Loss of Chk1 Function and Exogenous Expression of Cyclin A1/Cdk2 Results in Apoptosis after the MBT in Early *Xenopus laevis* Embryos

Ayesha D. Carter

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Jill C. Sible, Chair
Klaus D. Elgert
William R. Huckle
Charles L. Rutherford
John J. Tyson

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(ABSTRACT)

Early *Xenopus laevis* embryonic cell cycles exemplify rapid, non-pathological cell cycles without checkpoint pathways to arrest cell cycles in response to DNA assaults. There is no transcription or apoptosis during these cell cycles, and they continue unperturbed until the 12th cell cycle, marking a period called the midblastula transition (MBT). At the MBT, the embryo undergoes a period of developmental remodeling: gap phases are acquired, zygotic transcription is initiated, and the maternal mRNAs are degraded. After the MBT, checkpoint pathways can be activated in response to unreplicated DNA, and apoptosis initiates when continued embryonic survival is compromised. These studies examine how cell cycle regulation and apoptotic regulation are related. Specifically, the role of two cell cycle components, Chk1 and cyclin A1/Cdk2, during apoptosis was studied during early development of *Xenopus* embryos.

Chk1 is a serine/threonine kinase that inhibits the activity of cyclin-dependent kinases (Cdks) in response to unreplicated DNA. In the pre-MBT embryo, Chk1 is present, but inactive. Injection of mRNA encoding dominant-negative Chk1 (DN-Chk1) into single-celled embryos results in the initiation of apoptosis after the MBT. The loss of Chk1 function also results in the initiation of additional rapid rounds of DNA replication after the MBT. These results suggest that Chk1 has a required function for the embryo after the MBT, possibly through the regulation of a cyclin/Cdk complex responsible for the apoptotic checkpoint.

Cyclin A1 is a maternally provided mRNA that is degraded at the MBT. Prior to the MBT, cyclin A1 complexes exclusively with Cdc2 to regulate mitosis. When embryos are treated with ionizing radiation (IR), cyclin A1 activity and protein level persist after the MBT, and cyclin A1 complexes with Cdk2. When treated with aphidicolin, cyclin A1-associated activity and protein level persists. Injection of cyclin A1/Cdk2 into single-cell embryos results in apoptosis after the MBT; however, inhibition of cyclin A1 expression does not abrogate apoptosis. Therefore, cyclin A1/Cdk2 activity is sufficient, but not required, for the initiation of apoptosis in the early *Xenopus* embryo. These studies show that Chk1 and cyclin A1/Cdk2 have roles in regulating apoptosis in the post-MBT embryo.

As generic as it sounds,
this work is dedicated to
Mom and Dad,
who are anything but.

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Specific Aims

Specific Aim 1: To determine whether inhibition of Chk1 activity results in apoptosis in early *Xenopus laevis* embryos.

The Chk1 pathway is present in the early *Xenopus* embryo but is not active until the MBT. The embryo is unable to respond to a DNA replication block by phosphorylating Chk1 until the embryo reaches an approximate MBT content of DNA (Kappas et al. 2000). From fertilization through tadpole stage, Chk1 levels are constant (Nakajo et al. 1999; Kappas et al. 2000), and at the MBT, Chk1 is transiently activated by phosphorylation (Shimuta et al. 2002). Over expression of Chk1 in the early embryo results in tyrosine phosphorylation of Cdc2 and Cdk2, causing premature cell cycle arrest before the MBT (Kappas et al. 2000). Further, Chk1 function is required for the proper timing of Cdc25A degradation and for lengthening of the cell cycles post-MBT (Shimuta et al. 2002). To test the requirement of the embryo for Chk1 activity, single-celled embryos were injected with 18 ng mRNA encoding a dominant-negative Chk1 (DN-Chk1) protein. The DN-Chk1-expressing embryos underwent apoptosis at the MBT. These studies suggest that Chk1 is required for embryonic survival beyond the MBT and that the inhibition of Chk1 activity results in the initiation of apoptosis. (Chapter 3)

Specific Aim 2: To determine whether inhibition of Chk1 affects initiation of transcription and DNA synthesis.

