The Role of Chk2 and Wee1 Protein Kinases during the Early Embryonic Development of *Xenopus laevis*

Brian Noel Wroble

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Jill C. Sible, Chair
William R. Huckle
F.M. Anne McNabb
Charles L. Rutherford
Richard A. Walker

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(ABSTRACT)

In somatic cells, when DNA is damaged or incompletely replicated, checkpoint pathways arrest the cell cycle prior to M or S phases by inhibiting cyclin-dependent kinases (Cdks). In *Xenopus laevis*, embryonic cellular divisions (2-12) consist of rapid cleavage cycles in which gap phases, checkpoint engagement, and apoptosis are absent. Upon the completion of the 12th cellular division, the midblastula transition (MBT) begins and the cell cycle lengthens, acquiring gap phases. In addition, cell cycle checkpoint pathways and an apoptotic program become functional. The studies described here were performed to better understand the roles of two protein kinases, Chk2/Cds1 and Wee1, during checkpoint signaling in the developing embryo.

The DNA damage checkpoint is mediated by the Chk2/Cds1 kinase. Conflicting evidence implicates Chk2 as an inhibitor or promoter of apoptosis. To better understand the developmental function of Chk2 and its role in apoptosis, we expressed wild-type (wt) and dominant-negative (DN) Chk2 in *Xenopus* embryos. Wt-Chk2 created a pre-MBT checkpoint by promoting degradation of Cdc25A and phosphorylation of Cdks. Embryos expressing DN-Chk2 developed normally until gastrulation and then underwent apoptosis. Conversely, low doses of wt-Chk2 blocked radiation-induced apoptosis. These data indicate that Chk2 inhibits apoptosis in the early embryo. Therefore, Chk2 operates as a switch between cell cycle arrest and apoptosis in response to genomic assaults.

In *Xenopus laevis*, Wee1 kinase phosphorylates and inhibits Cdks. To determine the role of Wee1 in cell cycle checkpoint signaling and remodeling at the MBT, exogenous Wee1 was expressed in one-cell stage embryos. Modest overexpression of Wee1 created a pre-MBT cell cycle checkpoint, similar to Chk2, characterized by cell cycle delay and phosphorylation of Cdks. Furthermore, overexpression of Wee1 disrupted remodeling events that normally occur at the MBT, including degradation of Cdc25A, cyclin E, and Wee1. Interestingly, overexpression of Wee1 also resulted in post-MBT apoptosis. Taken together, these data suggest the importance of Wee1 as not only a Cdk inhibitory kinase, but also potentially as a promoter of apoptosis during early development of *Xenopus laevis*. The studies described here provide evidence that Chk2 and Wee1 have both similar and distinct roles in the developing embryo.

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List of Abbreviations

APC- anaphase-promoting complex
CAK- Cdk activating kinase
Cdks- cyclin-dependent kinases
CKI- cyclin-dependent kinase inhibitor
DN- dominant-negative
HCG- human chorionic gonadotropin
IP- immunoprecipitation
IR- ionizing radiation
MBT- midblastula transition
MPF- maturation promoting factor
ORC- origin replication complex
PF- post-fertilization
PMSG- pregnant mare serum gonadotropin
wt- wild-type

Chapter 1: Literature Review

Introduction

Oncogenic transformation of normal somatic cells is the direct result of successive genetic mutations. The emergence of cancerous cells, leading to unregulated proliferation, is marked by a number of acquired capabilities including an insensitivity to growth inhibitory signals, self-sufficiency in growth signals, limitless replicative potential, and the ability to evade apoptosis (Hanahan and Weinberg, 2000). Furthermore, the complexity of cancer cells along with their decreased sensitivity to chemotherapeutic agents provides scientists with the complicated task of deciphering these aberrations. Fortunately, during normal cell growth, cell cycle checkpoints and an apoptotic program exist as guards against abnormal growth and cellular proliferation, allowing the study of the molecular differences between cancerous and noncancerous cells.

A major area of cancer research involves the study of cell cycle controls including checkpoint activation, DNA repair, and apoptosis. In order to examine the molecular differences of these controls in cancer cells, it is pertinent to understand the fundamentals of the normal cell cycle and its regulation. In its simplest definition, the cell cycle is the process by which a normal cell grows, replicates its DNA, and then divides into two separate, genetically identical daughter cells. However, the process is obviously much more complicated. The normal somatic cell cycle consists of 5 phases including growth phase 1 (G1), DNA synthesis (S), growth phase 2 (G2), mitosis (M), and an unproliferative, quiescent phase (G0) (Fig. 1-1). Normal cell cycle progression is driven by the coordinated activity of two groups of proteins, the cyclin-dependent kinases (Cdks) and their associated regulatory cyclin partners.

Although cyclin/Cdk complexes directly determine in which phase a cell exists, many other important controls contribute to its regulation. Specifically, networks of signaling cascades consisting of growth factors, checkpoint kinases, and other activating and inhibitory proteins all contribute to the ultimate regulation of the cell and whether or not the cell cycle will progress into the next phase. When certain constituents of these

networks malfunction due to genetic mutations or epigenetic effects, and these problems are unable to be corrected through repair mechanisms or apoptosis, oncogenic transformation is likely.

The early embryonic development of our model system, *Xenopus laevis* (South African clawed frog), provides a unique context in which to study cell cycle regulation due to extensive cell cycle remodeling at the midblastula transition (MBT). The goal of the following study was to further elucidate the roles of two protein kinases, Chk2 and Wee1, in cell cycle checkpoint regulation and apoptosis during the early development of *Xenopus laevis*.

1.1 Cell Cycle Regulation

The progression and regulation of the cell cycle is controlled directly by a profile of cyclin-dependent kinases (Cdks) and their associated cyclin regulatory subunits. In proliferating cells, the normal somatic cell cycle consists of four phases, growth phase 1 (G1), DNA synthesis (S), growth phase 2 (G2), and mitosis (M). Non-proliferating cells exist in a fifth quiescent phase, G0 (Fig. 1-1). For a cell to progress through the proliferative cell cycle, proper growth factors or mitogens must initially be present.

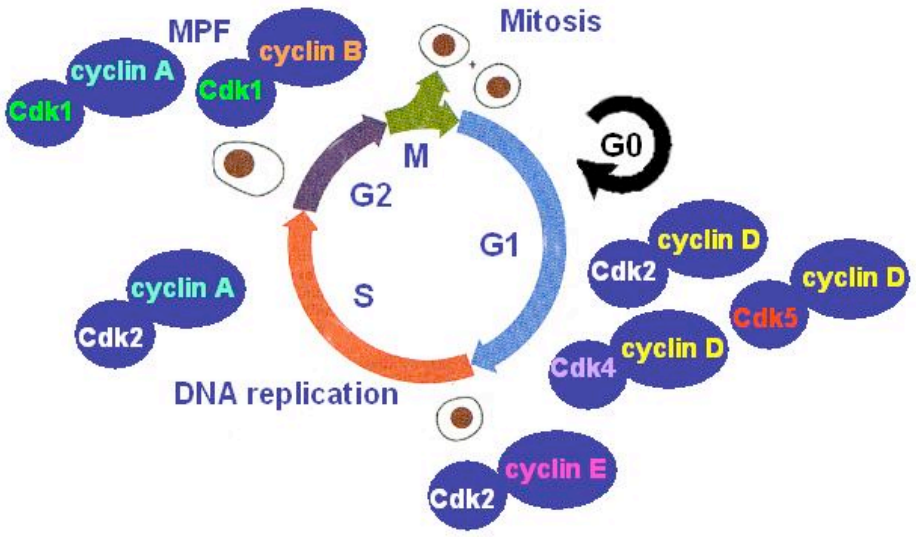

Figure 1-1. The Normal Somatic Cell Cycle. The normal somatic cell cycle consists of 5 phases: G1, S, G2, M, and G0. Specific cyclin/Cdk complexes regulate progression through each phase of the cell cycle.

G1 and S Phases

Upon the binding of growth factors to their receptors, the membrane bound GTPase Ras, is activated (Lavoie *et al.*, 1996; Winston *et al.*, 1996; Aktas *et al.*, 1997; Campbell *et al.*, 1998; McCormick, 1999). GTP-bound Ras actively signals the mitogen-activated protein kinase (MAPK) pathway, leading to the phosphorylation and activation of various transcription factors which induce multiple genes (Hill and Treisman, 1999). One of the important genes potently induced by Ras signaling is cyclin D (Filmus *et al.*, 1994; Liu *et al.*, 1995; Aktas *et al.*, 1997). A second pathway that contributes to cyclin D accumulation is the PI3K/Akt pathway (Gille and Downward, 1999). As cyclin D protein accumulates, it associates with Cdk 4 and 6 (Matsushime *et al.*, 1994), essentially initiating the beginning of the cell cycle in G1.

Cyclin D-associated Cdks are responsible for the progression of the cell cycle from G1 to S phase. Specifically, a cell must pass the restriction point in late-G1 to ensure the progression to the next phase. The restriction point is the critical point at which a cell commits to completing the rest of the cell cycle. Furthermore, once this commitment is made, progression through the rest of the cell cycle is growth factor-independent (Pardee, 1974). In order for the cell to pass the restriction point, Cyclin D-associated Cdks must phosphorylate the Retinoblastoma tumor suppressor protein, Rb, which when hypophosphorylated, binds the E2F family of cell cycle transcription factors necessary for the transcription of genes essential for the G1/S transition (Harbour and Dean, 2000). Specifically, when Rb is hyperphosphorylated, E2F transcription factors are liberated from Rb. This liberation enables the E2F-mediated activation of important target genes necessary for S phase entry and progression, such as cyclin E and cyclin A, respectively (DeGregori *et al.*, 1995). Once the E2F target genes are expressed, growth factors are no longer required for S phase entry or progression throughout the rest of the cell cycle.

Another important pathway that has been shown to be important for the G1/S phase transition involves the transcription factor Myc. Mitogen-induced Myc induces transcription of downstream targets such as cyclins D1/2, cyclin E, and Cdc25A in a parallel Rb/E2F-independent pathway (Santoni-Rugiu *et al.*, 2000). The induction of cyclin E and Cdc25A serves as a convergence point for these two parallel pathways, ultimately regulating cyclin E/Cdk2 activity and the G1/S transition. Furthermore, Myc has also been implicated specifically in cell growth and E2F-dependent transcription (Bouchard *et al.*, 1998; Elend and Eilers, 1999; Beier *et al.*, 2000).

The expression of cyclin E and its association with Cdk2 is crucial for S phase entry. Specifically, cyclin E/Cdk2, through a positive feedback loop, facilitates its own activation through the phosphorylation and subsequent inhibition of Rb, liberating E2F transcription factors (Bartek and Lukas, 2001b). In addition to the phosphorylation of Rb, cyclin E/Cdk2 targets its own cyclin-dependent kinase inhibitor (CKI), p27, for ubiquitination and subsequent proteasomal degradation (Weinberg, 1995; Sherr and Roberts, 1999; Nakayama *et al.*, 2001). Furthermore, cyclin E/Cdk2 activity stimulates DNA synthesis by facilitating origin firing through the Cdc45-dependent loading of DNA

polymerase and promoting histone synthesis (Bartek and Lukas, 2001a). Cyclin E/Cdk2 activity also triggers the induction of other genes necessary for DNA synthesis, including cyclin A (Zhang *et al.*, 2000). Specifically, for DNA replication to occur, cyclin A associated with Cdk2 must accumulate to levels which are essential for origin firing and subsequent DNA replication.

Prior to origin firing, pre-replication complexes (pre-RC's) must be assembled in a process known as replication origin licensing. This occurs through the stepwise assembly of the origin replication complex (ORC), Cdc6/7, Cdt1, and the Mcm 2-7 complex (Maiorano *et al.*, 2000; Nishitani *et al.*, 2000; Takisawa *et al.*, 2000). The anaphase-promoting complex (APC), an E3 ubiquitin ligase, is also necessary for proper replication origin licensing. Prior to the restriction point, during mid-G1, the inhibition of Cdk activity is essential for replication origin licensing (Diffley, 2001). Cdk activity is inhibited by the APC through the ubiquitination of G1 cyclins necessary for DNA synthesis and geminin, an inhibitor of Cdt1 (McGarry and Kirschner, 1998; Wohlschlegel *et al.*, 2000). Once replication origin licensing is complete during late G1, triggered by cyclin A accumulation resulting in the inhibition of pre-RC molecules and the inactivation of the APC by active Cdks, origin firing and subsequent DNA synthesis can occur. At this point, the reassembly of pre-RCs is prevented, ensuring that each chromosome is replicated just once during the passage through the cell cycle (Diffley, 2001; Coverley *et al.*, 2002)

G2 and M Phases

During S phase, mitotic cyclins are synthesized and associated with Cdk1 (Cdc2 in *Xenopus*), but activity is inhibited through phosphorylation at inhibitory sites until DNA replication is complete. The completion of DNA replication marks the beginning of G2 phase. G2 is characterized by the preparation of the cell for mitosis, which is largely dependent upon the accumulation of cyclin B and its subsequent association with Cdc2. Cdc2 and its associated regulatory subunit, cyclin B, form a heterodimer known as maturation promoting factor (MPF) (Newport and Kirschner, 1984; Dunphy *et al.*, 1988; Gautier *et al.*, 1990), originally identified in *Xenopus* oocytes (Newport and Kirschner, 1984). As cyclin B associates with Cdc2 and MPF accumulates to threshold

concentrations necessary for progression into mitosis, it becomes phosphorylated on three sites. Cdk activating kinase (CAK), a heterotrimer consisting of Cdk7, cyclin H, and MAT1, activates MPF through the phosphorylation of Cdc2 on Thr-161 (Solomon *et al.*, 1992; Morgan, 1995). However, inhibitory phosphorylations on Thr-14 and Tyr-15 of Cdc2, by Wee1 and Myt1/Wee1, respectively, render MPF inactive until the beginning of mitosis due to the inhibition of ATP binding (Parker *et al.*, 1992b; Mueller *et al.*, 1995b). When the cell is ready to undergo mitosis, Cdc25C phosphatase removes the inhibitory phosphates on Thr-14 and Tyr-15, rendering MPF phosphorylated only on Thr-161 and active (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991). MPF also phosphorylates Cdc25C, resulting in further activation and completion of a positive feedback loop (Hoffman *et al.*, 1993; Izumi and Maller, 1993). Additionally, MPF phosphorylates and inhibits Wee1, further contributing to MPF activation (Mueller *et al.*, 1995a).

Regulation of MPF at various levels ensures the proper timing of events that are necessary for mitosis. Once MPF is activated, it is able to phosphorylate downstream targets essential for mitosis. Specifically, MPF phosphorylates nuclear lamins which leads to nuclear-envelope breakdown (Miake-Lye *et al.*, 1983; Heald and McKeon, 1990). MPF activity is also responsible for chromosome condensation through the phosphorylation of condensin subunits (Kimura *et al.*, 1998). Furthermore, MPF phosphorylates microtubule-associated proteins, promoting assembly of the mitotic spindle assembly (Tombes *et al.*, 1991; Verde *et al.*, 1992).

Exit from mitosis requires sister chromatid separation, spindle disassembly, and lastly, cytokinesis. A key mitotic regulator, the anaphase promoting complex (APC), is an E3 ligase that polyubiquitinates anaphase inhibitors and cyclin B, leading to the onset of anaphase and the later events of mitosis, respectively. Specifically, the APC polyubiquitinates securin, the protein largely responsible for sister chromatid binding. Prior to anaphase, securin binds and inhibits the ubiquitous protease, separase. At the end of metaphase, the APC is directed by Cdc20 to polyubiquitinate securin, releasing separase. Once securin is degraded, separase is free to cleave the cohesin complex, which physically holds together sister chromatids (Lodish *et al.*, 2004). Prior to anaphase, MPF phosphorylates the APC, eventually targeting cyclin B for polyubiquitination, leading to its degradation. This negative feedback loop, resulting in

MPF inactivation, ensures chromosome decondensation, nuclear reformation, and cytokinesis, all of which are essential for mitotic exit (Peters, 2002). Following cytokinesis, daughter cells will enter G1 and continue to proliferate as long as mitogens are present. Alternatively, if mitogens are absent, progression into the quiescent, G0 phase will result.

1.2 Cell Cycle Checkpoints

Progression through the cell cycle is monitored at several checkpoints that function to arrest the cell cycle when genomic integrity is threatened. Mutations incapacitating checkpoint pathways resulting in defective cell cycle control are common in cancers (Bartek and Lukas, 2001a). Although cell cycle checkpoints exist during all phases of the cell cycle including S and M phases, the current study focuses on those pathways governing the G1/S and G2/M transitions (Fig. 1-2).

