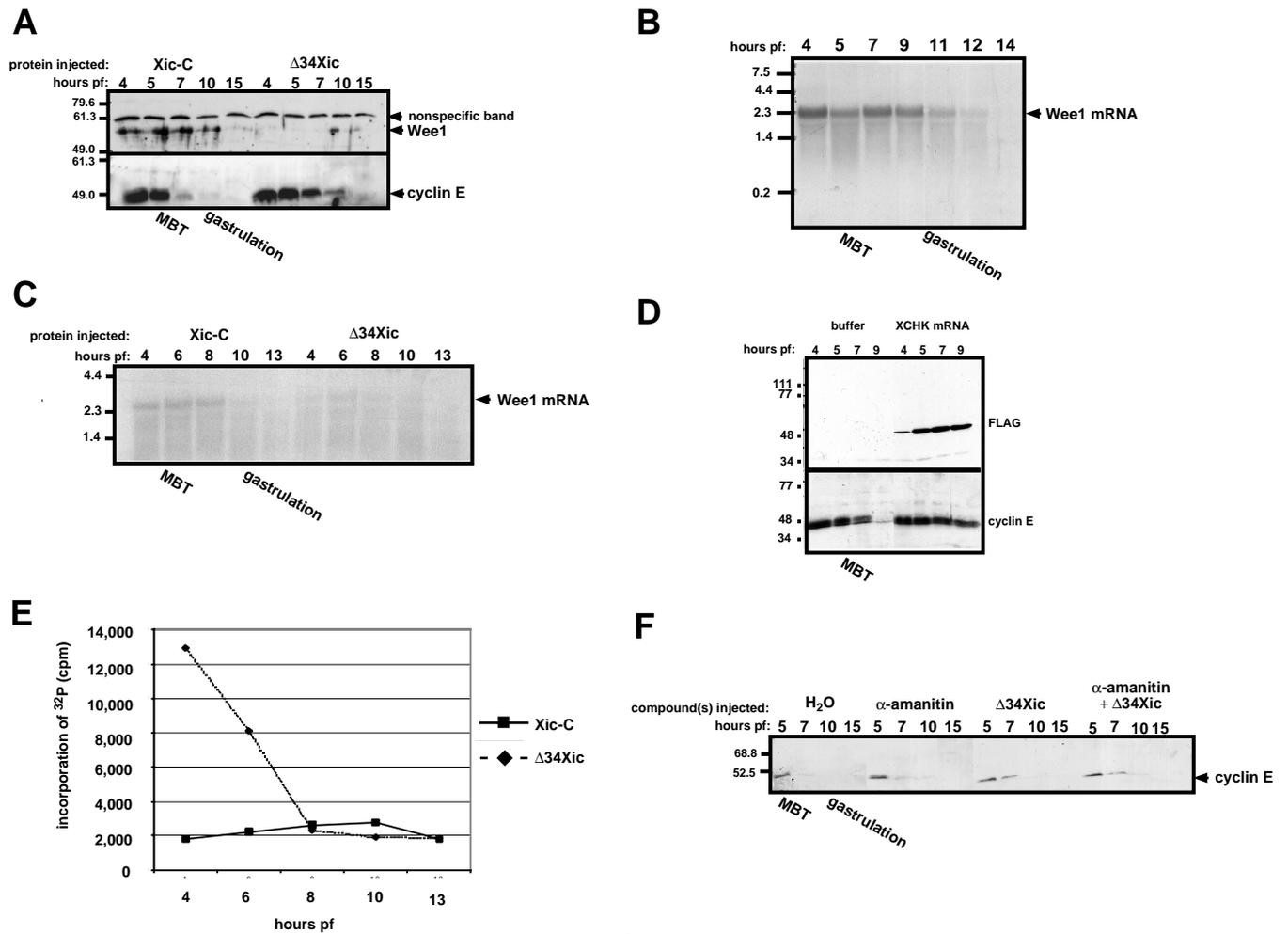
**(C) Bistability in the positive feedback module.** We plot  $dH/dt$  as a function of  $H$  for two values of  $k_{\text{off}}$ . The system is monostable (one steady state, where  $dH/dt = 0$ ) for  $k_{\text{off}} = 0.0001$  and bistable (three steady states) for  $k_{\text{off}} = 0.01$ .

**(D) Oscillations in the negative feedback module.** Symbols as in panel (B). The steady state is unstable between the two points of Hopf bifurcation at  $\tau = 5 \times 10^{-4}$  and 8 (see text).