Shimuta et al. (2002) have shown that embryos expressing a dominant-negative form of Chk1 possess two – three fold more genomic DNA than control embryos after the MBT. The increase in the amount of genomic DNA is reminiscent of *Drosophila* embryos lacking the Chk1 homolog, Grp1 which undergo additional rounds of replication (Fogarty et al. 1997; Sibon et al. 1997). Like Chk1 (Shimuta et al. 2001), Grp1 is required for cell cycle lengthening at the MBT (Fogarty et al. 1997; Sibon et al. 1997). Grp1 is also required for expression of zygotic genes at the MBT (Sibon et al. 1997). When *Xenopus* embryos are injected with α-amanitin, an RNA polymerase II inhibitor, the embryos divide normally until the MBT and then undergo apoptosis at the EGT (Sible et al. 1997); Carter and Sible 2003). Given that *Xenopus* and *Drosophila* embryos both develop without early zygotic transcription or growth phases, the goal of this aim was to determine the effect the loss of Chk1 function would have on zygotic transcription and regulation of DNA synthesis. This goal was determined by injection of single-celled embryos with the DN-Chk1 mRNA, and replication rates and initiation of transcription were

analyzed. There was no change in the initiation of transcription, but at the MBT, DNA replication underwent additional rapid rounds (Chapter 3).

Specific Aim 3: To determine whether there is a correlation between cyclin A1-associated kinase activity and apoptotic programs resulting from assault to the embryo.

Over expression of *Xenopus* Chk1 results in the premature tyrosine phosphorylation of Cdc2 and Cdk2 (Kappas et al. 2000). When the activity of human Chk1 is inhibited in cell culture by treatment with a Chk1 inhibitor, UCN-01, the cells undergo apoptosis and show increased Cdk2 activity (Wang et al. 1995). Anderson et al. (1997) have shown that when a pre-MBT embryo is treated with IR, the amount of cyclin A1 protein persists and forms a catalytically active dimer with Cdk2, a Cdk that cyclin A1 does not usually form a complex with except in irradiated embryos. The persistence of cyclin A1 protein contrasts with control embryos where cyclin A1 disappears at the early gasturula transition (EGT) soon after the MBT and is replaced with cyclin A2 (Howe et al. 1995; Anderson et al. 1997). To determine whether cyclin A1 is a common mediator of apoptosis induced at the MBT, cyclin A1 levels and cyclin A1-associated kinase activity were analyzed from embryos treated with aphidicolin to induce apoptosis. The levels of cyclin A1 persisted beyond the EGT and cyclin A1 associated kinase activity was increased in aphidicolin-treated embryos (Chapter 4).

Specific Aim 4: To determine whether cyclin A1/Cdk2 triggers apoptosis in *Xenopus* embryos.

Addition of recombinant cyclin A1/Cdk2 protein to *Xenopus* egg extracts results in the appearance of condensed apoptotic nuclei (Anderson et al. 1997). To determine the sufficiency of cyclin A1 in the initiation of apoptosis, single-celled embryos were injected with recombinant cyclin A1/Cdk2 protein and analyzed for the appearance of apoptotic characteristics, such as gross and nuclear morphology and caspase activity. To test the converse hypothesis, that cyclin A1 is required for the initiation of apoptosis at the MBT, embryos were injected with a morpholino specifically designed to inhibit cyclin A1 translation. The embryos were then treated with aphidicolin and IR to induce apoptosis. Apoptosis was not abrogated in either case, so cyclin A1 is not required for the induction of apoptosis (Chapter 4).

1 Literature Review

1.1 The Importance of the Cell Cycle

Treating cancer is one of modern medicine's greatest challenges. In order to maintain a healthy organism, metazoan tissues depend upon the ability of their constituent cells to modulate proliferation, differentiation and movement (Rich et al. 2000). A somatic cell receives external and internal signals telling it when to divide and when to remain quiescent. Cancer results, in part, when cells respond inappropriately to these signals and therefore, lose control over the timing of proliferation and divide when they should be quiescent, to the detriment of the organism. The survival of the organism is more important than that of one cell, so when the promiscuous division of one cell endangers the organism, external and internal mechanisms forcing the cell to self-destruct are engaged, initiating a suicide program called apoptosis. A second part of the cancer equation occurs when a cell acquires the ability to bypass these suicide mechanisms as well as the mechanisms regulating the timing of division (Hanahan and Weinberg 2000). Therefore, one part of treating cancer is related to understanding the molecular controls regulating cell proliferation and cell suicide. To do so, one must understand the fundamental controls regulating the cell cycle.

A proliferating cell enters the cell cycle at the first gap phase, G1. From there, the cell cycle transitions to DNA synthesis, to a second gap phase and the final equal allotment of DNA and cellular components to two daughter cells during mitosis. The timing of the process is orchestrated by the activity of cyclin/cyclin dependent kinase (Cdk) complexes. When activated, cyclin/Cdk complexes induce downstream processes by phosphorylating select target proteins (Pines and Hunter 1994). The activity of cyclin/Cdk complexes is tightly regulated by cell cycle checkpoint pathways that halt the cell cycle in response to factors

that threaten the integrity of cellular progeny. It is the disruption of these checkpoint pathways by mutation of their components that, in part, allows for unregulated cyclin/Cdk activity and the promiscuous cell proliferation that can result in cancer.