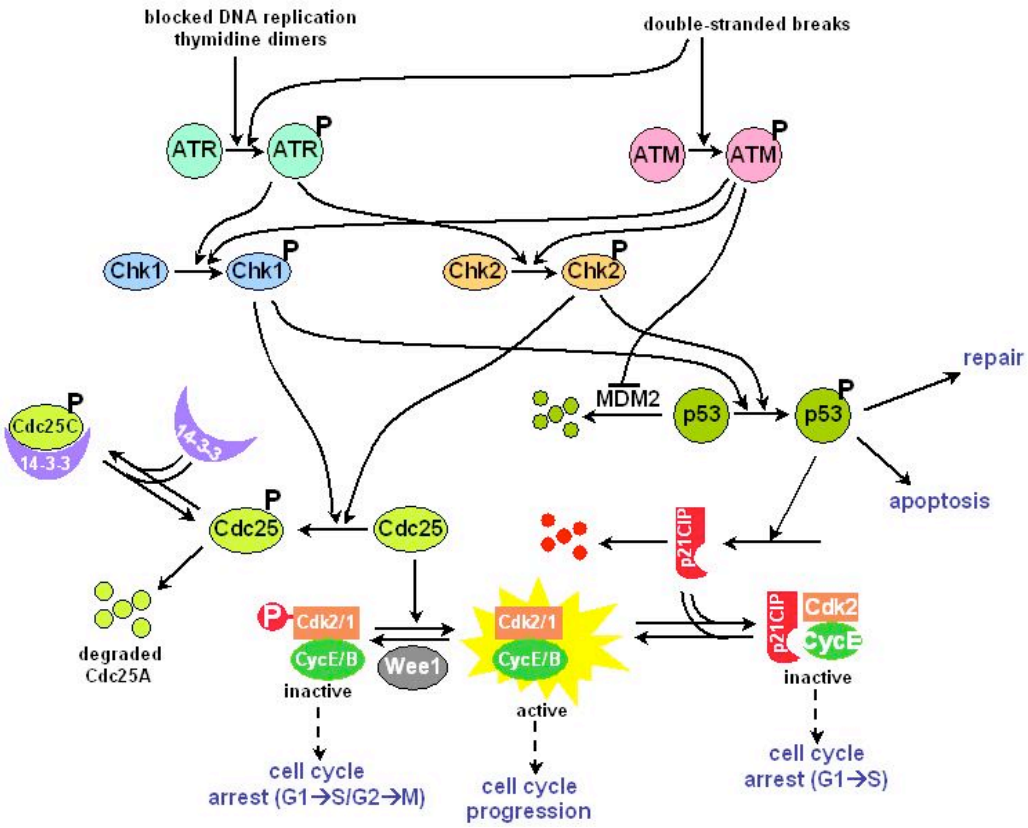

Figure 1-2. The G1/S and G2/M Checkpoint Signaling Pathways. Depending on the type of assault, specific cell checkpoint signaling is initiated, ultimately resulting in the inhibition of cyclin/Cdks, followed by cell cycle arrest, repair, and/or apoptosis.

G1/S Cell Cycle Checkpoint

When DNA is damaged in normal somatic cells, checkpoint pathways function to delay or arrest the cell cycle prior to S phase, preventing the replication of possibly deleterious mutations. Depending on the type and severity of assault, checkpoint activation ultimately results in the inhibition of Cdks, which leads to either DNA repair or apoptosis. Currently, two checkpoint pathways have been defined that ultimately result in checkpoint activation and the inhibition of S phase transition: Chk1/Chk2- and p53- dependent pathways.

Chk1/Chk2-dependent pathway

When a cell detects a threat to genomic integrity, either ataxia telangiectasia mutated (ATM) or ataxia telangiectasia related (ATR) serine/threonine kinases are activated, depending on the type of assault. Specifically, evidence has shown that both ATR and ATM respond to double-stranded breaks, possibly through the direct interaction with damaged DNA (Banin *et al.*, 1998; Abraham, 2001). On the other hand, ATR alone responds to UV-radiation and DNA replication blocks (Guo *et al.*, 2000) ATM and ATR can phosphorylate and activate either Chk1 or Chk2, which in turn phosphorylate and trigger the degradation of the Cdc25A phosphatase (Mailand *et al.*, 2000; Bartek and Lukas, 2001a; Shimuta *et al.*, 2002; Xiao *et al.*, 2003; Petrus *et al.*, 2004; Wroble and Sible, 2005). Cdc25A removes inhibitory phosphates from Thr-14 and Tyr-15 of Cdk2, activating cyclin E/Cdk2, resulting in the progression through the G1/S transition. Degradation of Cdc25A results in the inability to remove inhibitory phosphates from Thr-14 and Tyr-15 of Cdk2 (Costanzo *et al.*, 2000). The sustained inactivation of cyclin E/Cdk2 results in cell cycle arrest at the G1/S transition (Bartek and Lukas, 2001a).

p53-dependent pathway

In addition to Chk1 and Chk2, ATM/ATR can phosphorylate other downstream targets such as p53 on Ser-15 and its inhibitor, Mdm2, on the carboxyl-terminal half of the amino-terminal domain, leading to p53 stabilization. Furthermore, Chk1 and Chk2 can also phosphorylate p53 on Ser-20 (Banin *et al.*, 1998; Khosravi *et al.*, 1999; Rotman and Shiloh, 1999). Normally, Mdm2 interacts with p53 at the N-terminus, targeting it for

ubiquitination and subsequent degradation. However, when DNA is damaged, Chk1 and Chk2 phosphorylate p53 on Ser-20, inhibiting Mdm2 interaction. This results in the stabilization of p53, which induces transcription of p21, an inhibitor of Cdk2 (Khosravi *et al.*, 1999; Carr, 2000; Chehab *et al.*, 2000). p21-dependent inhibition inactivates cyclin E/Cdk2 resulting in cell cycle arrest at the G1/S transition.

G2/M Cell Cycle Checkpoint

One of the most well defined cell cycle checkpoints is that governing the G2/M transition. The most basic role of the G2/M checkpoint is to arrest the cell cycle when aberrations such as damaged or unreplicated DNA are present. This ensures that cells with altered genetic material are unable to undergo mitosis.

The G2/M cell cycle checkpoint is governed by cyclin B/Cdc2, also known as MPF in *Xenopus laevis*. As mentioned previously, during G2, MPF is phosphorylated on two inhibitory residues, Thr-14 and Tyr-15, and one activating residue, Thr-161. Prior to mitotic entry, the inhibitory phosphates on Thr-14 and Tyr-15 must be removed by the Cdc25C phosphatase. This activates MPF, promoting progression into mitosis. When a cell senses a threat to genomic integrity such as damaged or unreplicated DNA, checkpoint signaling is engaged. The type of genomic assault dictates the specific signaling that leads to cell cycle arrest. ATM and/or ATR are activated in response to various types of genomic assaults, leading to variation in downstream signaling.

Activated ATR and ATM directly phosphorylate the Chk1 and/or Chk2 kinases. Conflicting evidence indicates that various types of genomic assaults can activate both kinases or solely Chk1 or Chk2. This indicates the existence of overlap between G2/M checkpoint signaling. Specifically, the activation of the Chk1 signaling pathway at the G2/M checkpoint begins with the phosphorylation of Chk1 by ATR. Phosphorylated Chk1 then phosphorylates Cdc25C on Ser-285 (Furnari *et al.*, 1997; Sanchez *et al.*, 1997), creating a binding site for the 14-3-3 cytoskeletal protein. The binding of phosphorylated Cdc25C to 14-3-3, sequesters Cdc25 to the cytoplasm, inhibiting its accumulation in the nucleus (Peng *et al.*, 1997; Zeng *et al.*, 1998). Therefore, sequestered Cdc25C cannot remove inhibitory phosphates on the Thr-14 and Tyr-15 residues of Cdc2, located in the nucleus (Furnari *et al.*, 1997; Sanchez *et al.*, 1997). In

addition, similar to Cdk2/cyclin E inactivation, Chk1 phosphorylates Wee1 kinase which phosphorylates and inhibits Cdc2/cyclin B (Mueller *et al.*, 1995b; Raleigh and O'Connell, 2000; Lee *et al.*, 2001). Consequently, Cdc2 is inactivated, arresting the cell cycle in G2 phase and inhibiting its progression into mitosis.

In addition to checkpoint signaling, Chk1 activation is also important for development in *Xenopus*. Chk1 is transiently activated at the MBT in the absence of genotoxic stress, and then degraded following the MBT. It is believed that this transient activation is partially necessary to remove the maternal pool of Cdc25A (Shimuta *et al.*, 2002). However, further investigation is necessary to determine what triggers this activation and how Chk1 is subsequently degraded.

1.3 Early Embryonic Development of *Xenopus laevis*

The early embryonic development of *Xenopus laevis* is a rare example of a biological system that exhibits nonpathological cell cycles lacking checkpoints. Later in development at the midblastula transition (MBT), controls on cellular proliferation are acquired. Therefore, the embryonic development of *Xenopus* serves as a molecular model system for the comparison of cells that can initiate checkpoint pathways in response to damaged or unreplicated DNA (Newport and Dasso, 1989; Dasso and Newport, 1990) and those which cannot.

Upon fertilization, the *Xenopus* embryo begins the first embryonic cellular division, which takes roughly 1.5 hours. The next 11 cellular divisions occur in a very synchronous and rapid manner, averaging 30 minutes each. Upon the completion of the twelfth division, when the MBT begins, extensive cell cycle remodeling takes place.

Prior to the MBT, *Xenopus* development is under the control of a maternal program of development. Specifically, all of the proteins that are used to power these early cell cycles are translated from a maternal store of mRNAs, and transcription does not occur until the MBT. Furthermore, prior to the MBT, cellular divisions 2-12 lack gap phases, which are integral parts of the normal somatic cell cycle. Instead, the abbreviated cell cycle consists of the alternation between DNA replication (S) and mitosis (M) phases (Newport and Kirschner, 1982a) (Fig. 1-3). Additionally, functional cell cycle checkpoints, an apoptotic program, and cell growth are absent (Newport and Dasso,

1989; Anderson *et al.*, 1997; Hensey and Gautier, 1997).

Following the completion of the MBT, embryonic development is under control of a zygotic program. Specifically, zygotic transcription initiates, and cells gain motility as maternal mRNAs are degraded (Newport and Kirschner, 1982b; Frederick and Andrews, 1994). In addition, gap phases, operative cell cycle checkpoints, and a functional apoptotic program are all acquired (Dasso and Newport, 1990; Kappas *et al.*, 2000).

At the MBT, extensive cell cycle remodeling occurs, which is thought to be partially due to a critical nucleo-cytoplasmic ratio. The attainment of this critical nucleo-cytoplasmic ratio is necessary for the engagement of the new cell cycle program characteristic of post-MBT development (Newport and Kirschner, 1982b). At the MBT, a cytoplasmic factor that stoichiometrically interacts with increasing DNA during pre-MBT development is depleted when the cell reaches the critical nucleo-cytoplasmic ratio (Hara *et al.*, 1980; Newport and Kirschner, 1982a). Specifically, the nucleo-cytoplasmic ratio determines the following cell cycle remodeling events: acquisition of gap phases and checkpoints, initiation of transcription and cell motility, and the degradation of certain maternal proteins (Newport and Kirschner, 1982a, 1982b). In the *Xenopus* extract system, the concept of a critical nucleo-cytoplasmic ratio has been reproduced. Specifically, egg extracts containing a concentration of nuclei below the equivalent MBT nucleo-cytoplasmic ratio do not engage cell cycle checkpoints in response to DNA replication blocks (Dasso and Newport, 1990).

In addition to the nucleo-cytoplasmic factor, cell cycle remodeling and the onset of the zygotic program are partially due to a maternal cyclin E/Cdk2 developmental timer. One of these very important cell cycle remodeling events that is necessary for normal post-MBT development is the degradation of maternal cyclin E (Rempel *et al.*, 1995; Hartley *et al.*, 1996). While cyclin E levels are constant through pre-MBT development, cyclin E/Cdk2 activity oscillates twice per cell cycle independent of protein synthesis and the nucleo-cytoplasmic ratio (Hartley *et al.*, 1996; Howe and Newport, 1996; Hartley *et al.*, 1997) . The inhibition of Cdk2 by the specific Cdk2 inhibitor, Δ34Xic1, delays the cell cycle and the degradation of cyclin E, suggesting that the cyclin E/Cdk2 timer specifically regulates pre-MBT cell cycles. Furthermore, specific

inhibition of Cdk2 by 34Xic1 delays zygotic transcription, suggesting that the initiation of zygotic transcription is dependent on cyclin E/Cdk2 activity, specifically the degradation of cyclin E. However, injection of -amanitin (a drug that blocks zygotic transcription) does not affect cyclin E degradation, indicating that cyclin E degradation is maternally controlled. In fact, the degradation of cyclin E appears to be regulated by the oscillations in cyclin E/Cdk2 activity itself (Hartley *et al.*, 1997). These data suggest that the cyclin E/Cdk2 maternal timer specifically regulates some cell cycle remodeling events at the MBT.

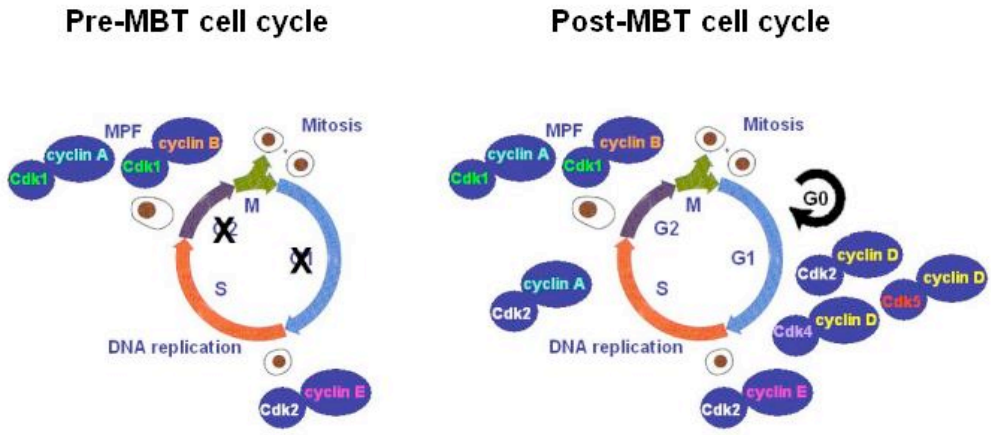

Figure 1-3. Pre-MBT verses Post-MBT Cell Cycles. The pre-MBT cell cycle consists of rapid oscillations between M and S phases. The post-MBT cell cycle is consistent with the normal somatic cell cycle. Progression through the post-MBT cell cycle is regulated by a complete set of cyclin/Cdk complexes, while the progression through the pre-MBT cell cycle is characterized by a much simpler profile of cyclin/Cdks complexes.

1.4 Comparison of Chk1 and Chk2 Kinases during *Xenopus* Development

The checkpoint kinases, Chk1 and Chk2, have been studied extensively in a wide range of organisms. Although the role of Chk2 has been shown to function redundantly to Chk1 in many studies across various model systems, key differences have also been identified. In *Schizosaccharomyces pombe*, both kinases are phosphorylated and activated in response to unreplicated DNA and the incurrence of DNA damage. Unlike Chk1 however, Chk2 is only activated during S phase in response to DNA damage (Lindsay *et al.*, 1998). This indicates that Chk2 is activated by DNA replication-specific structures. Chk1, on the other hand, functions to arrest mitosis in response to DNA damage. Following this, it is apparent that Chk1 is activated by DNA damage-specific structures in *S. pombe*. Although Chk1 and Chk2 kinases both function in a manner dependent on the checkpoint Rad pathway, this data indicates that two distinct pathways exist distinguishing Chk1 and Chk2 (Lindsay *et al.*, 1998).

In comparison to the yeast model system, research studies of Chk2 in *Xenopus laevis* have produced different results. As in the Chk1 signaling pathway, Chk2 functions in a similar way. In *Xenopus laevis* and most vertebrates, ATR/ATM is responsible for sensing DNA damage and unreplicated DNA and subsequently activating both Chk1 and Chk2 (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998). Both kinases inactivate Cdc25C through phosphorylation, leading to the inactivation of MPF (Cdc2/cyclin B), and arresting progression into mitosis (Zeng *et al.*, 1998). This is in contrast to *S. pombe* where Chk2 does not function in mitotic arrest induced by DNA damage (Lindsay *et al.*, 1998).