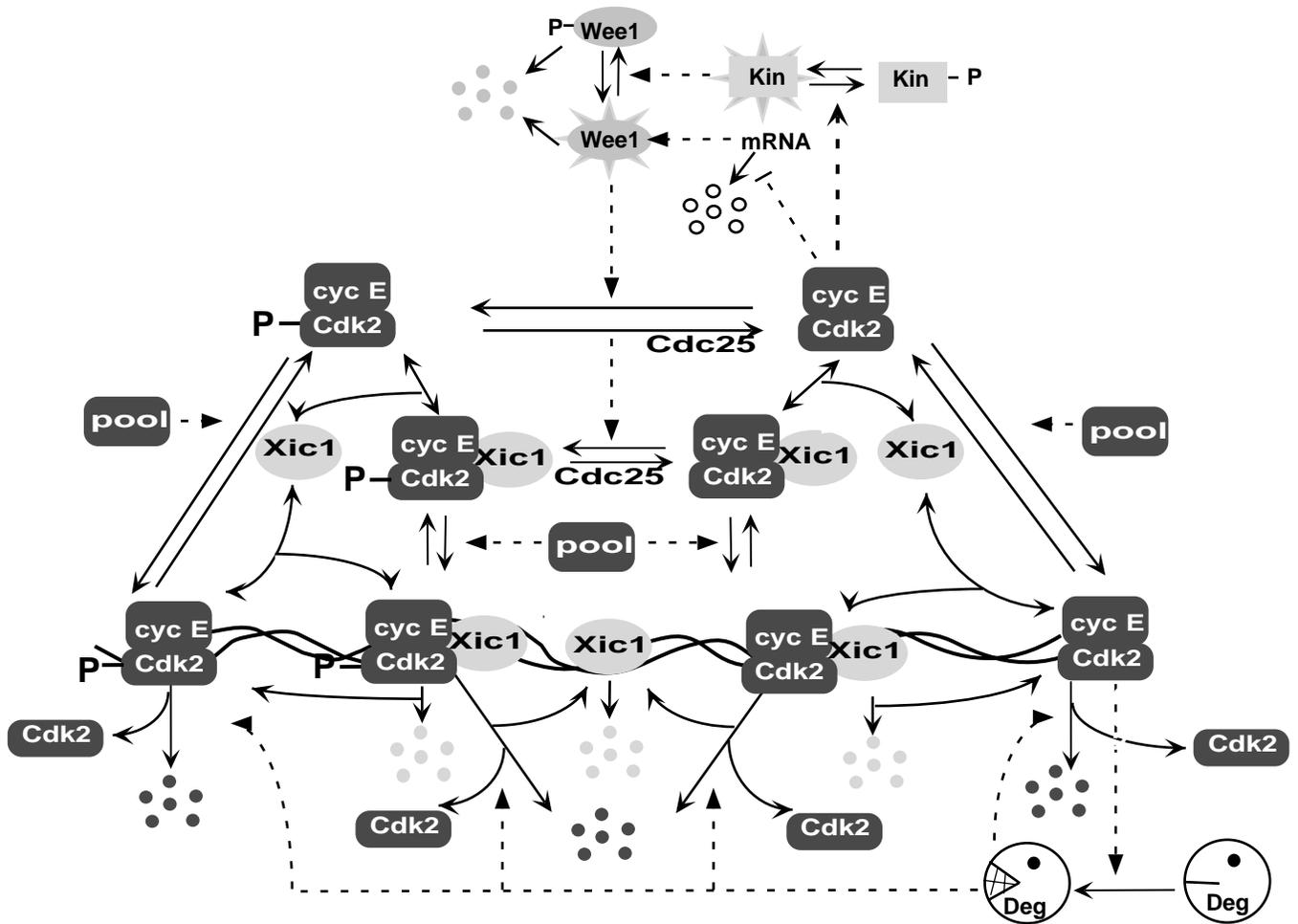
Ciliberto et al.  
Figure 1



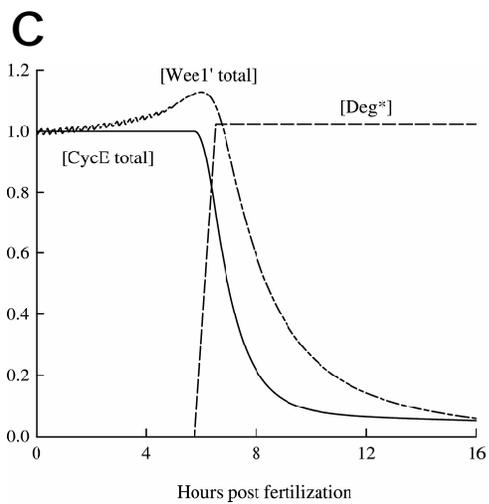
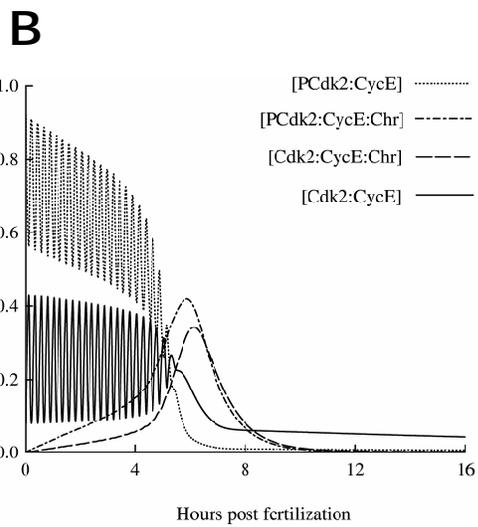