In order for the cell to proliferate promiscuously, it must bypass not only its own checkpoint pathways, but also the suicide pathways that will force the cell to self-destruct when checkpoint and/or repair pathways are no longer fully functional. Apoptosis is an active suicide program initiated by the cell in instances of cellular distress (Orrenius et al. 2003). Initiation of apoptosis allows metazoan organisms to favor organismal survival over cell survival by eliminating those cells that have abrogated their checkpoint pathways or are damaged beyond the cell's capability to repair (King and Cidlowski 1995). Apoptosis is designed to delete cells from tissues rapidly, by marking them for phagocytosis, and recycling the usable parts (Rich et al. 2000) all without instigating an inflammatory response in the surrounding tissues (Orrenius et al. 2003).

While apoptosis has been studied for over twenty years, the mechanisms governing initiation, regulation and execution of apoptosis are still not clearly understood (Wyllie and Golstein 2001), although evidence is mounting that indicates that the same pathways regulating the cell cycle are also important for the regulation of apoptosis (Pucci et al. 2000). When assaulted by agents that compromise cellular integrity, cells must 1) recognize the disrupted areas, 2) assess the extent the disruption, and 3) implement the appropriate response, repair or death. Cells use checkpoints to monitor the decision to suicide or repair (Rich et al. 2000). Checkpoints ensure that the execution of one cellular processes is dependent upon the successful completion of the previous process (Hartwell and Weinert 1989), barring the incorporation of genomic errors that can lead to the development of cancers (Hanahan and Weinberg 2000). By incorporating apoptotic pathways into their

checkpoint controls, metazoans are able to counter the effects that incorporating genomic errors can establish.

Therefore, in many instances, the same genes that are involved in regulating the cell cycle and checkpoint pathways also regulate apoptosis (Nagano et al. 2000). Mitosis and apoptosis display similar morphological features: cells lose substrate attachment, become rounded, shrink, and condense their chromatin (Pucci et al. 2000). The similarities between mitosis and apoptosis are partly due to Cdk activity during both processes (Shi et al. 1994). Some cyclin/Cdk complexes initiate dissolution of the nuclear membrane and promote chromatin condensation, events marking mitosis and apoptosis (Shi et al. 1994). Further, premature cyclin/Cdk activation may be a general mechanism by which cells induced to undergo apoptosis initiate the disruption of the nucleus. The following studies are an attempt to further our understanding of how cell cycle regulation is related to regulation of apoptosis. To that extent, the role of two cell cycle components, Chk1 and cyclin A1/Cdk2, during apoptosis were studied in the context of the early development of *Xenopus laevis* embryos.

1.2 Regulation of Cyclin/Cdk Complexes

The importance of cyclin/Cdks in the cell cycle is evidenced by the fact that the cell has evolved numerous ways to regulate the activity of these complexes. Cdk activity is positively and negatively regulated by association with specific cyclin partners, phosphorylation and binding with stoichiometeric inhibitors called CKIs (Novak et al. 2002). Cdk protein levels remain constant during most cell cycles. Cdks are only active as kinases when they form complexes with cyclins, which regulate Cdk activity and help form the substrate recognition site of the complex (Sandal 2002). In addition to regulating substrate recognition, cyclins

regulate cell phase specificity for the Cdks (Sandal 2002). For example, activity of the Cdk, Cdc2 (for Cell division cycle mutant 2; also called Cdk1) is restricted to the G2/M transition by localization and available levels of cyclin B (Owa et al. 2001). Generally, cyclin levels are regulated by synthesis at the level of transcription and quickly removed from the cell cycle via the proteosome-ubiquitin pathway, as is the case with cyclin A and cyclin B (Owa et al. 2001). However, the location of the cyclin can also play a part in regulating Cdk activity, as is the case with cyclin B.

Cyclin B synthesis begins during S-phase, where cyclin B is localized to the cytoplasm. The levels of cyclin B increase during G2 and the cyclin is not localized to the nucleus until the cell is ready to initiate mitosis (Pines and Hunter 1994). The N-terminal domain of cyclin B contains a cytoplasmic retention signal (CRS) that must be phosphorylated on a number of serine residues in order for cyclin B to be translocated to the nucleus. In addition, the CRS contains a nuclear export signal that ensures that cyclin B is transported out of the nucleus during interphase (Pines and Hunter 1994). The transport of cyclin B out of the nucleus is mediated by the nuclear export factor, CRM1, and the phosphorylation of cyclin B at the G2/M transition blocks the direct interaction of cyclin B with CRM1 (Hagting et al. 1998; Toyoshima et al. 1998). Mitotic phosphorylation of cyclin B also promotes nuclear import of the cyclin. At this time, the mechanism for localizing cyclin B into the nucleus is not well known since cyclin B does not contain a classical nuclear localization sequence (Smits and Medema 2001).