The MPF complex is the key target in both the Chk1 and the less well-understood Chk2 signaling pathways. Prior to fertilization, immature *Xenopus* oocytes are arrested at prophase I, or the late G2 phase. Release from prophase I arrest by progesterone, corresponding to the G2/M transition, results in the maturation of oocytes. Both Chk1 and Chk2 are involved in the physiological prophase I arrest through the inhibition of MPF via phosphorylation of Cdc25C (Nakajo *et al.*, 1999; Gotoh *et al.*, 2001) Furthermore, both Chk1 and Chk2 are believed to function under a different mechanism

in the absence of the G2/M checkpoint. Chk2 activation has been shown to occur independently of genomic assault and therefore G2/M checkpoint activation in immature oocytes, although the mechanism is unknown (Gotoh *et al.*, 2001). It is however hypothesized that Chk2 activation through a non-G2/M checkpoint mechanism could occur as a response for the need of intrinsic or basal kinase activity. Moreover, it is believed that Chk2 activation through a non-G2/M checkpoint mechanism is distinct from that of Chk1 (Gotoh *et al.*, 2001). This hypothesis is evident through the difference in localization of Chk1 in comparison to Chk2 within immature oocytes. In oocytes, Chk2 is expressed in the nucleus of the cell while Chk1 is expressed in both the cytosol and the nucleus suggesting separate roles for each protein (Gotoh *et al.*, 2001; Oe *et al.*, 2001).

In *Xenopus* embryos, both Chk1 and Chk2 protein kinases have been shown to function as checkpoint kinases in response to both DNA damage and unreplicated DNA. Specifically, Chk1 and Chk2 are phosphorylated and activated by ATM/ATR in response to IR and aphidicolin in post-MBT embryos (Kappas *et al.*, 2000; Gotoh *et al.*, 2001; Wroble and Sible, 2005). Similarily, Chk2 is responsive to DNA replication blocks caused by hydroxyurea treatment (Gotoh *et al.*, 2001).

In *Xenopus* egg extracts, Chk1 and Chk2 activation differs from that reported in *Xenopus* embryos. Embryo extracts incubated with DNA templates with double-stranded ends such as poly$(dT)_{40,}$ M13 DNA, or double-stranded oligonucleotides result in Chk2 activation and cell cycle delay. In contrast, Chk1 is not responsive to these assaults. Conversely, Chk1 is activated by DNA replication blocks induced by aphidicolin, while Chk2 is unresponsive to aphidicolin (Guo and Dunphy, 2000). Interestingly however, immunodepletion of Chk1 and/or Chk2 does not affect the cell cycle delay induced by double-stranded DNA ends. These data are suggestive of the overlapping functions of Chk1 and Chk2 and possibly the presence of another checkpoint kinase.

Although Chk1 and Chk2 function similarly during the regulation of the cell cycle, differences exist. Based on the aforementioned, it should be noted that both the similarities and differences of Chk1 and Chk2 function could be model system-specific.

1.5 The Role of Chk2 during Development: An Inhibitor or Promoter of Apoptosis?

The transition from checkpoint-unregulated to checkpoint-regulated cell cycles makes *Xenopus* embryos an excellent system for studying the molecular mechanisms that regulate cell cycle arrest and apoptosis in response to damaged DNA. As discussed previously, in eukaryotes, Chk1 and Chk2 kinases are central components of signaling networks activated in response to damaged and unreplicated DNA. Activation of Chk1 or Chk2 can promote cell cycle arrest (via phosphorylation of Wee1, Cdc25A, Cdc25C, and p53), repair (via phosphorylation of BRCA1), and apoptosis (via phosphorylation of p53, E2F1, and PML) (Novak *et al.*, 2002; Ahn *et al.*, 2004). What determines whether cells respond to DNA damage by arresting the cell cycle or committing apoptosis is not well understood.

Chk1 is required for *Xenopus* embryos to survive beyond gastrulation, even in the absence of explicitly damaged DNA (Shimuta *et al.*, 2002; Carter and Sible, 2003). Likewise, Chk1 is essential for early development of mice (Liu *et al.*, 2000; Takai *et al.*, 2000). In contrast, Chk2 is not essential in mice (Takai *et al.*, 2002), but has been implicated as both a promoter and inhibitor of apoptosis (Hirao *et al.*, 2002; Jack *et al.*, 2002; Peters *et al.*, 2002; Takai *et al.*, 2002; Yang *et al.*, 2002; Stevens *et al.*, 2003; Wu and Chen, 2003; Brodsky *et al.*, 2004; Castedo *et al.*, 2004; Hong and Stambrook, 2004; Jack *et al.*, 2004; Rogoff *et al.*, 2004). Specifically, mice lacking the Chk2 gene are viable, but deficient in IR-induced apoptosis (Hirao *et al.*, 2002; Jack *et al.*, 2002; Takai *et al.*, 2002; Jack *et al.*, 2004). In the *Drosophila* eye, Chk2 promotes the induction of apoptosis in response to DNA damage (Peters *et al.*, 2002). In both cases, Chk2 promotes apoptosis via phosphorylation of p53. In human breast carcinoma cells, Chk2 promotes DNA damage-induced apoptosis by phosphorylating the transcription factor E2F-1 (Stevens *et al.*, 2003) and in lymphoma cells by phosphorylating the scaffolding protein PML (Yang *et al.*, 2002). Thus, in response to DNA damage, Chk2 can phosphorylate at least three targets to promote apoptosis (Ahn *et al.*, 2004).

In contrast, other studies have implicated Chk2 as an inhibitor of apoptosis. Mouse embryonic stem cells are deficient in a G1 checkpoint and highly susceptible to

IR-induced apoptosis. Ectopic expression of Chk2 in these cells confers protection from IR-induced apoptosis. This protection may be p53-independent, since the p53 target, p21, is not induced (Hong and Stambrook, 2004). Expression of DN-Chk2 in human heterokarya and HCT116 colon carcinoma cells promotes apoptosis in response to mitotic catastrophe (Castedo *et al.*, 2004). Furthermore, exogenous wt-Chk2 blocked IR-induced apoptosis, while DN-Chk2 promoted apoptosis in the early *Xenopus* embryo. These data implicate Chk2 as an inhibitor of apoptosis (Wroble and Sible, 2005).

Studies in early *Xenopus* embryos may shed light upon the apparent paradox regarding the relationship between Chk2 and apoptosis. Early embryos contain p53, but development is not noticeably affected by expression of either wt- or DN-p53 until well past gastrulation (Wallingford *et al.*, 1997). Furthermore, p53 cannot function as a transcription factor before the MBT because transcription is silent (Newport and Kirschner, 1982b). Therefore, early embryos provide a context in which the p53-signaling pathway is not fully functional, and may explain why Chk2 does not promote apoptosis. On the other hand, it has been shown that Chk2 and Chk1 are capable of activating cell cycle checkpoints in the early embryos by triggering tyrosine phosphorylation of cyclin-dependent kinases (Kappas *et al.*, 2000; Wroble and Sible, 2005). By causing cell cycle delay in response to IR, Chk2 may allow for repair and may also promote repair pathways through its interaction with proteins such as BRCA1 and Mus81 (McGowan, 2002). Since many tumors lack functional p53, it will be important to determine whether Chk2 truly blocks apoptosis in a p53-negative background, in order to decide when drugs that target Chk2 are appropriate chemotherapeutics. These studies are of particular importance to the current project and will be addressed in Chapter 3.

1.6 The Role of the Wee Kinases during Development

The key complexes that control cell cycle progression in S phase and M phase are cyclin E/Cdk2 and MPF, respectively. In addition to the checkpoint kinases Chk1 and Chk2, other upstream regulators, including the Wee1 tyrosine kinase, regulate control of these complexes. Initially identified in *Schizosaccharomyces pombe* (Russell and Nurse, 1987a), Wee1 homologs have been identified in many eukaryotes including humans, *Drosophila*, and *Xenopus* (Igarashi *et al.*, 1991; Campbell *et al.*, 1995; Mueller *et al.*,

1995a; Watanabe *et al.*, 1995). In *Xenopus*, Wee1 belongs to a group of proteins termed the Wee family of kinases, consisting of Wee1, Wee2, and Myt1 (Leise and Mueller, 2002). In early *Xenopus* development, the maternal Wee1 kinase is present in pre-MBT embryos, but degraded following the MBT (Murakami and Woude, 1998). Myt1, the other MPF tyrosine kinase expressed during pre-MBT development, persists through post-MBT development, although at lower levels (Leise and Mueller, 2002). In addition to cyclin synthesis and degradation, Wee1 and Myt, along with the Cdc25 phosphatases, control the periodicity of cyclin/Cdk activation and subsequent deactivation, characteristic of pre-MBT development (Kim *et al.*, 1999). The zygotic Wee2 kinase, expressed exclusively in post-MBT embryos, is a much more active kinase than the maternal Wee1, in terms of Cdc2 inactivation (Leise and Mueller, 2002). Following the completion of the MBT, the onset of Wee2 expression coincides with cell cycle lengthening, zygotic transcription, and the acquisition of gap phases. Additionally, microinjection of exogenous Wee2 DNA in one blastomere of a two-cell stage embryo results in cell cycle arrest in post-MBT cells localized near the site of injection. Taken together, these data suggest that the switch from maternal to zygotic isoforms of Wee may partially account for cell cycle remodeling. In post-MBT embryos, Wee2 expression is confined specifically to embryonic regions absent of mitotic cells, such as the involuting dorsal endomesoderm and dorsal mesoderm. On the other hand, Myt1 is expressed in regions of high proliferation such as the neural ectoderm (Leise and Mueller, 2002). These data suggest an important role for the Wee kinases in both cell cycle regulation and morphogenesis.

Although Myt1 and Wee2 have various roles in both cell proliferation and morphogenesis during the post-MBT development of *Xenopus*, Wee1 functions exclusively during the rapid, synchronous cellular divisions of pre-MBT development. Wee1 is present in early *Xenopus* embryos in a hypophosphorylated form during interphase, but undergoes extensive phosphorylation, and subsequent down-regulation upon entry into mitosis. In *Xenopus* egg extracts, Wee1 is degraded upon mitotic entry (Michael and Newport, 1998). In budding yeast, Cdk1 (Cdc2) phosphorylates and activates Wee1, which phosphorylates Cdk1, its own activator, maintaining Cdk1 in an inhibited state. This double-negative feedback loop is disrupted upon mitotic entry when

the dephosphorylation of Cdk1 triggers the hyperphosphorylation and inactivation of Wee1 (Harvey *et al.*, 2005). Hence, a negative feedback loop contributes to the activation of Cdk1 and ensuing entry into mitosis. Specifically, Wee1 acts in opposition to Cdc25C, inhibiting MPF through phosphorylation of Tyr-15 on Cdk1 (Cdc2) (Parker *et al.*, 1992b; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993; Mueller *et al.*, 1995a). During checkpoint signaling in *Xenopus*, Chk1 also phosphorylates Wee1 on Ser-549, allowing 14-3-3 binding and activating Wee1, inhibiting M phase entry (Lee *et al.*, 2001).

In mammalian cells, Wee1 inhibits cyclin E/Cdk2 through phosphorylation of Tyr-15 on Cdk2 (Watanabe *et al.*, 1995; Wu *et al.*, 2001). Currently, it is unknown whether Wee1 inhibits cyclin E/Cdk2 in *Xenopus*. This and other questions regarding the role in which Wee1 has in cell cycle regulation during *Xenopus* development were addressed in current study and will be discussed in Chapter 4.

1.7 Cell Cycle Regulation and Apoptosis.

Regulation of not only cellular proliferation, but also cell death is essential for normal development and tissue homeostasis in eukaryotes. Programmed cell death, or apoptosis, is a highly conserved process that eliminates unwanted cells from an organism while preserving others (Jacobson *et al.*, 1997). The regulation of apoptosis is of particular importance in the investigation of potential therapeutics for diseases such as cancer. In many cancers, genes responsible for executing pro-apoptotic function are commonly mutated and dysfunctional. Specifically, the gene encoding the tumor suppressor, p53, is mutated in half of all cancers (Pucci *et al.*, 2000). Normally, p53 is involved in modulating many aspects of cellular proliferation including cell cycle arrest, DNA repair, and apoptosis. During apoptotic signaling, p53 induces the transcription of pro-apoptotic genes such as Bax and IgF-Bp3. Additionally, p53 induces the transcription of the cell surface receptor Fas, which when associated with its ligand, FasL, induces apoptosis (Owen-Schaub *et al.*, 1995). Synergistically, p53 represses the transcription of genes that function to inhibit apoptosis such as Bcl-2 (Miyashita *et al.*, 1994).

As discussed in 1.2, progression through the cell cycle, mediated by the activity of specific Cdks and their associated cyclins, is monitored by checkpoints. Furthermore, cyclin/Cdks have also been implicated in apoptotic signaling. Several studies have demonstrated cyclin A/Cdk2 activity as a positive regulator of apoptosis. Specifically, IR-induced apoptosis elevates cyclin A1/Cdk2 activity in *Xenopus laevis* embryos (Anderson *et al.*, 1997). In DO11.10 T-cell hybridomas, cyclin A/Cdk complexes are activated during apoptosis (Harvey *et al.*, 1998). In HeLa cells, cyclin A expression restores TNF-induced apoptosis that was blocked by the expression of dominant-negative mutants of Cdk1/2/3 (Meikrantz *et al.*, 1994). The primary G1-phase cyclin, cyclin D, has also been demonstrated to promote apoptosis. Specifically, cyclin D1 overexpression causes apoptosis in neuronal cells and fibroblasts (Kasten and Giordano, 1998), while cyclin D3 promotes TNF-induced apoptosis (Janicke *et al.*, 1996).

In addition to the involvement of specific cyclin/Cdks during apoptotic signaling, cyclin-dependent kinase inhibitors (CKIs) have been implicated. CKIs such as p21 and p27 have been shown to have both pro- and anti-apoptotic function. Specifically, p21, a target of p53, represses DNA damage-induced apoptosis by X-ray irradiation in human colorectal carcinoma cells (Lu *et al.*, 1998). Similarly, the expression of p27, a Cdk2 inhibitor, rescues the apoptotic effect of serum starvation in p27 -/- mesangial and fibrobast cells (Hiromura *et al.*, 1999). Conversely, the transfection of breast cancer cells with a degradation-resistant form of p27 results in increased growth arrest and apoptosis (Zhang *et al.*, 2005). The aforementioned studies demonstrate the involvement of cyclins, Cdks, and CKIs in both cellular proliferation and apoptosis in a wide-range of cell types. The specific mechanisms by which these complexes can function to promote both survival and/or apoptosis are only partially understood.

One specific system that provides a unique context in which to study apoptotic mechanisms is the early *Xenopus* embryo. During *Xenopus* development, the MBT is characterized by cell cycle remodeling events that are necessary for post-MBT development. As the cell cycle lengthens at the MBT, cells gain the ability to initiate a functional program of apoptosis (Hensey and Gautier, 1997). During pre-MBT *Xenopus* development, a functional apoptotic program is absent. In response to genotoxic stress, the pre-MBT embryo will continue to divide normally without any physical aberrations

(Newport and Dasso, 1989; Anderson *et al.*, 1997; Hensey and Gautier, 1997; Sible *et al.*, 1997). Shortly after reaching the MBT however, these compromised cells undergo a well-defined apoptotic death (Anderson *et al.*, 1997; Hensey and Gautier, 1997; Sible *et al.*, 1997). In contrast, post-MBT embryos initiate cell cycle checkpoint signaling in response to identical genomic assaults (Anderson *et al.*, 1982; Kappas *et al.*, 2000; Wroble and Sible, 2005). Specifically, pre-MBT embryos treated with aphidicolin and IR will divide normally before undergoing apoptosis just prior to gastrulation (Anderson *et al.*, 1997). Similarly, other genotoxic stresses such as blocked transcription and protein synthesis will also result in a similar apoptotic death (Hensey and Gautier, 1997; Sible *et al.*, 1997; Stack and Newport, 1997). Conversely, post-MBT embryos initiate checkpoint responses to these stresses (Anderson *et al.*, 1997; Hensey and Gautier, 1997; Sible *et al.*, 1997; Kappas *et al.*, 2000).

Another unique characteristic of *Xenopus* development is the absence of transcription until the MBT. As the cell cycle remodels at the MBT, transcription is initiated for the first time in development (Newport and Kirschner, 1982b). The delayed onset of transcription provides an interesting context in which to study apoptotic signaling. Specifically, although p53 is expressed in pre-MBT embryos and essential for development (Wallingford *et al.*, 1997), it cannot function as a transcription factor. Therefore, pre-MBT development provides a p53-transcription factor-independent background in which to study apoptosis. However, studies have implicated transcription factor-independent p53 activity in the induction of apoptosis (Schuler and Green, 2005). Further investigation is necessary to determine the relationship between p53 activity and apoptosis in *Xenopus* development.