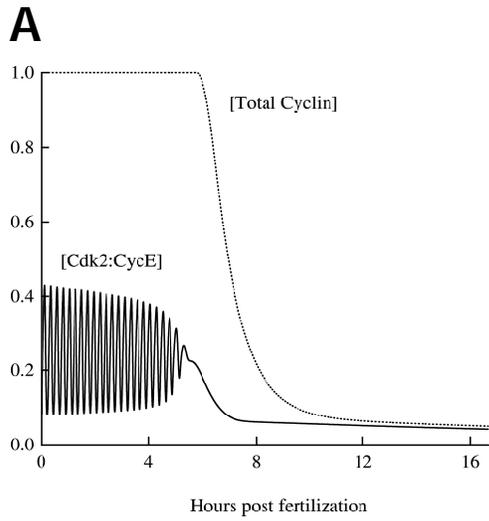
Ciliberto et al.  
Figure 2



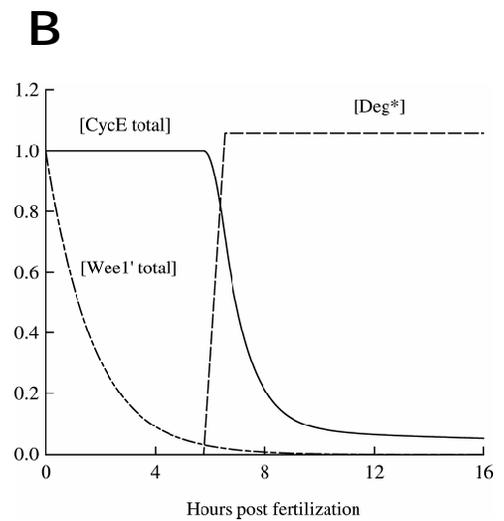
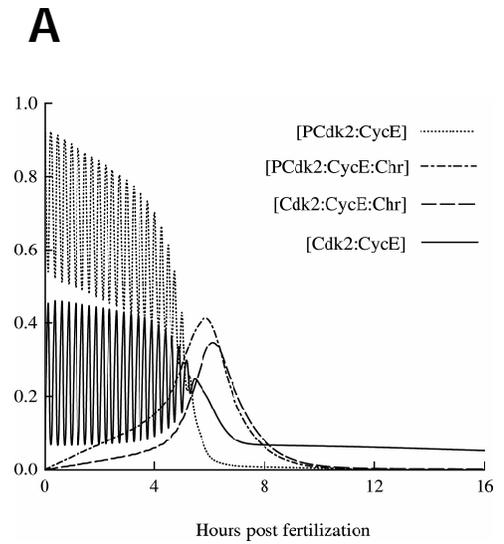
Ciliberto et al.  
Figure 3