In addition to regulated synthesis and localization, cyclin B protein is regulated by timed degradation. In order for the cell to exit mitosis, cyclin B must be degraded by ubiquitination (Murray and Kirschner 1989; Holloway et al. 1993; Stemmann et al. 2001). The E3 ligase that targets cyclin B, as well as cyclin A, for degradation is the anaphase

promoting complex (APC) in association with its activators, Cdc20 and Cdh1, which are responsible for recognition of cyclin B by the APC (Clute and Pines 1999). Cyclin B/Cdc2 activates Cdc20, while inactivating Cdh1 (Castro et al. 2005). As cyclin B levels decrease as a result of Cdc20 activity in metaphase, Cdh1 is dephosphorylated, and its levels increase. The APC^{Cdh1} complex targets its other activator, Cdc20, for degradation, therefore inactivating the APC^{Cdc20} complex, and increasing the amount of active APC^{Cdh1}. The change from Cdc20 to Cdh1 further decreases the mitotic cyclin levels to prevent premature accumulation of these cyclins and the early initiation of S-phase (Zachariae and Nasmyth 1999).

In addition to cyclin partner availability and localization, cyclin/Cdk complex activity is also regulated by phosphorylation and dephosphorylation of activating sites by cyclin activating kinases (Novak et al. 2002). For example, in order for Cdc2 to be active, it must be phosphorylated on a conserved threonine (Thr 161) in the T-loop by CAK (Solomon et al. 1992; Smits and Medema 2001). CAK is itself a cyclin/Cdk complex, comprised of cyclin H/Cdk7, and stabilized by MAT1 (Fisher and Morgan 1994; Makela et al. 1994; Devault A 1995). The binding of cyclin B to Cdc2 stabilizes Cdc2, allowing for the activating phosphorylation of Thr 161 by CAK. Other phosphorylation events on Tyr 15 and Thr 14 hold the complex inactive until needed for initiation of mitosis (Parker et al. 1992). These phosphorylation events will be detailed further in the discussion on mitosis.

The activity of cyclin/Cdk complexes is also regulated by stoichiometeric inhibitors (CKIs), like the members of the Cip/Kip family. The first Cip/Kip family member to be isolated was $p21^{Cip1}$ (Coqueret 2003). Cip/Kip family members bind to cyclin/Cdk complexes and hold them inactive until the cyclin/Cdk complex is released. In order to inhibit cyclin/Cdk activity, the Cip/Kip protein first initiates contact with

the cyclin subunit via an α-helix, and then a second helix inserts itself into the catalytic subunit of the Cdk, interfering with ATP loading (Coqueret 2003). Release of the cyclin/Cdk is mediated via phosphorylation of the Cdk, causing a conformational shift that makes Cip/Kip binding no longer possible (Coqueret 2003).

It is through these three methods of regulation: level of available cyclin, phosphorylation and stoichiometeric inhibition, that checkpoint pathways control the activity of cyclin/Cdk complexes during a cell cycle checkpoint. Checkpoint pathways are highly conserved inhibitory pathways responsible for ensuring that critical cell cycle events, DNA replication and repair and mitotic spindle assembly, are completed with high fidelity (Pucci et al. 2000). Activation of cell cycle checkpoint signaling pathways by internal cellular sensors generates a signal to arrest cells at specific cell cycle transitions. Entry into S-phase is arrested when DNA is damaged. Damaged DNA or blocked replication will also cause the G2/M transition to arrest (Novak et al. 2002). Further, at the beginning of mitosis, the cell must determine that the chromosomes are properly aligned on mitotic spindles and that the centrosome has been duplicated once and only once, otherwise resulting in an arrest at the metaphase checkpoint (Stearns 2001). Progression through and exit from mitosis are both, in part, regulated by the spindle checkpoint. This checkpoint ensures that anaphase only occurs once all the chromosomes have formed stable bipolar attachments to microtubules. In addition, it ensures that exit from mitosis is delayed until the chromosomes have been properly partitioned to both daughter cells (Tan et al. 2005). The following sections will describe the mechanisms pushing the cell cycle and then the checkpoints responsible for maintaining genomic fidelity during the cell cycle.