The unique characteristics of *Xenopus* development provide an interesting context in which to study apoptosis from both a developmental and therapeutic standpoint. It is however important to consider differences in cell type when comparing cellular proliferation and apoptosis. The following chapters further discuss the regulation of apoptosis in *Xenopus laevis* embryos, while providing perspective on apoptotic signaling in other cell types.

Chapter 2: Materials and Methods

2.1 Induced Ovulation of Female *Xenopus laevis*

To induce ovulation, female *Xenopus laevis* frogs were primed with 75 IU pregnant mare serum gonadotropin (PMSG) (Calbiochem, La Jolla, CA) injected subcutaneously into the dorsal lymph sac 3-5 days prior to fertilization. Frogs subsequently received another subcutaneous injection of 550 IU human chorionic gonadotropin (HCG) (Sigma) the evening prior to fertilization.

2.2 Embryo Maintenance and Manipulation

Eggs from wild-type *Xenopus laevis* (*Xenopus* Express, Plant City, FL) 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 $MgSO_4$, 0.2 mM $CaCl_2$, 0.01 mM EDTA), and maintained in 0.1X MMR. Embryos were staged (Nieuwkoop and Faber, 1975) and subjected to manipulation. Embryos were subjected to DNA damage with ionizing radiation (IR) emitted from a TFI Mini Shot X-ray chamber at specific stages. To block DNA replication, embryos were incubated at specific stages in 0.1X MMR containing 100 g/mL aphidicolin (Calbiochem) and 1% dimethyl sulfoxide (DMSO). Control embryos were incubated in 0.1X MMR, 1% DMSO. Embryos were injected at the one-cell stage with specific concentrations of Chk1, DN-Chk1, Chk2, DN-Chk2, Wee1 and luciferase mRNA dissolved in 25-30 nL TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and 34-Xic and Xic1 mutant protein dissolved in buffer (20mM Hepes, 88mM NaCl, 7.5mM $MgCl_2$, 10mM BME). Embryos were observed with an Olympus SZX12 stereomicroscope and photographed with an Olympus DP10 digital camera.

2.3 Western Analysis

Embryos were lysed in EB buffer (20 mM HEPES, pH 7.5, 80 mM -glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 g/mL leupeptin, 1 mM microcystin). Samples were then resolved on modified SDS polyacrylamide gels (separating gel =10% acrylamide, 0.1% bis-acrylamide, 0.37 M Tris,

pH 8.7, 0.1% SDS; running buffer = 0.05 M Tris, pH 8.3, 0.384 M glycine, 0.2% SDS), transferred to a nitrocellulose membrane (Schleider and Schuell, Keene, NH) on semi-dry transfer apparatus (BioRad, Hercules, CA), and blocked in 10% nonfat dry milk in PBS (phosphate buffered saline- 20 mM $Na_2HPO_4 \cdot 7H_2O$, 20 mM $NaH_2PO_4 \cdot H_2O$, 100 mM NaCl), 10% nonfat dry milk in TBS-Tween (tris-buffered saline- 20mM Tris-Base, 0.14 M NaCl, pH-7.6) or 5% BSA in TBS. Membranes were incubated in primary antibody αFLAG M2 (1:1000) (Sigma), and αPARP (1:5000) (Calbiochem) diluted in 10% nonfat dry milk in PBS; αChk2 (1:1000) (a gift from Paul Mueller) in 5% nonfat dry milk in PBS; αPhospho-Cdk (1:1000) (Cell Signaling Technology, Beverly, MA), αCdc25A (1:15) (a gift from Jim Maller), αcyclin E (1:2) (a gift from Jim Maller), and αWee1 (1:1000) (Zymed) diluted in 5% BSA-TBS- 0.1% Tween overnight at 4°C. Membranes were washed in PBS- 0.1% Tween or TBS- 0.1% Tween, followed by incubation in secondary antibody (Peroxidase-conjugated AffiniPure Donkey Anti-Rabbit IgG or Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG for αFLAG (Jackson Immuno Research Laboratories Inc., West Grove, PA) 1:10,000 in 10% nonfat dry milk in PBS or TBS- 0.1% Tween (αPhospho-Cdk). Alternatively, membranes were incubated in secondary antibody (HRP-linked αRabbit 1:2000) (Cell Signaling Technology) in 5% BSA in TBS- 0.1% Tween (αCdc25A, αCyclin E, and αPhospho-Cdk). Following secondary antibody incubation, membranes were washed in PBS- 0.1% Tween or TBS- 0.1% Tween. Immunoreactive proteins were detected by chemilluminescence using an ECL Plus kit (Amersham, Arlington Heghts, Il).

2.4 Cloning WT and Mutant Chk2 into pSP64polyA Plasmid

pCD47 (harbors wt Chk2) and pCD51 (harbors mutant dominant-negative Chk2 (DN-Chk2):kinase dead Chk2 with S324A) vectors (gifts from William Dunphy) were resuspended in TE to a concentration of 50 ng/μL and transformed into chemically competent DH5α bacteria cells (Invitrogen, Carlsbad, CA) using kanamycin as the selection antibiotic. Five milliliter cultures and subsequent 100 mL cultures (LB) were grown from selected colonies. Plasmid DNA using QIAfilter Plasmid Maxi Kit (Qiagen, Valencia, CA) was prepared from 100 mL cultures. Plasmid DNA was quantified by spectrophotometry. Test restriction digestions were performed to insure the correct DNA

was isolated. Plasmid DNA was linearized using *Sma* I (Cell Signaling Technology) and used as templates for PCR. PCR was run using Elongase DNA polymerase (Invitrogen) and the following two primers:
5'-CCCCTGCAGCTCTCGTGATACTAAAACAGAGT-3' and
3'-CCCGGATCCTTATCTTTTTGCTCTCTTTTCG-5'. Samples were subjected to 30 cycles at 94^{o}C for 30 seconds, 58^{o}C for 30 seconds, and 68^{o}C for 6 minutes. PCR products were resolved on a 0.8% agarose gel, and purified using a PCR purification kit (Qiagen). PCR products and pSP64polyA plasmid (Promega, Madison, WI) were digested overnight using *Bam* HI (Cell Signaling Technology), phenol/chloroform extracted, and then digested overnight with *Pst* I (Cell Signaling Technology). Digestion of pSP64polyA plasmid was followed by calf intestinal alkaline phosphate (CIP) treatment. PCR products (WT and mutant Chk2) were ligated into pSP64polyA vector, transformed into DH10 electrocompetent cells (Invitrogen), cultured and purified as Maxiprep DNA (Qiagen).

2.5 *In Vitro* Transcription of Chk2 and Wee1 mRNA

FLAG-tagged wt-Chk2, catalytically inactive FLAG-tagged DN-Chk2, and Wee1 constructs were linearized using *Eco* RI (Cell Signaling Technology) and used as templates to produce polyadenylated mRNA encoding FLAG-tagged wt-Chk2, FLAG-tagged DN-Chk2, and Wee1 respectively, using the Ambion SP6 mMessage mMachine *in vitro* transcription kit (Ambion, Austin, TX).

2.6 TUNEL Assay

One-cell stage embryos were injected with DN-Chk2 or luciferase mRNA and fixed at desired stages (post-gastrulation) in MEMFA (100 mM MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1-2 hours at room temperature on a nutator. Embryos were washed twice in ethanol with shaking on a Nutating mixer (Shelton Scientific, Peosta, IA) (all washes and incubation used Nutating mixer unless noted) and then rehydrated through an ethanol series and washed 2 X 15 min in PBS-Tween (0.2% Tween in 1X PBS). Embryos were then washed for 30 min in 1X TdT buffer. Embryos were then incubated overnight in 1X TdT buffer containing 0.5 M

DIG-dUTP (Boehringer Mannheim, Indianapolis, IN) in 150 U/mL TdT (Invitrogen). Following incubation, embryos were washed consecutively with PBS/EDTA at 65°C, PBS and PBT (2 mg/mL BSA, 0.1% Triton X-100 in PBS), and then incubated with PBT and 20% goat serum. Embryos were incubated overnight in 1:2000 dilution of anti-DIG antibodies in PBT and 20% goat serum. The next day, embryos were washed in PBT six times at room temperature for 1 hour and then washed with PBT overnight at 4°C. The next day, embryos were washed consecutively with alkaline phosphate buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 □□ NaCl, 0.1% Tween 20, 5 mM levamasol), alkaline phosphate buffer containing 4.5 □L NBT (Boehringer Mannheim) in 70% DMF (dimethyl formamide) and 3.5 □L BCIP (Boehringer Mannheim). Embryos were rocked in tubes until staining was apparent, transferred into a dish with alkaline phosphate buffer, fixed in MEMFA, and then photographed.

2.7 Assay for Cleavage of PARP

Embryos previously injected with DN-Chk2, Wee1, and luciferase mRNA or □34-Xic1 and p27XicCK- proteins were collected at desired stages (post-gastrulation), snap frozen on dry ice, and assayed for the cleavage of exogenous PARP (Carter and Sible, 2003). Specifically, embryos were homogenized in caspase extraction buffer (10 □l/embryo) (CEB: 80mM □-glycerophosphate, 15mM $MgCl_2$, 20 mM EGTA, 10 mM DTT). Lysates were incubated with 2 ng/□L recombinant human PARP (Alexis Biochemicals, Carlsbad, CA) at 30°C for 30 min, resolved on a 7.5% Anderson SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. □PARP (1:5000) and HRP-conjugated donkey anti-rabbit (Jackson Immuno Research Laboratories, Inc.) (1:10000) diluted in 10% nonfat dry milk in PBS were used as primary and secondary antibodies, respectively. Both full-length and cleaved PARP proteins were detected by chemiluminescence using an ECL Plus kit (Amersham, Piscataway, NJ).

2.8 IP Western Analysis

Embryos were injected with Wee1 and luciferase mRNA, collected at various timepoints, and lysed in EB. An antibody against cyclin E was used to immunoprecipitate cyclin E/Cdk2 as previously described in Kappas et al, 2000.

Specifically, embryos lysates were precleared with protein G Sepharose beads (Sigma, St. Louis, MO) for 30 min, mixed with cyclin E antiserum (gift from Jim Maller), and incubated overnight on ice. Protein G Sepharose beads were added the next day and incubated with the immunoprecipitates for 1 h with rotation. Beads were then washed twice in low-salt buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 100 mM NaCl) and twice in high-salt buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 1 M NaCl). Protein G beads were then mixed with 12.5 μl 2X gel loading buffer (1X = 0.6 mM Tris base, 2% glycerol, 3% SDS, 0.002% bromphenol blue) containing 10 mM *n*-ethylmaleimide, and resolved by electrophoresis on a 15% polyacrylamide gel for Western analysis. Western analysis was performed as described in 2.3 using a primary αPhospho-Cdk antibody (Cell Signaling Technology) diluted 1:1000 in 5% BSA-TBS-Tween and a secondary HRP αRabbit antibody (Cell Signaling Technology) diluted 1:2000 in 5% BSA-TBS- 0.1% Tween.

2.9 DNA Content Assay

Embryos were injected with Wee1 and luciferase mRNA, or treated with IR, lysed in DNA digestion buffer (500 μl/10 embryos) (0.01 mM Tris, 100 mM EDTA, 50 μg/mL RNase, 0.5% SDS). Lysates were incubated at 37°C for 2 hrs. Proteinase K was then added to a final concentration of 100 μg/mL with continued incubation at 50°C for 4 hrs with slight intermittent manual agitation. Genomic DNA was phenol/chloroform extracted, and precipitated overnight at 20°C in 100% ethanol. Samples were resuspended in TE, and resolved on a 0.7% agarose gel.

2.10 Protein Purification of p27XicCK-

The recombinant pGEX vector containing the GST-p27XicCK- mutant fusion containing 4 mutations (R33A, L35A, F65A, F67A) (gift from Carla Finkielstein) was electorporated into competent Rosetta cells (Novagen, San Diego, CA), incubated at 37°C for 1 hour in SOC media, plated on LB ampicillin plates, and incubated overnight at 37°C.

Recombinant colonies were selected and used to inoculate 50 mL LB media containing 1 mM ampicillin. Cultures were grown for 12 hrs at 37°C. One liter cultures

were then inoculated at 1:500 and incubated at 37°C until reaching an optical density of 0.6 nm at which point 0.4 mM IPTG was added to induce expression. Cultures were incubated for 3 hrs at 37°C, and then centrifuged at 10,000g for 15 minutes at 4°C.

Pellets were resuspended in an appropriate volume (40 mL) of resuspension buffer (50 mM Tris pH 7.3, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA) containing 1 tablet of Complete protease inhibitors (Roche, Indianapolis, IN) and 5 mM DTT. Cell suspension was incubated at room temperature with 1 mg/mL lysozyme for 20 minutes with stirring. Following, cell suspension was incubated for an additional five minutes with 1/20 volume 10% Triton X. Cell suspension was then sonicated 2 X 30 seconds until no longer viscous, and then spun down at 17510 x g on Sorvall SA-60 rotor for 1 hr at 4°C.

The supernatant fraction was added to a column of glutathione-agarose beads (Sigma) previously equilibrated with 5 volumes of extraction buffer (50 mM Tris pH 7.3, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA). Supernatant was passed through the column twice. The column was then washed with 10 volumes of buffer A (50 mM Tris pH 8.0, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X, 0.5% Tween-20, 5 mM DTT) and 10 volumes of buffer B (20 mM Tris pH 8.0, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT). p27XicCK- protein was then eluted with 5 mL of elution buffer (20 mM Tris pH 7.0, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 mM glutathione (GSH) (Sigma)). Purified p27XicCK- was then concentrated by buffer exchange using Amicon Ultra Ultracel low binding regenerated cellulose tubes (Millipore, Bedford, MA), resuspended in buffer (20 mM Hepes pH 7.5, 88 mM NaCl, 7.5 mM $MgCl_2$, 10 mM □-mercaptoethanol) and stored at -80°C.

Chapter 3: Chk2/Cds1 Protein Kinase Blocks Apoptosis during Early Development of *Xenopus laevis*

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3.1 Abstract

Early *Xenopus laevis* embryos possess cell cycles that do not arrest at checkpoints in response to damaged DNA. At the midblastula transition (MBT), embryos with damaged DNA undergo apoptosis. After the MBT, DNA damage triggers cell cycle arrest rather than apoptosis. The transition from checkpoint-unregulated to checkpoint-regulated cycles makes *Xenopus* embryos compelling for studying mechanisms regulating response to genomic damage. The DNA damage checkpoint is mediated by the Chk2/Cds1 kinase. Conflicting evidence implicates Chk2 as an inhibitor or promoter of apoptosis. To better understand the developmental function of Chk2, we expressed wild-type (wt) and dominant-negative (DN) Chk2 in *Xenopus* embryos. Wt-Chk2 created a pre-MBT checkpoint due to degradation of Cdc25A and phosphorylation of cyclin-dependent kinases. Embryos expressing DN-Chk2 developed normally until gastrulation and then underwent apoptosis. Conversely, low doses of wt-Chk2 blocked radiation-induced apoptosis. Therefore, Chk2 operates at a switch between cell cycle arrest or apoptosis in response to genomic assaults.

3.2 Introduction

Checkpoints that arrest the cell cycle in response to DNA damage are conserved features among eukaryotes. Mutations incapacitating these checkpoints are frequent in cancers (Bartek and Lukas, 2003). Early embryonic cell cycles of *Xenopus laevis* lack checkpoints (Newport and Dasso, 1989; Anderson *et al.*, 1997; Hensey and Gautier, 1997). Following fertilization, the embryo begins twelve rapid cleavage cycles that alternate between DNA replication and mitosis without cell growth, gap phases, or checkpoints (Newport and Dasso, 1989; Anderson *et al.*, 1997; Hensey and Gautier, 1997). Completion of the twelfth cleavage marks the MBT when transcription initiates, cells become motile, an apoptotic program is functional, and cell cycles lengthen as they acquire the gap phases characteristic of somatic cell cycles (Newport and Kirschner, 1982a; Frederick and Andrews, 1994). Furthermore, checkpoints become operative after

the MBT (Newport and Dasso, 1989). The transition from checkpoint-unregulated to checkpoint-regulated cycles makes *Xenopus* embryos an excellent system for studying the molecular mechanisms that regulate cell cycle arrest and apoptosis in response to damaged DNA.