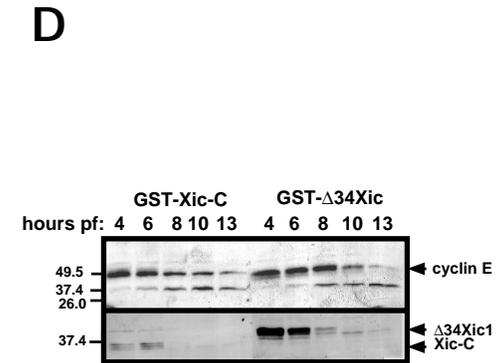
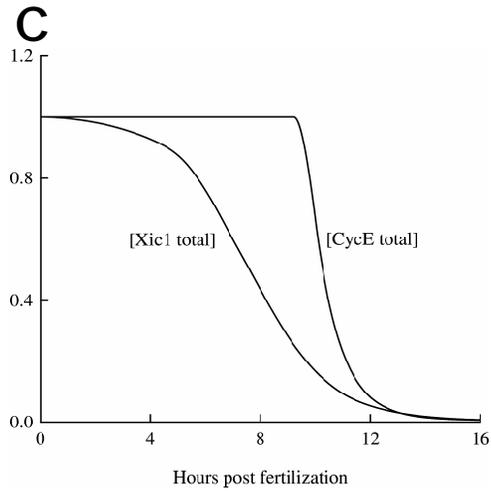
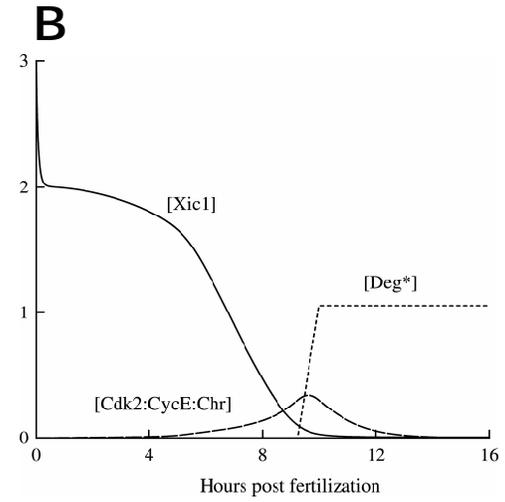
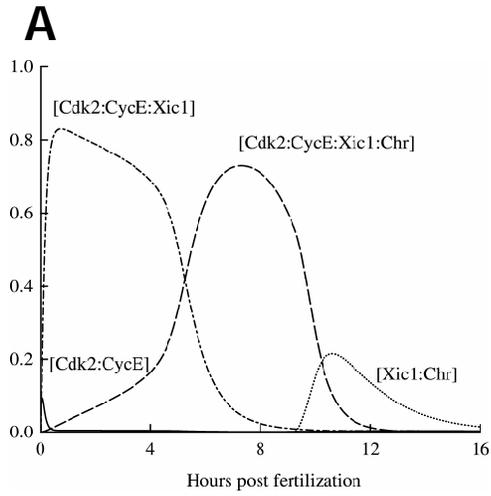
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Figure 4



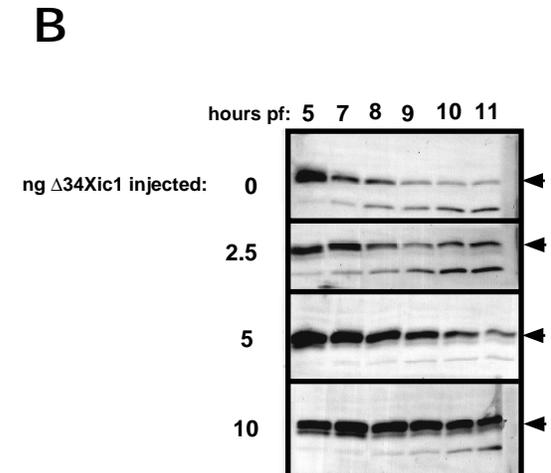
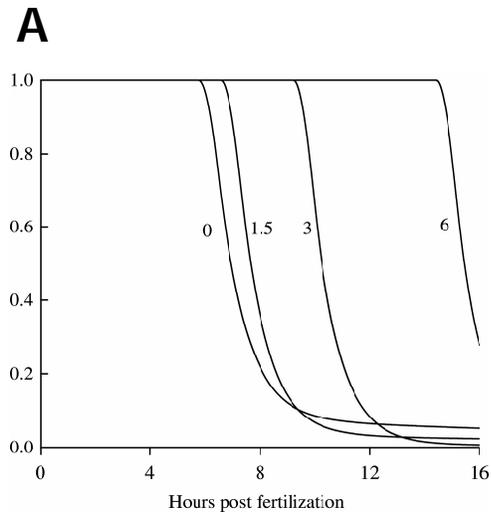
Ciliberto et al.  
Figure 5