1.3 The Somatic Cell Cycle

The somatic cell cycle is divided into four phases: gap phase 1 (G1-phase), DNA synthesis (S-phase), gap phase 2 (G2-phase), and mitosis (M-phase). Each phase of the cell cycle is driven by the tightly regulated activity of various cyclin/Cdk complexes. In order for the cell cycle to progress, a quiescent cell must be stimulated by external growth factors. In the presence of growth factors, Ras, a membrane bound GTPase, is activated, leading to the activation of the Raf-MEK-ERK/MAPK pathway (Lavoie et al. 1996; Winston et al. 1996; Aktas et al. 1997). The activation of this pathway ultimately leads to the transcription of cyclin D through the activation of transcription factors like Fra1, Fra2, c-Jun and JunB (Balmanno and Cook 1999; Treinies et al. 1999). Cyclin D accumulates, complexes with Cdks 4 and 6, and must reach a threshold level before the cell cycle can initiate (Coleman et al. 2004).

It is the accumulation of cyclin D protein that pushes the cell past the restriction point. The restriction point is the time within the G1 phase which, once passed, cells can complete the division cycle even in the absence of growth factors (Pardee 1974). Before the restriction point is passed, the cell is not committed to entering the cell cycle and the initial cell cycle machinery can be stopped by withdrawal of growth signal. After the restriction point, the cell is committed to entering the cell cycle, withdrawal of growth factors has no effect on the cell cycle, and cyclin D is no longer required for the commitment of the cell to division (Novak et al. 1998).

In order to initiate the cell cycle, the cyclin D/Cdk4, 6 complexes partially phosphorylate RB family proteins, facilitating the release of E2F transcription factors from inhibition by RB family members. The release of E2F transcription factors allows for the transcription of genes, such as cyclin E (Sutherland and Musgrove 2004), required for the G1/S transition (Coleman et al. 2004).

The partial phosphorylation of pRB results in the induction of cyclin E protein in mid-to late-G1 and the formation of active cyclin E/Cdk2 complexes to drive the transition to S-phase (Sutherland and Musgrove 2004). Cyclin E accumulation drives a positive feedback loop by completing the phosphorylation of pRB and releasing additional E2F activity (Owa et al. 2001; Obaya and Sedivy 2002). To further promote its own activity, Cyclin E/Cdk2 phosphorylates its inhibitor, p27, promoting the inhibitor's degradation (Muller et al. 1997; Sheaff et al. 1997; Vlach et al. 1997). Cyclin E/Cdk2 activity regulates the transcription of genes that activate the replication machinery. Of great importance to these studies is the transcription of cyclin A. Cyclin E/Cdk2 activity promotes transcription of cyclin A by disrupting the pRB-hSWI/SNF complexes that repress the cyclin A1 promoter (Zhang et al. 2000). Partial activation of pRB at the beginning of G1-phase makes the transcription of cyclin A dependent upon the transcription of cyclin E, ensuring the proper sequence of events for the G1/S transition.

The end of the G1-phase is marked by the accumulation of cyclin E/Cdk2 levels to a permissive level, opening a "window of opportunity" for the assembly of replication complexes at the replication origins (Coverley et al. 2002). While the exact mechanism is unclear, cyclin E/Cdk2 promotes the loading of the pre-replication complex (pre-RC) onto DNA with the help of Cdc6. Cdc6 is a highly conserved replication regulatory protein that is essential for the initiation of DNA replication (Carpenter et al. 1996; Cocker et al. 1996; Coleman et al. 1996; Hateboer et al. 1998). Cdc6, in cooperation with the origin recognition complex (ORC) and the MCM family of proteins, initiate a process called replication licensing (Botchan 1996), a process that is terminated by the accumulation and activity of cyclin A/Cdk2 (Coverley et al. 2002). The ORC recruits Cdc6 to the replication origin, then Cdc6 facilitates the loading of MCM proteins onto the origin, by mechanisms as yet unclear

(Coverley et al. 2000). The beginning of S-phase is marked when cyclin A levels rise to a particular threshold and cyclin A/Cdk2 phosphorylates Cdc6, targeting it for degradation and ensuring that further pre-RC loading cannot occur (Coverley et al. 2002). This event ensures that replication is limited to once per cell cycle. The continued events of DNA synthesis are carried out by cyclin A/Cdk2, mainly the initiation of DNA replication, through origin firing, and the duplication of the centrosome (Girard et al. 1991; Pagano et al. 1992).

The completion of DNA synthesis ends S-phase and marks the beginning of the second gap phase, G2-phase. The initiation of M-phase is regulated by high levels of cyclin B/Cdc2 activity. Once synthesized and localized to the nucleus, cyclin B/Cdc2 is activated by CAK, which phosphorylates Cdc2 on threonine 161 (Solomon et al. 1992), as detailed earlier. The cyclin B/Cdc2 complex is held inactive by further phosphorylation on residues Thr 14, predominately by Myt1, and Tyr 15, by Wee1 and Myt1 (Parker et al. 1992; Mueller et al. 1995). The phosphate group on Thr 14 interferes with ATP binding in the active site (Li et al. 1995), while the phosphate group on Tyr 15 interferes with phosphate transfer within the ATP binding site (Parker et al. 1992). The cyclin B/Cdc2 complex cannot drive the mitotic machinery until the Thr 14 and Tyr 15 phosphates are removed by the Cdc25C phosphatase (Kumagai and Dunphy 1991).