In eukaryotes, Chk1 and Chk2 kinases are central components of signaling networks activated in response to damaged and unreplicated DNA. Activation of Chk1 or Chk2 can promote cell cycle arrest (via phosphorylation of Cdc25A, Cdc25C, and p53), repair (via phosphorylation of BRCA1), and apoptosis (via phosphorylation of p53, E2F1, and PML) (Novak *et al.*, 2002; Ahn *et al.*, 2004). What determines whether cells respond to DNA damage by arresting the cell cycle or committing apoptosis is not well understood.

It has been shown previously that Chk1 is required for *Xenopus* embryos to survive beyond gastrulation, even in the absence of explicitly damaged DNA (Carter and Sible, 2003). Likewise, Chk1 is essential for early development of mice (Liu *et al.*, 2000; Takai *et al.*, 2000). In contrast, Chk2 is not essential in mice (Takai *et al.*, 2002) but has been implicated as both a promoter and inhibitor of apoptosis (Hirao *et al.*, 2002; Jack *et al.*, 2002; Peters *et al.*, 2002; Takai *et al.*, 2002; Yang *et al.*, 2002; Stevens *et al.*, 2003; Wu and Chen, 2003; Brodsky *et al.*, 2004; Castedo *et al.*, 2004; Hong and Stambrook, 2004; Jack *et al.*, 2004; Rogoff *et al.*, 2004).

With the goal of better understanding the developmental function and regulation of Chk2 in the remodeling cell cycles of the *Xenopus* embryo, we overexpressed wild-type (wt-) or dominant-negative (DN-) Chk2 during early development. Our results indicate that Chk2 functions to inhibit developmentally regulated and DNA damage-induced apoptosis, suggesting that both Chk1 and Chk2 kinases operate at developmental switches that determine whether cells arrest or die in response to genomic assaults.

3.3 Results and Discussion

Overexpression of Wild-Type *Xenopus* Chk2 (wt-Chk2) Triggers a Cell Cycle Checkpoint prior to the MBT

Prior to the MBT, *Xenopus* embryos do not arrest cell cycles in response to damaged or unreplicated DNA (Newport and Dasso, 1989; Anderson *et al.*, 1997; Hensey and Gautier, 1997) even though Chk1 and Chk2 kinases are expressed (Nakajo *et al.*, 1999; Kappas *et al.*, 2000; Gotoh *et al.*, 2001). Chk1 does not become activated by unreplicated DNA until the MBT or by ionizing radiation (IR) until early gastrulation (Kappas *et al.*, 2000), correlating with the time at which embryos arrest cell cycles in response to unreplicated or damaged DNA. However, overexpression of Chk1 triggers degradation of Cdc25A, tyrosine phosphorylation of Cdks, and cell cycle arrest in pre-MBT embryos (Kappas *et al.*, 2000; Shimuta *et al.*, 2002; Petrus *et al.*, 2004). When embryos are treated at the MBT with agents that block DNA replication, Chk2 likewise is activated (Gotoh *et al.*, 2001).

To determine whether pre-MBT cell cycles respond to exogenous Chk2, mRNA encoding FLAG-tagged, wild-type Chk2 (wt-Chk2) or FLAG-tagged luciferase (as a control) was microinjected into one-cell stage embryos. Western analysis with a FLAG antibody confirmed expression of exogenous proteins (Fig. 3-1). Embryos expressing exogenous wt-Chk2 displayed a dose-dependent delay of cleavage cycles. Embryos injected with 2 ng wt-Chk2 mRNA exhibited slower cleavage cycles compared to controls. At 4.5 hr post-fertilization (pf), control embryos had reached developmental Stage 6.5, corresponding to 64 cells (Nieuwkoop and Faber, 1975), whereas embryos expressing wt-Chk2 were approximately one cycle delayed (Stage 6 = 32 cells; Fig. 3-1B). At 8 hr pf, embryos expressing wt-Chk2 completed the MBT and remained delayed by approximately one cell cycle compared to controls (Fig. 3-1B). These embryos proceeded to gastrulate and survived through neurulation (not shown; see Fig. 3-3B). Embryos injected with a higher dose of 8-10 ng wt-Chk2 mRNA arrested cell division at the 2-4 cell stage, whereas control embryos injected with the same dose of luciferase mRNA divided normally (Fig. 3-1C).

To determine whether the cell cycle arrest induced by wt-Chk2 resulted from engagement of known checkpoint events, embryos expressing luciferase or wt-Chk2 were assayed for tyrosine phosphorylation of Cdks by Western analysis with a phosphoCdk antibody. In metazoans, Chk2 inactivates Cdc25 phosphatases, resulting in Cdks in their tyrosine-phosphorylated, inactive state (Bartek *et al.*, 2001; McGowan, 2002). In untreated embryos, periodic tyrosine phosphorylation of Cdks occurs but is low until after the MBT (Ferrell *et al.*, 1991; Hartley *et al.*, 1996; Kim *et al.*, 1999). Likewise, the level of tyrosine phosphorylation in control embryos was low until after the MBT (Fig. 3-1D; 7-9 hr pf). In contrast, Cdks were phosphorylated on tyrosine well before the MBT, by 3 hr pf in embryos expressing exogenous wt-Chk2, indicating that the cell cycle delay resulted from reduced activity of Cdks. Therefore, pre-MBT embryos, which do not arrest cleavage cycles in response to damaged DNA (Anderson *et al.*, 1997; Hensey and Gautier, 1997), can arrest the cell cycle in response to exogenous wt-Chk2. These data fit an emerging model that embryos possess the necessary components of checkpoint signaling pathways, but require threshold amounts of DNA to effectively transduce a signal resulting in cell cycle arrest (Kappas *et al.*, 2000; Conn *et al.*, 2004). The extent of tyrosine phosphorylation in these embryos expressing wt-Chk2 was approximately the same as in control embryos at 9 hr post-fertilization (Fig. 3-1D), when cell cycles are asynchronous, averaging >200 min in length (Howe *et al.*, 1995). Since the embryos expressing this dose of wt-Chk2 exhibited only modestly delayed cleavage cycles (Fig. 3-1B), additional changes at the MBT (such as changes in the pool of cyclin mRNAs (Audic *et al.*, 2002), the switch from less active Wee1 to more active Wee2, and the expression of the Cdk inhibitor, Xic1 (Su *et al.*, 1995)), are likely to contribute to the longer cell cycles at the MBT.

Another event of the MBT that may contribute to cell cycle lengthening is the degradation of Cdc25A, leading to enhanced tyrosine phosphorylation of Cdks (Kim *et al.*, 1999). To determine whether expression of exogenous wt-Chk2 triggered premature degradation of Cdc25A, embryos were assayed for steady-state levels of Cdc25A (Fig. 3-1E). As shown in Figure 3-1E, Cdc25A content was decreased in embryos expressing exogenous wt-Chk2 compared to control embryos. These data provide further evidence

that exogenous Chk2 disrupts cleavage cycles by perturbing known cell cycle checkpoint signaling pathways, leading to decreased activity of Cdks.

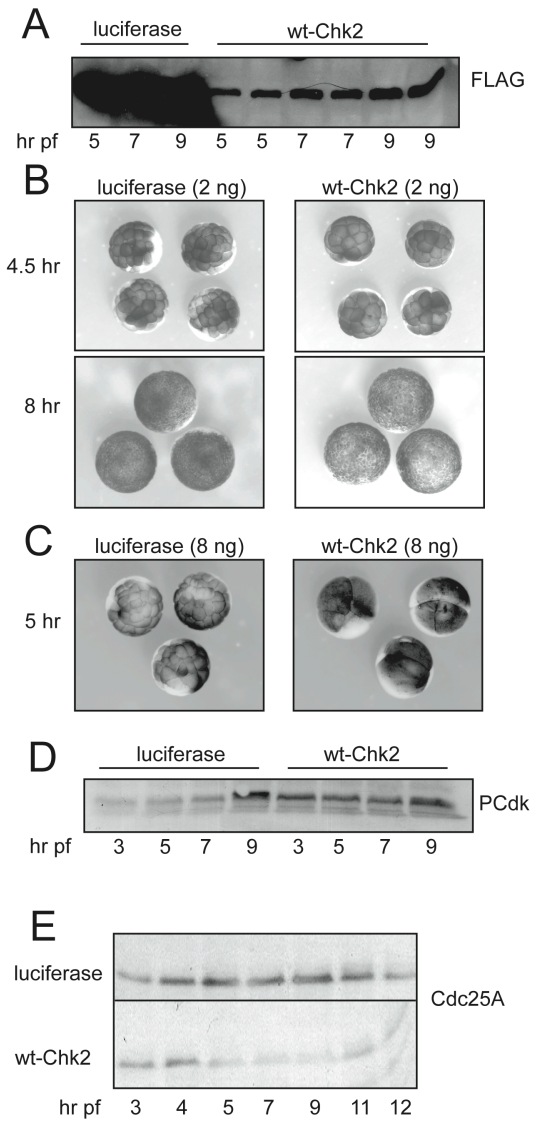

Figure 3-1. Overexpression of wt-Chk2 leads to cell cycle lengthening prior to the MBT. One-cell stage embryos were microinjected with FLAG-tagged wt-Chk2 or luciferase (control) mRNA. **A**: Embryos injected with 2 ng mRNA were collected at indicated times post-fertilization (hr pf) and analyzed for the FLAG tag by Western blotting. Duplicate collections were made of embryos expressing Chk2 to verify consistent expression. **B**:

Xenopus embryos injected with either 2 ng luciferase or wt-Chk2 mRNA were photographed at 4.5 hr pf, when embryos expressing exogenous wt-Chk2 were delayed approximately one cell cycle compared to luciferase controls. Embryos were photographed again at 8 hr. **C**: *Xenopus* embryos were injected with either 8 ng luciferase or wt-Chk2 mRNA. Embryos expressing exogenous wt-Chk2 arrested at the 2-4 cell stage and were photographed at 5 hr pf, when control embryos possessed approximately 64 cells. **D**,**E**: Overexpression of wt-Chk2 results in premature inhibitory phosphorylation of Cdks on tyrosine 15 and premature degradation of Cdc25A. One-cell stage *Xenopus* embryos were microinjected with either 2 ng luciferase (control) or wt-Chk2 mRNA. Embryos were collected at indicated times post-fertilization (hr pf), and subjected to Western blotting with antibodies against tyrosine-phosphorylated Cdks (**D**) and against Cdc25A (**E**).

Expression of Dominant-Negative Chk2 (DN-Chk2) Triggers a Post-MBT Apoptotic Death

Although pre-MBT embryos do not engage checkpoints in response to damaged DNA, Chk1 and Chk2 are expressed throughout early development (Nakajo *et al.*, 1999; Kappas *et al.*, 2000; Gotoh *et al.*, 2001). Chk1 is transiently activated at the MBT (Shimuta *et al.*, 2002) and is required for embryonic survival beyond gastrulation (Hensey and Gautier, 1997; Shimuta *et al.*, 2002; Carter and Sible, 2003). To determine whether Chk2 is required for early development, one-cell stage embryos were microinjected with either 8 ng luciferase or catalytically inactive DN-Chk2 mRNA, and development was observed. Both embryos expressing luciferase or DN-Chk2 developed normally through the MBT. During gastrulation, ~90% of embryos (52/58) expressing DN-Chk2 appeared abnormal with loss of organization and cell-cell attachment, whereas all embryos expressing luciferase appeared normal. At neurulation, embryos expressing luciferase continued to develop normally, whereas the embryos expressing DN-Chk2 had lost attachments and appeared abnormal (Fig. 3-2A). The morphology of embryos expressing DN-Chk2 resembled that of embryos that had undergone apoptosis in response to IR (Anderson *et al.*, 1997; Hensey and Gautier, 1997), aphidicolin, and expression of DN-Chk1 (Carter and Sible, 2003).

To determine whether DN-Chk2 induced apoptosis, albino embryos expressing luciferase or DN-Chk2 were collected at gastrulation and subjected to whole-mount

TUNEL assays, which detect fragmented DNA, characteristic of apoptotic cells (Sible *et al.*, 1997). Approximately 90% (50/55) of embryos expressing DN-Chk2 exhibited a positive TUNEL reaction indicated by punctate, deep purple staining. By comparison, ~2% (1/52) of embryos expressing luciferase exhibited positive TUNEL staining (Fig. 3-2B).

As a biochemical assay of apoptosis, embryos expressing luciferase or DN-Chk2 were collected at the times indicated and assayed for caspase activity by the cleavage of PARP protein (Carter and Sible, 2003). Embryos expressing DN-Chk2 were positive for caspase activity beginning at gastrulation (Fig. 3-2C), indicating that these embryos had undergone apoptosis. Therefore, *Xenopus* embryos may require Chk2 function in order to survive beyond the MBT.

Previously, Shimuta et al. 2002 observed death in embryos expressing DN-Chk1 but not DN-Chk2. The death of embryos expressing DN-Chk1 corresponded with a delayed increase in tyrosine phosphorylation of Cdks and degradation of Cdc25A and could be mimicked by expressing nondegradable Cdc25A (Shimuta *et al.*, 2002). Because different mutations were generated to create the dominant-negative constructs (S324A vs. D340A), the proteins expressed by their group may have functioned differently in the embryo or may have been expressed at lower levels. To test whether our DN-Chk1 and DN-Chk2 created the same effects on cell cycle remodeling at the MBT, we compared the effects of each (Fig. 3-2D). Our data indicate that both DN-Chk1 and DN-Chk2 delay the increase in tyrosine phosphorylation of Cdks and degradation of Cdc25A, which normally begins at the MBT. Therefore, expression of our DN-Chk2 construct disrupts cell cycle remodeling at the MBT and triggers a program of apoptosis.

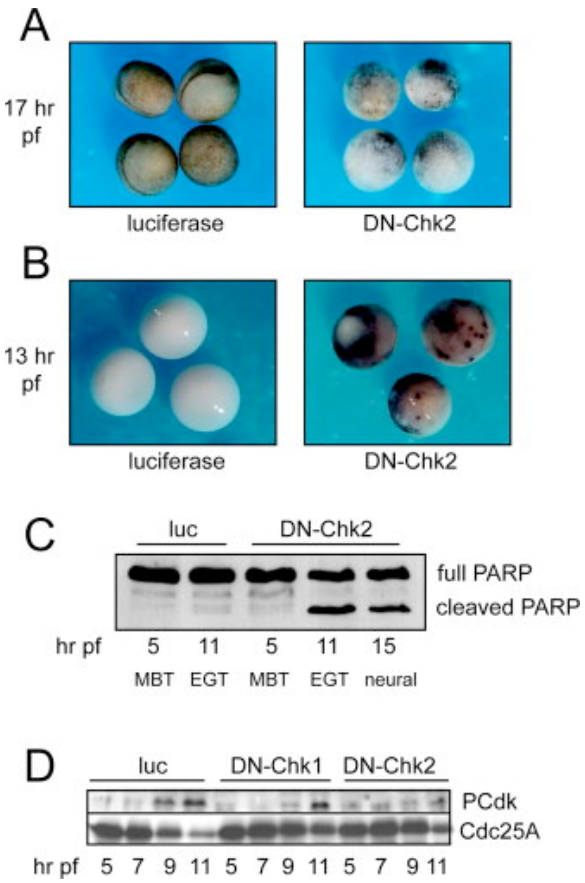

Figure 3-2. Expression of DN-Chk2 results in apoptosis after the MBT. One-cell stage *Xenopus* embryos were microinjected with either 8 ng luciferase (control) or dominant-negative Chk2 (DN-Chk2) mRNA. **A**: Embryos expressing DN-Chk2 and luciferase developed normally until the early gastrula stage. Embryos shown were photographed when controls were neurulating (17 hr pf). **B**: Whole-mount TUNEL assays were performed on albino embryos expressing luciferase or DN-Chk2. When abnormal morphology was evident at gastrulation (13 hr pf), embryos were collected and processed. A positive TUNEL reaction is indicated by deep purple staining. Control (luciferase) embryos exhibit normal morphology but are white because they are albino. **C**: Embryo extracts were collected at the times indicated and incubated with recombinant PARP, a substrate for caspase 3 (Carter and Sible, 2003). The presence of a cleaved PARP fragment indicates caspase activity. **D**: Embryo extracts were collected at the times indicated. Western blotting was performed with antibodies against tyrosine-phosphorylated Cdks (PCdk) and Cdc25A.