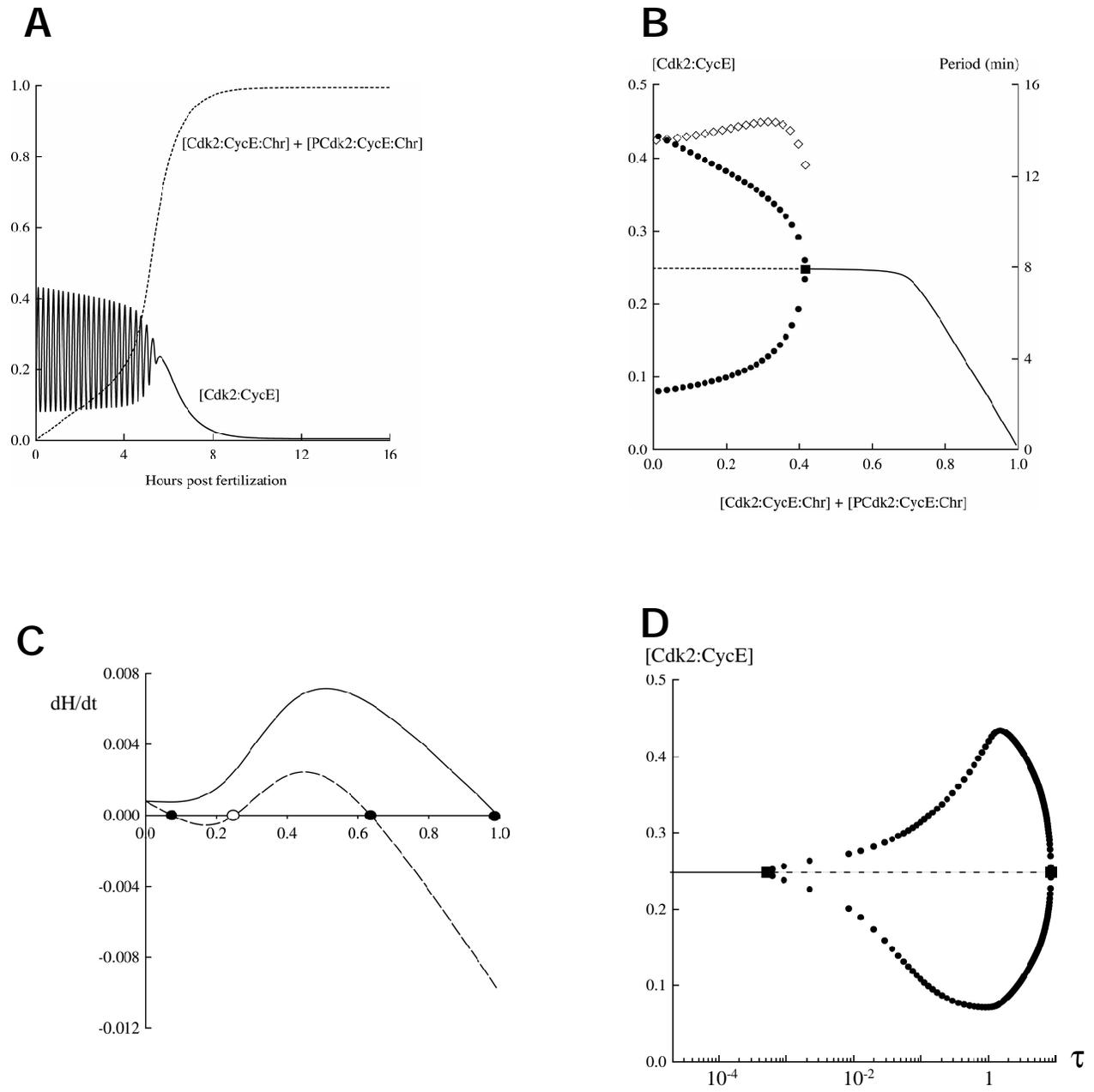
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Figure 6



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Figure 7



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Figure 8



## Vita

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Matthew J. Petrus  
309 Apartment Heights Dr. Apt. U2  
Blacksburg, VA 24060  
email: [mapetrus@vt.edu](mailto:mapetrus@vt.edu)  
Work Phone: (540) 231-3576  
Home Phone: (540) 961-0592

**Position:** Research Assistant

**Education:** B.S. from Radford University in 1997, Biology  
M.S. from Virginia Polytechnic Institute and State University in 2002,  
Molecular Cell Biology

**Professional Experience and Awards:**

1996-1997 Research Assistant Radford University  
1997-1998 Intramural Research Training Award (pre-IRTA)  
National Cancer Institute, National Institutes of Health  
1998-1999 Cancer Research Training Award (CRTA)  
National Cancer Institute, National Institutes of Health  
5) Graduate Teaching/Research Assistant, Virginia Polytechnic Institute and State University  
2002-Current Research Assistant, Virginia Polytechnic Institute and State University

**Research Projects:**

"Genetic Analysis of *Drosophila mauritiana*"

Principal Investigator: Richard M. Kliman, Ph.D.