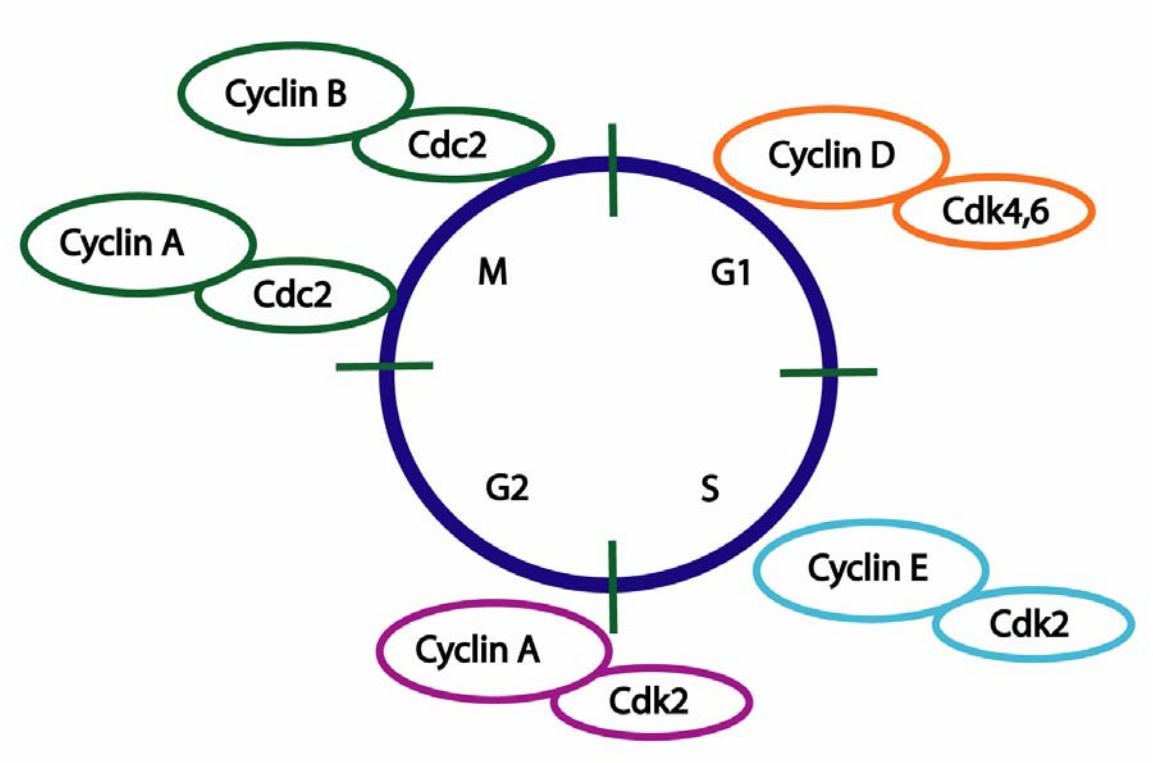

Figure 1. The somatic cell cycle. Cyclin D/Cdk4,6 are the effector kinases of G1, initiating the synthesis of cyclin E/Cdk2. Cyclin E/Cdk2 initiates DNA replication origin loading and promotes the synthesis of cyclin A/Cdk2, which initiates DNA synthesis. During G2, cyclin B levels steadily increase until they reach a threshold amount, signaling the beginning of mitosis. The abrupt degradation of cyclin B brings mitosis to a close.

Once the inhibitory phosphates are removed from Thr 14 and Tyr 15, cyclin B/Cdc2 is now capable of phosphorylating the substrates responsible for driving the morphologic events that mark mitosis. This includes, but is not limited to, cytoskeletal proteins, including the nuclear lamina, and histone H1. The phosphorylation of nuclear lamins results in the dissolution of the nuclear envelope, while phosphorylation of histone H1 leads to chromatin condensation (Smits and Medema 2001).