Exogenous wt-Chk2 Inhibits Apoptosis following Ionizing Radiation (IR)

Embryos exposed before the MBT to a variety of assaults to the genome, including IR, develop normally through the MBT and then die by apoptosis during early gastrulation (Anderson *et al.*, 1997; Hensey and Gautier, 1997; Sible *et al.*, 1997; Stack and Newport, 1997). When embryos are exposed to these same assaults after the MBT, they are resistant to apoptosis (Anderson *et al.*, 1997; Hensey and Gautier, 1997). To see how susceptibility to apoptosis correlated with activation of Chk2, embryos were exposed to IR before (3 hr pf) or after (14 hr pf) the MBT, then collected at several times points and analyzed by Western blotting for activation of Chk2, as determined by a shift in electrophoretic mobility, indicative of the activating phosphorylation (McSherry and Mueller, 2004). When exposed to 30 or 60 Gy IR before the MBT, no shift in Chk2 mobility was observed after 1 or 4 hr, suggesting that little or no Chk2 was activated in these embryos, which ultimately died by apoptosis during early gastrulation (Fig. 3-3A). In contrast, when embryos were irradiated after the MBT, a fraction of the Chk2 was shifted 1 or 2 hr later. The amount of shifted Chk2 was greater in embryos treated with 60 Gy versus 30 Gy IR, indicating a dose-dependent effect (Fig. 3-3A). These experiments indicate an inverse correlation between activation of Chk2 and susceptibility to apoptosis in embryos.

Based on these results as well as our observation that DN-Chk2 triggers apoptosis during gastrulation of *Xenopus* embryos (Fig. 3-2), it was hypothesized that wt-Chk2 could play a dominant role in blocking apoptosis. To test this hypothesis, one-cell stage embryos were microinjected with 2 ng luciferase or wt-Chk2 mRNA, then exposed to 30 Gy IR before the MBT, at 3.5 hr pf. All embryos (20/20) expressing luciferase exhibited abnormal morphology, consistent with apoptosis, at gastrulation (Fig. 3-3B). In contrast, only 10% of embryos (2/20) expressing exogenous wt-Chk2 exhibited abnormal morphology at gastrulation, and 25-30% completed gastrulation and formed a neural groove (Fig. 3-3B). Therefore, irradiated embryos expressing exogenous Chk2 developed normally, well beyond the developmental stage at which irradiated embryos usually die by apoptosis. These data indicate that Chk2 inhibits IR-induced apoptosis in the early *Xenopus* embryo.

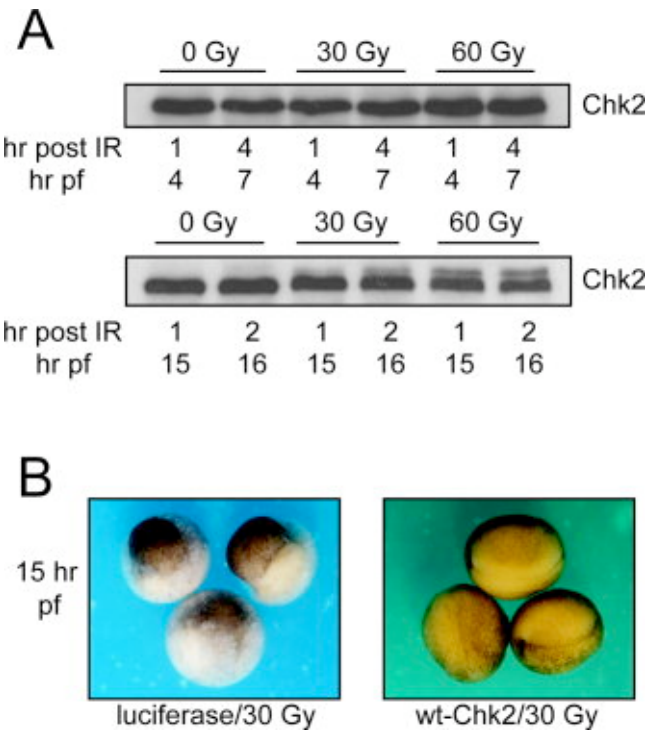

Figure 3-3. Expression of exogenous wt-Chk2 inhibits apoptosis following IR. **A**: Endogenous Chk2 becomes activated after the MBT in response to IR. Embryos were treated at 3 hr (pre-MBT; top) or 14 hr (post-MBT; bottom) with the indicated dose of IR (Gy), then collected at the times indicated, and subjected to Western analysis with a Chk2 antibody. The phosphorylated form of Chk2 is retarded in electrophoretic mobility (McSherry and Mueller, 2004). **B**: One-cell stage *Xenopus* embryos were microinjected with either 2 ng of luciferase (control) or wt-Chk2 mRNA. Embryos were subjected to 30 Gy IR prior to the MBT, at 3.5 hr pf. One hundred percent of embryos expressing luciferase exhibited abnormal morphology, consistent with apoptosis, at gastrulation (11 hr pf). In contrast, only 10% of embryos expressing exogenous wt-Chk2 exhibited abnormal morphology at gastrulation. Furthermore, 25-30% of IR-treated embryos expressing exogenous wt-Chk2 completed gastrulation and proceeded to neurulate at 15 hr pf (as shown).

Does Chk2 Inhibit or Promote Apoptosis?

We provide evidence that Chk2 inhibits apoptosis during early development of *Xenopus laevis*. However, other studies have indicated that Chk2 promotes apoptosis (Ahn *et al.*, 2004). Mice lacking the Chk2 gene are viable, but deficient in IR-induced apoptosis (Hirao *et al.*, 2002; Jack *et al.*, 2002; Takai *et al.*, 2002; Jack *et al.*, 2004). In the *Drosophila* eye, Chk2 promotes the induction of apoptosis in response to DNA damage (Peters *et al.*, 2002). In both cases, Chk2 promotes apoptosis via phosphorylation of p53. In human breast carcinoma cells, Chk2 promotes DNA damage-induced apoptosis by phosphorylating the transcription factor E2F-1 (Stevens *et al.*, 2003), and in lymphoma cells by phosphorylating the scaffolding protein PML (Yang *et al.*, 2002). Thus, in response to DNA damage, Chk2 can phosphorylate at least three targets to promote apoptosis (Ahn *et al.*, 2004).

In contrast, other studies have implicated Chk2 as an inhibitor of apoptosis. Mouse embryonic stem cells are deficient in a G1 checkpoint and highly susceptible to IR-induced apoptosis. Ectopic expression of Chk2 in these cells confers protection from IR-induced apoptosis. This protection may be p53-independent, since the p53 target, p21, is not induced (Hong and Stambrook, 2004). Expression of DN-Chk2 in human heterokarya and HCT116 colon carcinoma cells promotes apoptosis in response to mitotic catastrophe (Castedo *et al.*, 2004). Likewise, we show both that exogenous wt-Chk2 blocked IR-induced apoptosis (Fig. 3-3B) and DN-Chk2 promoted apoptosis (Fig. 3-2) in the early *Xenopus* embryo, implicating Chk2 as an inhibitor of apoptosis.
Our studies may shed light upon the apparent paradox regarding the relationship between Chk2 and apoptosis. Early embryos contain p53, but development is not noticeably affected by expression of either wt- or DN-p53 until well past gastrulation (Wallingford *et al.*, 1997). Furthermore, p53 cannot function as a transcription factor before the MBT because transcription is silent (Newport and Kirschner, 1982b). Therefore, early embryos provide a context in which the p53-signaling pathway is not fully functional, and may explain why Chk2 does not promote apoptosis. On the other hand, we have shown that Chk2 and Chk1 are capable of activating cell cycle checkpoints in the early embryos by indirectly triggering tyrosine phosphorylation of cyclin-dependent kinases (Fig. 3-1) (Kappas *et al.*, 2000). By causing cell cycle delay in response to IR, Chk2 may allow for

repair and may also promote repair pathways through its interaction with proteins such as BRCA1 and Mus81 (McGowan, 2002). Since many tumors lack functional p53, it will be important to determine whether Chk2 truly blocks apoptosis in a p53-negative background, in order to decide when drugs that target Chk2 are appropriate chemotherapeutics.

Interestingly, induction of apoptosis by DN-Chk2 did not require exposure to IR or any other overt assault to the embryo. At this stage, it is unknown why *Xenopus* embryos require Chk2 and Chk1 (Shimuta *et al.*, 2002; Carter and Sible, 2003) during early development, but we suggest two possibilities: (1) inhibition of Chk2 during the 12 rapid cycles, which lack checkpoints before the MBT inevitably results in DNA damage that triggers apoptosis post-MBT, or (2) the lengthening of cell cycles at the MBT requires functional Chk2 and/or Chk1 (Shimuta *et al.*, 2002; Carter and Sible, 2003). As these possibilities are explored in the *Xenopus* embryo, we will build a better understanding of how checkpoint controls are coordinated with other aspects of metazoan development.

Chapter 4. Wee1 Kinase Promotes Apoptosis during the Early Embryonic Development of *Xenopus laevis*

4.1 Abstract

In somatic cells, when endogenous DNA is damaged or unreplicated, checkpoint pathways function to arrest the cell cycle prior to M or S phases through the inhibition of cyclin-dependent kinases (Cdks). In *Xenopus laevis*, the early embryonic cellular divisions 2-12 consist of rapid cleavage cycles in which gap phases, cell cycle checkpoint engagement, and an apoptotic program are absent. Upon the completion of the 12th cellular division, the midblastula transition (MBT) begins and cell cycle lengthens, acquiring gap phases. In addition, cell cycle checkpoint pathways and an apoptotic program become functional. While some cell cycle remodeling events are dependent upon a critical nucleo-cytoplasmic ratio, others depend on an autonomous maternal timing mechanism controlled by cyclin E/Cdk2 activity. These events include degradation of maternal cyclin E itself. Another key cell cycle remodeling event that occurs at the MBT is the degradation of maternal Wee1. In order to determine the role of Wee1 during cell cycle remodeling at the MBT, exogenous Wee1 was expressed in one-cell stage embryos. Overexpression of Wee1 created a pre-MBT cell cycle checkpoint, characterized by cell cycle delay and inhibitory phosphorylation of Cdks on tyrosine 15. Furthermore, overexpression disrupted key developmental events that normally occur at the MBT such as the degradation of Cdc25A, cyclin E, and Wee1. Similarly, ionizing radiation (IR) also delayed the degradation of these proteins. The overexpression of Wee1 also partially inhibited DNA synthesis. Interestingly, overexpression of Wee1 also resulted in post-MBT apoptosis, as determined by gross morphology and activation of caspases. Furthermore, apoptosis was shown to correlate with the inhibition of Cdk2. To determine whether Cdk2 was required specifically for the survival of the embryo, the specific cyclin E/Cdk2 inhibitor, Δ34-Xic1, was expressed in embryos and also shown to induce apoptosis. Taken together, these data suggest that Wee1 triggers apoptosis through the disruption of the cyclin E/Cdk2 timer.

4.2 Introduction

Cell cycle checkpoints that function to arrest the cell cycle in the presence of damaged or unreplicated DNA are highly conserved among eukaryotes. Genetic mutations compromising these checkpoints are common among cancers (Bartek and Lukas, 2003). Similarly, the first twelve cell cycles of early *Xenopus laevis* development lack functional checkpoints (Newport and Dasso, 1989; Anderson *et al.*, 1997; Hensey and Gautier, 1997). At the midblastula transition (MBT), the cell cycle lengthens and acquires gap phases and operable cell cycle checkpoints. Additionally, transcription initiates and an apoptotic program gains functionality, both of which are absent during pre-MBT development (Newport and Kirschner, 1982a; Frederick and Andrews, 1994). The rapid transition from a highly unregulated to a highly regulated cell cycle makes *Xenopus* an excellent model system for studying cell cycle remodeling and regulation.

In *Xenopus*, three major complexes that regulate the cell cycle include cyclin A/Cdk2, cyclin B/Cdc2 (MPF), and cyclin E/Cdk2. MPF and cyclin E/Cdk2 are the key complexes that regulate the G2/M and G1/S phase transitions of the cell cycle, promoting mitosis and DNA replication, respectively (Strausfeld *et al.*, 1994; Jackson *et al.*, 1995). Various upstream regulators, including the Wee1 tyrosine kinase, regulate these complexes. In *Xenopus*, Wee1 is present in pre-MBT embryos, but degraded following the MBT (Murakami and Woude, 1998). Wee1 acts in opposition to Cdc25C, inhibiting MPF through phosphorylation of Tyr-15 on Cdc2 (Parker *et al.*, 1992b; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993; Mueller *et al.*, 1995a). In mammalian cells, Wee1 has also been shown to inhibit cyclin E/Cdk2 through phosphorylation of Tyr-15 on Cdk2 (Watanabe *et al.*, 1995; Wu *et al.*, 2001). It is however unknown if Wee1 maintains this function in *Xenopus*.

In *Xenopus laevis*, a maternal developmental timer, driven by oscillations in cyclin E/Cdk2 activity, is partially responsible for cell cycle remodeling that takes place at the MBT (Hartley *et al.*, 1997). One of these very important cell cycle remodeling events is the degradation of maternal cyclin E (Howe and Newport, 1996). Although cyclin E levels are constant throughout pre-MBT development, cyclin E/Cdk2 activity oscillates twice per cell cycle, independently of protein synthesis and the nucleo-cytoplasmic ratio (Hartley *et al.*, 1996; Howe and Newport, 1996; Hartley *et al.*, 1997).

The oscillatory activity of cyclin E/Cdk2 governed by changes in phosphorylation state, drive the early *Xenopus* cell cycles. Furthermore, the degradation of cyclin E at the MBT is probably triggered by the cyclin E/Cdk2 timer itself (Hartley *et al.*, 1997; Ciliberto *et al.*, 2003). The inhibition of Cdk2 by the specific Cdk inhibitor, p34Xic1, delays the onset of the MBT and the degradation of cyclin E. However, other inhibitors of the MBT such as α-amanitin (blocks zygotic transcription) and cycloheximide (blocks protein synthesis) do not affect the timing of cyclin E degradation (Hartley *et al.*, 1997). Presently, the overexpression of Chk1 in *Xenopus* embryos is the only other manipulation known to inhibit both the MBT and cyclin E degradation (Petrus *et al.*, 2004). Furthermore, the overexpression of Chk1 causes the premature degradation of Cdc25A (Petrus *et al.*, 2004), the activating phosphatase of cyclin E/Cdk2. Importantly, Cdc25A degradation, which normally coincides with the degradation of cyclin E at the MBT, is another cell cycle remodeling event that is necessary for post-MBT development.

Since Wee1 is the opposing kinase of Cdc25, degraded at the MBT, and functions to inhibit both MPF in metazoans and cyclin E/Cdk2 in mammals, we overexpressed Wee1 in *Xenopus* embryos to more closely identify its role in cell cycle remodeling during the early embryonic development of *Xenopus*. Our results indicate that Wee1 functions to promote apoptosis, most likely through the disruption of the cyclin E/Cdk2 timer. These data suggest that like cyclin E, Wee1 may play a role in the timing mechanism of the MBT.

4.3 Results

Overexpression of Wee1 Triggers a Cell Cycle Delay prior to the MBT

Prior to the MBT, *Xenopus* embryos do not arrest cell cycles in response to damaged or unreplicated DNA (Newport and Dasso, 1989; Anderson *et al.*, 1997; Hensey and Gautier, 1997) despite expression of the cell cycle checkpoint proteins, Chk1 and Chk2 (Nakajo *et al.*, 1999; Kappas *et al.*, 2000; Gotoh *et al.*, 2001). At the MBT, Chk1 (Kappas *et al.*, 2000) and Chk2 (Gotoh *et al.*, 2001) first become activated by unreplicated DNA or ionizing radiation (IR) (Kappas *et al.*, 2000; Wroble and Sible,

2005). However, overexpression of either exogenous Chk1 and Chk2 results in a pre-MBT cell cycle checkpoint (Kappas *et al.*, 2000; Wroble and Sible, 2005), indicating intact signaling downstream of the checkpoint kinases.

To determine whether exogenous Wee1 affects pre-MBT cell cycles, mRNA encoding wild-type Wee1 or luciferase (control) was microinjected into one-cell stage embryos. Western analysis confirmed expression of exogenous Wee1 (Fig. 4-1A). Embryos injected with ~2.5 ng Wee1 mRNA exhibited slower cleavage cycles compared to controls. At 4.5 hrs post-fertilization (hrs pf), embryos expressing exogenous Wee1 were delayed by approximately one cell cycle compared to controls (Fig. 4-1B) (Stage 6 = 32 blastomeres compared to Stage 7 = 64 blastomeres) (Nieuwkoop and Faber, 1975). Otherwise, these embryos developed normally through the MBT.