Period: 1996-1997

Worked in a team to sequence the alcohol dehydrogenase gene in *Drosophila mauritiana*.  
Actively participated in DNA purification, DNA sequencing, and animal husbandry.

"New Immune Therapy Approaches for the treatment of Chronic Lymphocytic Leukemia"

Principal Investigator: Daniel H. Fowler, M.D.

Period: 1997-1999

Worked independently on a project evaluating the role of fludarabine-based immunosuppressive regimens on the prevention of murine bone marrow graft rejection. Clinical trials are currently being developed to further investigate performing allogeneic bone marrow transplants in the absence of total body irradiation.  
Worked in a group investigating the role of type I and II cytokines in the fas/fasL cytolytic pathway.

"Mechanisms of cell cycle remodeling at the MBT during development of *Xenopus laevis* embryos"

Principal investigator: Jill C. Sible

Period: 1999-2002

We have worked collaboratively in a group investigating the timing of the midblastula transition (MBT). The cell cycle is remodeled at the MBT as the division time is extended and checkpoint controls are acquired. After the MBT, in response to damaged and unreplicated DNA, the protein kinase XChk1 becomes activated and prevents progression into mitosis. We hypothesize that XChk1 may play a role in altering the timing of the MBT in *Xenopus* embryos.

### **Recent Presentations at Meetings**

Williams JF, MJ Petrus, RE Gress, DH Fowler, C. Mackall. Regulation of the Fas cytolytic pathway in CLL cells: differential role of type I vs type II cytokines. Annual Meeting of the American Society of Hematology, Miami Beach, FL. December 4-8 1998

Husebekk A, VS Fellowes, EJ Read, JF Williams, MJ Petrus, RE Gress, DH Fowler, C Mackall. Selection and expansion of T cells that mediate Fas-based lysis of autologous CLL cells. Annual Meeting of the American Society of Hematology, Miami Beach, FL December 4-8 1998

Petrus MJ, JF Williams, MA Eckhaus, RE Gress, DH Fowler. A non-myeloablative regimen of fludarabine and cyclophosphamide prevents murine marrow graft rejection independent of GVHD. Annual Meeting of the American Society of Blood and Marrow Transplantation. Keystone, CO March 4, 1999

Sible, JC, NC Kappas, M Petrus, A Carter. The role of XChk1 in cell cycle remodeling during development of *Xenopus laevis*. Houston, TX November 2000.

Petrus, MJ, NC Kappas, and JC Sible. *Xenopus* Chk1 (XCHK1) Alters Cell Cycle Remodeling at the MBT. American Society of Cell Biology, San Francisco, CA December 9-13 2000.

Petrus, MJ, NC Kappas, and JC Sible. *Xenopus* Chk1 (XCHK1) Alters Cell Cycle Remodeling at the MBT. Virginia Tech Graduate Research Symposium. March 2001.

Petrus, MJ, NC Kappas, and JC Sible. *Xenopus* Chk1 (XChk1) alters the cell cycle remodeling at the MBT. South East Regional Developmental Biology Meeting. May 2001.

Petrus, MJ, BB Halsey and JC Sible. Altered Chk1 Expression Stabilizes Wee1 During *Xenopus* Development. American Society of Cell Biology, Washington D.C. December 8-12 2001.

## **Publications**

Williams JF, Petrus MJ, Wright JA, Husebekk A, Fellowes V, Read EJ, Gress RE, Fowler DH (1999) Fas-mediated lysis of chronic lymphocytic leukaemia cells: role of type I versus type II cytokines and autologous fasL-expressing T cells. *British Journal of Haematology*. 107(1):99-105.

Petrus MJ, Williams JF, Eckhaus MA, Gress RE, Fowler DH (2000) An immunoblative regimen of fludarabine and cyclophosphamide prevents fully MHC-mismatched murine marrow graft rejection independent of GVHD. *Biol. Blood Marrow Transplant*. 6(2A):182-9.

## **Submissions for Publication**

Ciliberto A., Petrus MJ, Tyson JJ, and Sible JC (2002) A kinetic model of the cyclin E/Cdk2 developmental timer in *Xenopus laevis* embryos.