In addition to phosphorylating the mitotic machinery, active cyclin B/Cdc2 regulates its own activity during mitosis. In a positive feedback loop, cyclin B/Cdc2 phosphorylates and activates its activator, Cdc25C,

increasing the levels of active cyclin B/Cdc2, while at the same time, it phosphorylates its inhibitors, Wee1 and Myt1, inhibiting the inhibitory activity of these molecules (Kumagai and Dunphy 1991; Izumi and Maller 1993). Further contributing to the levels of active cyclin B/Cdc2, the complex autophosphorylates on the serine residues located within the CRS of cyclin B, allowing for the accumulation of more cyclin B protein in the nucleus (Smits and Medema 2001).

In contrast to the positive feedback activity of cyclin B/Cdc2, the complex has a negative feedback action that leads to the ultimate degradation of cyclin B. Active cyclin B/Cdc2 indirectly activates Cdc20, the early mitosis APC activator. Cdc20 targets cyclin B for degradation by the ubiquitin-proteasome pathway, which will ultimately lead to the inhibition of Cdc2 activity (Felix et al. 1990; Lorca et al. 1998). As a consequence of Cdc2 inhibition, sister chromatids are separated through ubiquitination of securin, which is targeted by the APC^{Cdh1} complex (Castro et al. 2005). Secruin prevents the activity of separase, the protein that cleaves the complex cohesin, which holds sister chromatids together (Hagstrom and Meyer 2003). Other events marking exit out of mitosis are the disassembly of the mitotic spindle, chromatin decondensation, and the nuclear envelope reformation. Therefore, entry into mitosis is dependent upon the synthesis and nuclear accumulation of cyclin B, while exit out of mitosis is regulated by the degradation of cyclin B (Murray et al. 1989; Holloway et al. 1993; Stemmann et al. 2001).

1.4 The DNA Damage/Replication Checkpoint

As described in the preceding section, each phase of the cell cycle is regulated by the activity of a cyclin/Cdk complex. The entire point of the cell cycle is the passing down of a complete, intact genome to each

daughter cell. Unfortunately, there are many points within the cell cycle where the replication and segregation of the genome is problematic, making the process of passing down the genome imprecise (Hartwell and Weinert 1989). To decrease the chances of problems during this process, cells have evolved a series of pathways called checkpoints (Hartwell and Weinert 1989). Of greatest interest to these studies are the responses of a cell to DNA damage and inhibition of DNA replication.

There are two distinct pathways regulating the response to DNA damage and blocks to DNA replication. ATM and ATR are members of the mammalian phosphatidylinositol-3 kinase (PI3K)-related family of protein kinases and have been shown to be essential components of the mechanisms controlling the DNA damage response (Tibbetts et al. 2000). There are ATM and ATR homologs in all eukaryotic cell types currently studied. The primary differences between ATM and ATR are the form of DNA damage that will result in the phosphorylation and activation of ATM or ATR and the subsequent kinases ATR and ATM activate (Abraham 2001). ATM is activated in response to damage induced by IR; ATR is activated in response to damage induced by ultraviolet radiation and treatment with hydroxyurea (Zhou and Elledge 2000). In order for ATR to be activated, DNA replication must already be underway, and it is possible that the activation of ATR is in response to the stalling of the replication forks by anomalies in the DNA structure (Lupardus et al. 2002). ATR also responds to ionizing radiation, but the response is weak in comparison to the ATM response, suggesting overlapping functions for both pathways in response to damage inducing agents (Zhao and Piwnica-Worms 2001). Further, ATR is activated by explicit blocks to DNA replication (Guo and Dunphy 2000).