To determine whether the cell cycle lengthening induced by exogenous Wee1 resulted from cell cycle checkpoint engagement, embryos expressing exogenous Wee1 and luciferase were assayed for phosphorylation of Cdks on tyrosine 15 by Western analysis using a phosphoCdk primary antibody (Fig. 4-1C). In untreated embryos, low-level tyrosine phosphorylation of Cdks occurs prior to the MBT (Ferrell *et al.*, 1991; Hartley *et al.*, 1996; Kim *et al.*, 1999). Similarly, control embryos expressed low levels of Cdk phosphorylation until the MBT. However, Cdks were phosphorylated on tyrosine 15 as early as 3 hrs pf in embryos expressing exogenous Wee1, suggesting the cell cycle delay resulted from the inhibition of Cdks.

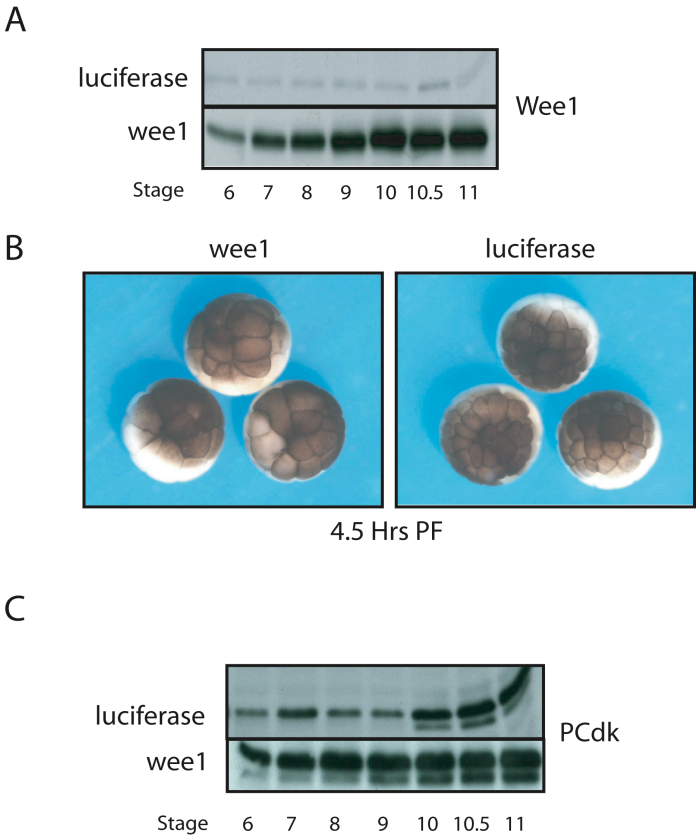

Figure 4-1. Overexpression of Wee1 triggers a cell cycle checkpoint prior to the MBT. One-cell stage embryos were microinjected with Wee1 or luciferase (control) mRNA. **A**: Embryos injected with 2.5 ng mRNA were collected at indicated times post-fertilization (hr pf) and analyzed for the Wee1 expression by Western analysis with a Wee1 antibody (Zymed). **B**: *Xenopus* embryos injected with either 2.5 ng Wee1 or luciferase mRNA were photographed at 4.5 hr pf, when embryos expressing exogenous Wee1 were delayed approximately one cell cycle compared to luciferase controls. **C**: Overexpression of Wee1 results in premature inhibitory phosphorylation of Cdks on tyrosine 15. Embryo lysates in **A** were subjected to Western analysis using a phosphoCdk primary antibody. The presence of two bands is suggestive of the higher molecular weight p34Cdc2 and the lower molecular weight p32Cdk2, which are both recognized. Phosphorylation of Cdks was apparent as early as Stage 6 (3 hrs pf) in embryos injected with Wee1.

Overexpression of Wee1 Triggers Apoptosis after the MBT

Xenopus embryos expressing exogenous Chk1, Chk2, and Wee1 exhibit a similar cell cycle delay resulting from premature Cdk phosphorylation (Kappas *et al.*, 2000; Wroble and Sible, 2005). Furthermore, embryos expressing exogenous Chk2 develop normally through neurulation and beyond (Wroble and Sible, 2005). Similarly, embryos expressing Wee1 developed normally through the MBT until early gastrulation (~Stage 10.5). However, during gastrulation, all embryos expressing exogenous Wee1 appeared abnormal, exhibiting a loss of cellular attachment and overall organization, compared to normal controls (Fig. 4-2A). The gross morphology of embryos expressing exogenous Wee1 appeared consistent with that of embryos that have undergone apoptosis in response to IR (Anderson *et al.*, 1997; Hensey and Gautier, 1997), unreplicated DNA, expression of catalytically inactive DN-Chk1 (Carter and Sible, 2003) and DN-Chk2 (Wroble and Sible, 2005).

To determine whether overexpression of Wee1 induced apoptosis, embryos were assayed for caspase activity by the cleavage of the PARP protein (Carter and Sible, 2003). Embryos expressing exogenous Wee1 were positive for caspase activity at gastrulation (Stage 11) indicated by the presence of a cleaved PARP fragment, compared to controls, which were negative for caspase activity (Fig. 4-2B).

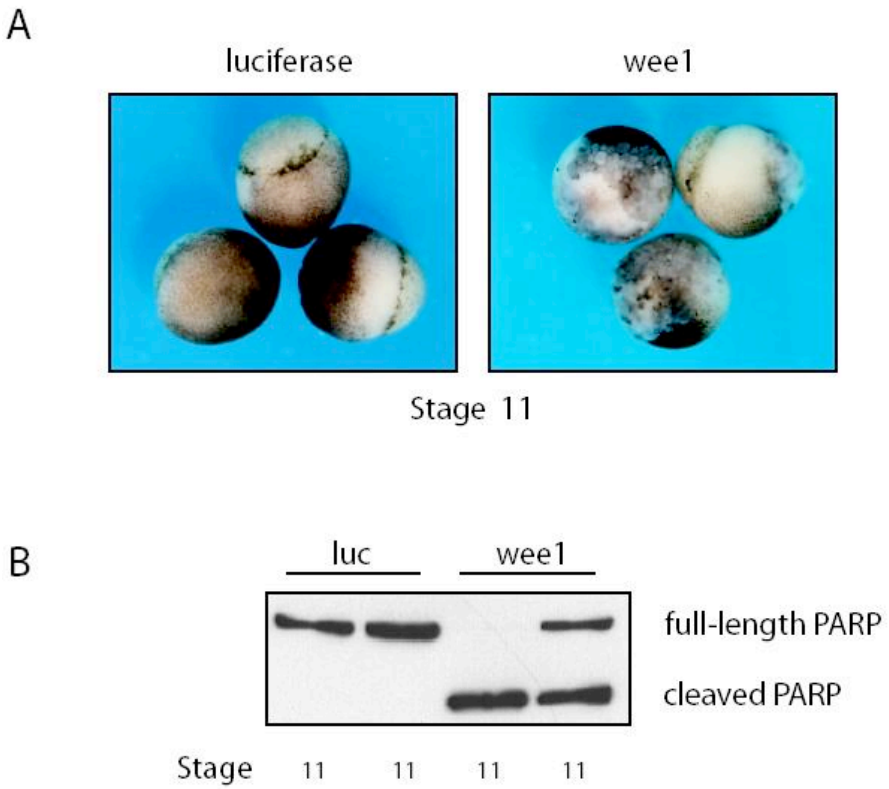

Figure 4-2. Overexpression of Wee1 triggers a post-MBT apoptotic death. One-cell stage *Xenopus* embryos were microinjected with either 2.5 ng Wee1 or luciferase (control) mRNA. **A**: Embryos expressing Wee1 and luciferase developed normally until the early gastrula stage, when embryos expressing exogenous Wee1 exhibited abnormal morphology consistent with apoptosis. Embryos shown were photographed at gastrulation. **B**: Embryo extracts were collected at Stage 11 (gastrulation) and incubated with recombinant PARP, a substrate for caspase 3 (Carter and Sible, 2003). The presence of a cleaved PARP fragment indicates caspase activity.

Overexpression of Wee1 Delays the Degradation of Cdc25A and Cyclin E

In *Xenopus* embryos, the MBT marks an important point during development when a host of cell cycle remodeling events occur. Two of these important events include the degradation of both Cdc25A (Izumi and Maller, 1995; Hartley *et al.*, 1996; Kim *et al.*, 1999) and cyclin E (Rempel *et al.*, 1995; Hartley *et al.*, 1996). Overexpression of Chk1 (Petrus *et al.*, 2004) and Chk2 (Wroble and Sible, 2005) in embryos results in the premature degradation of Cdc25A. Furthermore, the transient activation of Chk1 at the MBT is required for the degradation of Cdc25A (Shimuta *et al.*, 2002). Since Chk1, Chk2, and Wee1 overexpression all result in the premature phosphorylation of Cdks, we wanted to determine whether Wee1 overexpression would also result in the premature degradation of Cdc25A.

To determine the effect of overexpression of Wee1 on Cdc25A levels, mRNA encoding wild-type Wee1 (wee1) or luciferase (control) was microinjected into one-cell stage embryos. Embryos were then collected at the indicated times and subjected to Western analysis using a primary Cdc25A antibody. Surprisingly, Wee1 overexpression resulted in the delay of Cdc25A degradation, compared to controls (Fig. 4-3A). Thus, Chk1, Chk2, and Wee1 overexpression all delay the cell cycle and cause premature phosphorylation of Cdks. However, Chk1 and Chk2 overexpression triggers premature degradation of Cdc25A compared to controls, whereas Wee1 overexpression delays the degradation of Cdc25A compared to controls. Furthermore, Chk1 and Chk2 function as inhibitors of apoptosis in *Xenopus* embryos (Carter and Sible, 2003; Wroble and Sible, 2005).

Due to similar apoptotic effects of both Wee1 overexpression and pre-MBT IR treatment (Anderson *et al.*, 1997), it was important to determine what effect, if any, IR treatment had on the normal degradation of Cdc25A. To determine the effect of IR treatment, pre-MBT (2 hrs pf) and MBT (6 hrs PF) embryos were treated with 0 (control) or 30 Gy IR and subjected to Western analysis using a primary Cdc25A antibody (Fig. 4-3B). Compared to controls (0 Gy and 30 Gy treated MBT (6 hrs PF) embryos), Cdc25A degradation was delayed in embryos treated with 30 Gy IR pre-MBT.

As mentioned previously, cyclin E degradation is another key MBT event that occurs during normal development. Maternal cyclin E, when associated with Cdk2, acts

as a developmental timer that drives the early embryonic cell cycles until the MBT (Hartley *et al.*, 1997). Presently, only the expression of exogenous truncated *Xenopus* Cdk2 inhibitor, Δ34Xic1 (Hartley *et al.*, 1997), and Chk1 (Petrus *et al.*, 2004) have been shown to alter the degradation of Cyclin E. Since Cdc25A is the activating phosphatase of cyclin E/Cdk2, and Wee1 overexpression and IR treatment delayed the degradation of Cdc25A, it was important to determine whether these manipulations had any effect on the degradation of cyclin E.

To determine the effect of Wee1 overexpression and IR treatment on Cyclin E degradation, embryo lysates from Figure 4-3A/B were assayed for cyclin E levels by Western analysis. Wee1 overexpression and IR treatment significantly delayed the degradation of cyclin E until mid-gastrulation (Fig. 4-3C/D), similar to Chk1 overexpression (Petrus *et al.*, 2004) and the expression of exogenous Δ34-Xic1 (Hartley *et al.*, 1997). These results suggest that in *Xenopus*, overexpression of Wee1 and pre-MBT DNA damage, induced by IR, disrupt cell cycle remodeling that takes place at the MBT. Furthermore, the disruption of the cyclin E/Cdk2 timer, indicated by the delay in maternal cyclin E degradation might trigger the post-MBT apoptotic death that occurs at early gastrulation.

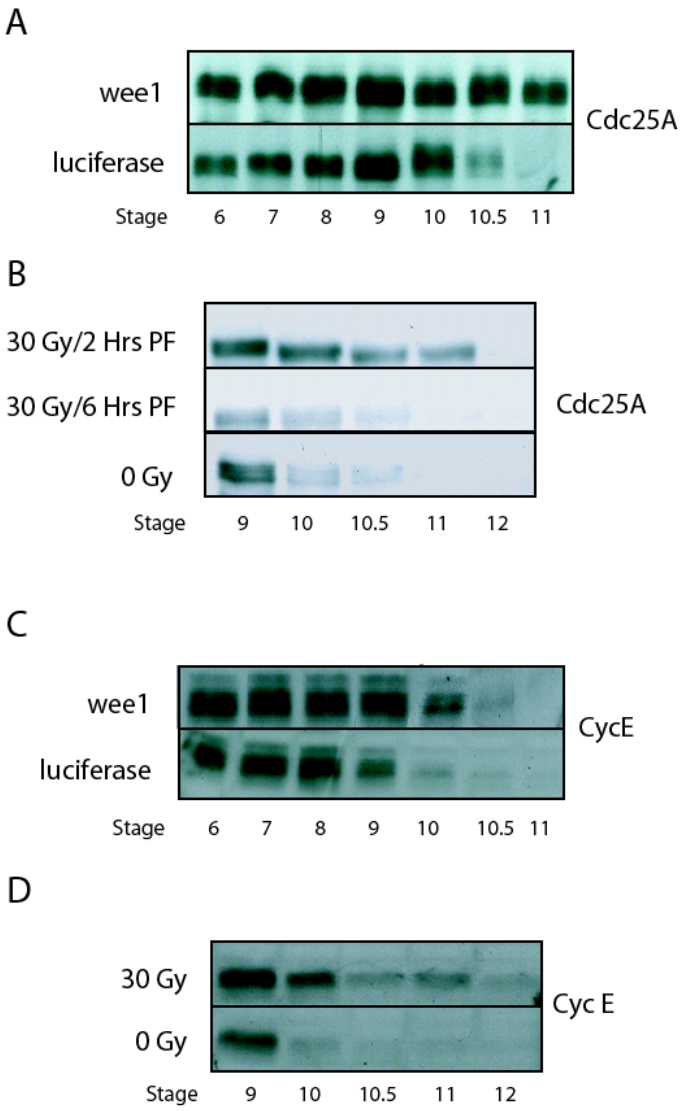

Figure 4-3. Overexpression of Wee1 and IR-induced DNA damage delays the degradation of Cdc25A and Cyclin E. Embryos were **A/C:** injected at the one-cell stage with 2.5 ng of Wee1 or luciferase (control) mRNA or **B/D:** treated with 30 or 0 Gy IR at 2 and 6 hrs pf. Embryos were collected at the indicated stages and lysates were subjected to Western analysis using **A/B:** Cdc25A and **C/D:** cyclin E antibodies.

Wee1 Inhibits Cdk2

Wee1 specifically interacts with Cdk1 (Cdc2), thereby inhibiting entry into mitosis (Russell and Nurse, 1987b; Featherstone and Russell, 1991; Parker *et al.*, 1992a; Parker and Piwnica-Worms, 1992). In human HeLa cells, Wee1 phosphorylates Cdk2, inhibiting entry into S-phase (Watanabe *et al.*, 1995; Wu *et al.*, 2001). In the current study, overexpression of Wee1 delayed the degradation of Cdc25A and cyclin E, suggesting inhibition of Cdk2, since it has been previously shown that Cdk2 inhibition disrupts the timing of cyclin E degradation (Hartley *et al.*, 1997; Ciliberto *et al.*, 2003).

In order to determine whether Wee1 specifically inhibits Cdk2 in *Xenopus*, embryos expressing exogenous Wee1 and luciferase (control) were subjected to IP (immunoprecipitation) Western analysis. Since cyclin E only associates with Cdk2, embryo lysates were immunoprecipitated with cyclin E antiserum, and subsequently used for Western analysis using a PhosphoCdk primary antibody to detect phosphorylated Cdk2. Embryos overexpressing Wee1 expressed higher levels of phosphorylated Cdks (PCdk) indicating inhibition of Cdk2 by Wee1 (Fig. 4-4). These results suggest that the delays in degradation of cyclin E and Cdc25A by the overexpression of Wee1 could be due to the inhibition of Cdk2.

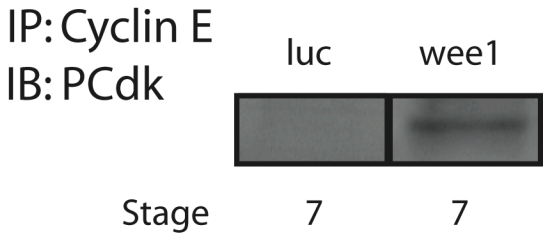

Figure 4-4. Exogenous Wee1 causes tyrosine phosphorylation of Cdk2. Embryos were injected at the one-cell stage with 2.5 ng of Wee1 or luciferase (control) mRNA. Stage 7 embryo lysates were immunoprecipitated with cyclin E serum and subjected to Western analysis using a primary phosphoCdk antibody (Cell Signaling Technology).

Wee1 Overexpression Inhibits DNA Accumulation

Overexpression of Wee1 results in the inhibition of Cdks, triggering a pre-MBT cell cycle delay. Based upon the disruption of normal cell cycle remodeling (Figs. 4-3A/B) and inhibition of Cdks (Fig. 4-4), overexpression of Wee1 may inhibit DNA accumulation resulting in a nucleo-cytoplasmic ratio below the critical concentration necessary for the engagement of a new cell cycle program characteristic of post-MBT development (Newport and Kirschner, 1982a). Furthermore, this sub-critical nucleo-cytoplasmic ratio could partially account for the post-MBT apoptotic death in embryos expressing exogenous Wee1 since aphidicolin (inhibits DNA replication) (Carter and Sible, 2003) and cycloheximide (inhibits protein synthesis) (Sible *et al.*, 1997) treatments both inhibit DNA accumulation and induce post-MBT apoptosis. To test this hypothesis, embryos expressing Wee1 or luciferase, were subject to a DNA content assay. Embryos expressing exogenous Wee1 and contained less DNA throughout early development, compared to luciferase controls (Fig. 4-5). These results suggest that the overexpression

of Wee1 inhibits DNA accumulation, contributing to the induction of post-MBT apoptosis.

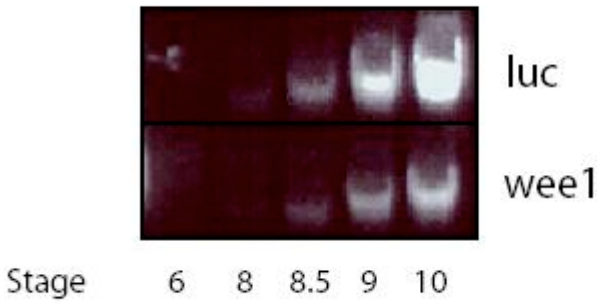

Figure 4-5. Wee1 overexpression inhibits DNA accumulation. Embryos were injected at the one-cell stage with Wee1 or luciferase (control) mRNA. Embryos (n=10) were collected at the indicated stages and subject to a DNA content assay. Embryos expressing exogenous Wee1 contained less DNA at each indicated stage compared to their respective controls.

Inhibition of Cdk2 Triggers Apoptosis

The overexpression of Wee1 in *Xenopus* embryos results in a post-MBT apoptotic death with 100% efficiency (40/40 embryos). Since the overexpression of Wee1 not only alters cell cycle remodeling events (Fig. 4-3A/C) and alters DNA concentration at the MBT (6 Hrs PF) (Fig 4-5), but also interacts with Cdk2 (Fig. 4-4), it was important to determine whether the inhibition of Cdk2 contributed to the induction of apoptosis. In order to determine whether specific inhibition of Cdk2 resulted in apoptosis, one-cell stage embryos were injected with either 5 ng Δ34-Xic1 or p27XicCK- (mutant control) proteins. Δ34-Xic1 is a truncated form of the full length-Xic1 that inhibits the activity of *Xenopus* cyclin E/Cdk2 complexes without affecting other cyclin/Cdk complexes (Su *et al.*, 1995). In *Xenopus* embryos, the expression of Δ34-Xic1 delays the degradation of

cyclin E, thereby disrupting the cyclin E/Cdk2 timer. The p27XicCK- construct contains 4 mutations in the Cdk2 binding site (See 2.10), inhibiting Xic1/Cdk2 interaction.

Embryos expressing Δ34-Xic1 were delayed slightly (~1 cell cycle) compared to Xic1 mutant controls (Fig. 4-6A), however developed otherwise normally through the MBT. Prior to gastrulation, embryos expressing Δ34-Xic1 died by apoptosis, indicated by gross morphology (Fig. 4-6B) and PARP cleavage (Fig. 4-6D). In contrast, embryos expressing p27XicCK- protein persisted through gastrulation and neurulated (Fig. 4-6C). This suggests that specific inhibition of Cdk2 directly results in apoptosis in *Xenopus* embryos. Furthermore, these data also provide evidence that the incidence of apoptosis due to the overexpression of Wee1 in embryos is at least partially due to the inhibition of Cdk2 by Wee1.

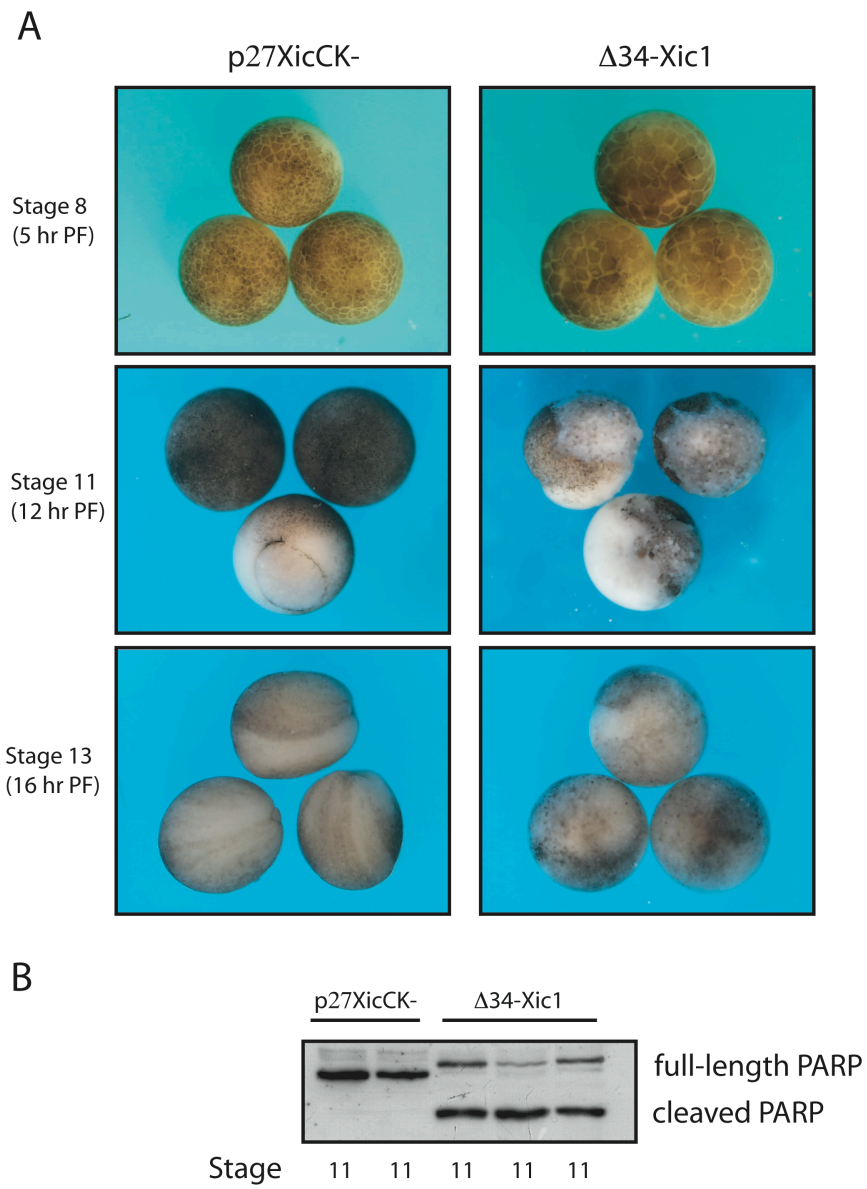

Figure 4-6. Inhibition of Cdk2 triggers apoptosis. One-cell stage embryos were injected with 5 ng of Δ34-Xic1 or p27XicCK- (control). **A:** Embryos expressing Δ34-Xic1 were delayed approximately one cell cycle at Stage 8 (5 hr PF) compared to p27XicCK- controls. At Stage 11 (12 hr PF), embryos expressing Δ34-Xic1 exhibited abnormal morphology consistent with apoptosis compared p27XicCK- controls, which gastrulated. p27XicCK- controls continued to develop through gastrulation and formed a neural groove, evident at Stage 13 (16 hr PF). **B:** Stage 11 embryos extracts were incubated with recombinant PARP, a substrate for caspase 3 (Carter and Sible, 2003). The presence of a cleaved PARP fragment indicates caspase activity.

4.3 Discussion

The current study provides evidence that altered expression of Wee1 promotes apoptosis during the early development of *Xenopus laevis*. Wee1 has been well defined in a wide range of eukaryotes as a specific cell cycle regulator of the G2/M transition. Acting in opposition to Cdc25C, Wee1 phosphorylates the Cdc2 subunit of MPF on Tyr-15, inhibiting the progression of the cell into mitosis. Wee1 itself is phosphorylated by Chk1 during cell cycle checkpoint signaling (Lee *et al.*, 2001) and further regulated by Cdc2 through both positive and negative feedback loops (Harvey *et al.*, 2005). Specifically, in budding yeast, phosphorylation of Wee1 by Cdc2 activates Wee1, which renders Cdc2 inactive. However, the dephosphorylation of Cdc2 leads to the hyperphosphorylation and inhibition of Wee1, rendering Cdc2 active (Harvey *et al.*, 2005). In the current study, it was demonstrated that Wee1 plays a key role during cell cycle remodeling at the MBT. Specifically, the overexpression of Wee1 disrupted the cyclin E/Cdk2 timer, indicated by the delayed degradation of cyclin E (Fig. 4-3B). It is possible that this delay could help to explain the incidence of post-MBT apoptosis, since cyclin E degradation was also delayed in embryos treated with 30 Gy IR (Fig. 4-3D). Furthermore, Wee1 overexpression inhibited DNA synthesis resulting in a nucleo-cytoplasmic ratio below the critical concentration necessary for cell cycle remodeling at the MBT (Fig. 4-5), suggesting altered cyclin E/Cdk2 activity.

In addition to the regulation of MPF and cell cycle remodeling in *Xenopus*, Wee1 contributes to the regulation of cyclin E/Cdk2 activity in mammalian cells. In rat aortic smooth muscle cells, endothelin-dependent proliferation is associated with N-terminal phosphorylation and suppression of Wee1, which results in a significant increase in Cdk2 activity (Chen and Gardner, 2004). In human epithelial cells, a Cdk2 interacting protein, Cables, increases the level of Tyr-15 phosphorylation of Cdk2 by Wee1. Furthermore, loss of Cables expression is associated with 50-60% of colon and head and neck cancers (Wu *et al.*, 2001). In the current study, it was demonstrated that Wee1 inhibits not only Cdc2, but also Cdk2 in *Xenopu*s embryos (Fig. 4-1C, 4-4). To determine whether apoptosis was at least partially due to Cdk2 inhibition, the specific Cdk2 inhibitor, Δ34-Xic1, which disrupts the cyclin E/Cdk2 timer in Xenopus embryos, was utilized (Hartley *et al.*, 1997). One hundred percent of embryos expressing Δ34-Xic1 exhibited a pre-

MBT cell cycle delay and underwent a post-MBT apoptotic death, compared to controls that developed normally through neurulation. Similarly, in breast cancer cells, transduction of a non-degradable form of the human homolog of Xic1, Kip1, induced cell cycle arrest, thereby inhibiting cellular proliferation. Furthermore, Kip1 transduction resulted in a significant increase in the number of apoptotic cells (Zhang *et al.*, 2005).

In addition to the inhibition of Cdk2 by Wee1, other apoptotic mechanisms involving Wee1 have been reported. It has previously been demonstrated that Wee1 accelerates apoptosis in *Xenopus* egg extracts through the interaction with the SH2 domain of the Crk-adaptor protein. Furthermore, Wee1 can restore apoptosis in extracts depleted of SH2 domain interactors (Smith *et al.*, 2000). In the current study, it was addressed whether Wee1 interacts with Crk through IP Western analysis. Although an interaction between Wee1 and Crk in embryos expressing exogenous Wee1 was apparent, it was no more prevalent than in control embryos (data not shown). This lack of distinction could be due to the interaction of endogenous Wee1 with Crk in normal cells, not just during apoptotic signaling. Furthermore, it is possible that the Wee1 interacting SH2 domains of Crk become saturated from endogenous pools of Wee1. In other words, the overexpression of Wee1 would not alter the levels of Crk interacting Wee1. Regardless, Wee1 does interact with Crk in *Xenopus* embryos. However, to what extent this interaction occurs remains to be determined.

Although the majority of Wee1 research focuses on the control of mitotic entry, the current and aforementioned studies demonstrate the importance of Cdk2 regulation by Wee1. We have provided evidence that the altered expression of Wee1 promotes apoptosis through the disruption of the cyclin E/Cdk2 timer, most likely due to the inhibition of Cdk2. Furthermore, based upon the role in which Wee1 has during pre-MBT development and its pattern of expression, it is possible that Wee1 functions as a regulator of the cyclin E/Cdk2 timer. Taken together, these data suggest that Wee1 functions to determine when a cell "switches" from an arrested state to a committed program of apoptosis. Future studies will hopefully elucidate these mechanisms, contributing to the ultimate goal of determining appropriate targets for chemotherapeutic agents.

Chapter 5: Conclusion

The current studies have examined two cell cycle regulators that play important, but very different roles in early *Xenopus laevis* development. In Chapter 3, it was demonstrated that Chk2 functions as an inhibitor of apoptosis. In the literature, conflicting evidence suggests that Chk2 can function as both an inhibitor and promoter of apoptosis. It is likely that specific cell type determines Chk2 function. Specifically, Chk2 has been shown to function as a promoter of apoptosis in somatic cells in *Drosophila* eye, human breast cancer cells, and lymphoma cells (Peters *et al.*, 2002; Yang *et al.*, 2002; Stevens *et al.*, 2003). In contrast, other studies have implicated Chk2 as an inhibitor of apoptosis. Ectopic expression of Chk2 in mouse embryonic stem cells confers protection from IR-induced apoptosis (Hong and Stambrook, 2004). Similarly, it was shown that exogenous Chk2 expression in early *Xenopus* embryos also inhibits IR-induced apoptosis (Wroble and Sible, 2005). Additionally, expression of dominant negative Chk2 (DN-Chk2) in human heterokarya, colon carcinoma cells, and early *Xenopus* embryos promotes apoptosis (Castedo *et al.*, 2004; Wroble and Sible, 2005). Furthermore, whether Chk2 functions as a promoter or inhibitor of apoptosis seems to be dependent on the status of p53. These studies provide some perspective on how the same gene can assume various roles across a wide range of organisms and cell types.

The second half of this study began in order to determine the role of another cell cycle regulator, Wee1, during early *Xenopus* development. Specifically, we wanted to determine whether Wee1 was able to inhibit IR-induced apoptosis as previously demonstrated by Chk2. Surprisingly, the overexpression of Wee1 resulted in post-MBT apoptosis. Further investigation revealed that the overexpression of Wee1 disrupted important cell cycle remodeling events and the cyclin E/Cdk2 timer, providing possible explanations for apoptosis. In addition to the overexpression of Wee1, various other manipulations of the embryo result in similar inhibition of cell cycle remodeling and apoptosis. Specifically, the exogenous expression of proteins such as Δ34-Xic, non-degradable Cdc25A, DN-Chk1, and DN-Chk2 all disrupt cell cycle remodeling and result in post-MBT apoptosis in embryos (Hartley *et al.*, 1997; Shimuta *et al.*, 2002; Carter and Sible, 2003; Wroble and Sible, 2005). Furthermore, IR and aphidicolin treatments have

similar effects (Wroble and Sible, 2005; our unpublished data). This suggests a strong correlation between aberrant cell cycle remodeling and apoptosis, but not a distinct mechanism. In contrast, others and we provide evidence that the specific inhibition of Cdk2 promotes apoptosis (Wroble *et al.*, 2005; Zhang *et al.*, 2005). This suggests the possibility of Cdk2 as a potential target for chemotherapeutic agents.

In the current study, we provide evidence that two cell cycle regulators, which share common downstream targets, play very different roles during the development of *Xenopus laevis*. It should be noted that although these characterizations of both Chk2 and Wee1 are specific to *Xenopus*, they have implications in higher eukaryotes, including humans. *Xenopus laevis* provides researchers with a unique model system where a very unregulated cancerous like cell cycle becomes highly regulated in a relatively short period. This attribute, in addition to the manipulability of *Xenopus*, enable the in depth study of the differences in the regulation of these two very different cell cycles. Hopefully, further investigation of early *Xenopus* development will contribute to our understanding of the mechanisms that promote the oncogenic transformation of normal cells.

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