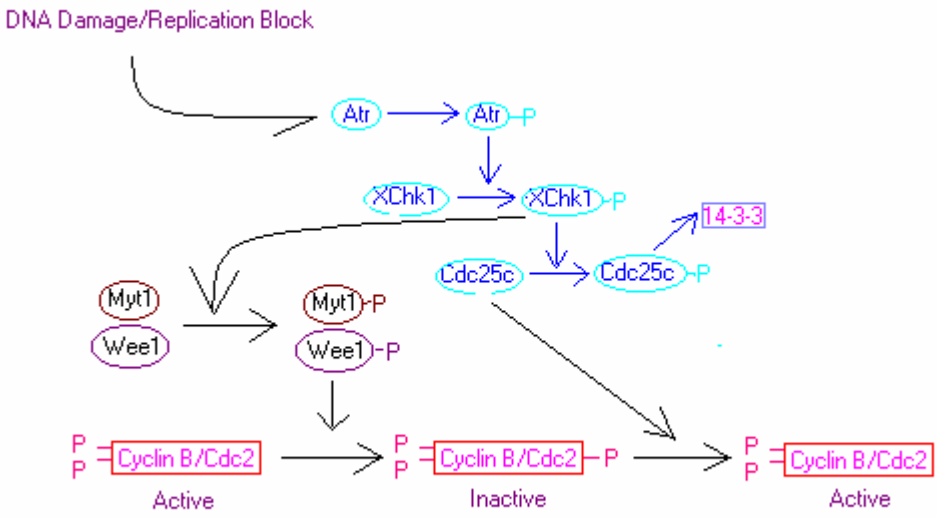

Figure 2. The Chk1 pathway in response to stalled replication forks. DNA damage (e.g. thymdine dimers) or toxic assaults (e.g. aphidicolin) which cause the replication fork to stall, activate the ATR kinase, a PI3K-related kinase, through phosphorylation. Active ATR phosphorylates the Chk1 kinase on Ser345, activating that kinase. Active Chk1 has two functions in this pathway, phosphorylation and activation of Wee1 and Myt1, inhibitory kinases of cyclin B/Cdc2, and phosphorylation and activation of Cdc25C, the activating phosphatase of cyclin B/Cdc2. Wee1 phosphorylates Cdc2 on the inhibitory Tyr 15 site, and Cdc25C removes that phosphate group. Without active Cdc25C, the cell remains arrested in the G2/M transition.

In *Xenopus* egg extracts, once ATR is activated in response to DNA damaging agents or replication blocks, it phosphorylates and activates Chk1 kinase (Guo and Dunphy 2000). Active Chk1 phosphorylates Cdc25C, causing a conformational change that results in enhanced binding of Cdc25C to the 14-3-3 cytoskeletal proteins (Yaffe 2002), sequestering Cdc25C in the cytoplasm away from the nucleus (Conklin et al. 1995; Chen et al. 1996; Kumagai et al. 1998). The primary function for Cdc25C is to remove the inhibitory phosphates on threonine 14 and tyrosine 15 to allow activation of the cyclin B/Cdc2 complex, which drives the events of mitosis. To further inhibit the G2/M transition,

Chk1 phosphorylates and activates Wee1, the active inhibitor of cyclin B/Cdc2 (Lee et al. 2001).

Chk1 functions in both the G2/M checkpoint and the G1/S-phase checkpoint. As well as phosphorylating Cdc25C for sequestration away from the nucleus, Chk1 phosphorylates Cdc25A, an event that targets Cdc25A for degradation (Zhao et al. 2002). Cdc25A dephosphorylates cyclin E/Cdk2 so that the complex can drive S-phase (Sagata 2002). Initiation of rapid Cdc25A degradation is an overlapping function that Chk1 shares with Chk2. ATM activates Chk2 in response to ionizing radiation and Chk2 phosphorylates Cdc25A to arrest the cell at the G2/M transition or during the G1/S-phase transition.

In mammals, ATM has a second mechanism to enforce cell cycle arrest at a checkpoint. As well as activating Chk2, ATM phosphorylates MDM2 so that it can no longer associate with p53 and target p53 for degradation (Novak et al. 2002). p53 accumulates and initiates the transcription of genes promoting cell cycle arrest, such as $p21^{Cip1}$, a stoichiometeric inhibitor of cyclin/Cdk complexes (Novak et al. 2002; Pietenpol and Stewart 2002). Another function of p53 is to induce transcription of genes for DNA repair. p53 is also phosphorylated by Chk1 and Chk2, to further stabilize p53 for continued expression of genes involved in repair, cell cycle arrest and, if needed, apoptosis (Shieh et al. 2000).

1.5 Early *Xenopus laevis* Embryonic Development and the Cell Cycle

The phases of the mammalian somatic cell cycle are highly complex with many cyclin/Cdk complexes regulating the progression from G1-phase, to S-phase, to G2-phase and finally to M-phase. Without the gap phases, the mechanisms leading from DNA synthesis to

mitosis are in a much simpler context, simplifying our understanding of what is required for these two phases to occur. This can be done by studying the early cell cycles of the *Xenopus laevis* embryo, which starts as a large egg and does not grow for several cycles. These early cell cycles provide a non-pathological setting in which to study rapid cell cycles alternating between DNA synthesis and mitosis without any gap phases (Newport and Kirschner 1982a; Kimelman et al. 1987). Therefore, the early *Xenopus* embryo is a simpler context in which to study the cell cycle and cell cycle checkpoints.

Immature *Xenopus* oocytes replicate their DNA once and then arrest in G2. During the G2 arrest, the oocyte stockpiles maternally provided mRNAs that will regulate the first twelve post-fertilization cell cycles, as there is no transcription in the early *Xenopus* embryo (Newport and Kirschner 1982a). Upon stimulation with progesterone, the oocyte undergoes Meiosis I where half its chromosome content is ejected from the oocyte in the first polar body (Murray and Hunt 1993). The unfertilized egg emerges from the female frog arrested in metaphase of Meiosis II with high cyclin/Cdk activity and is released from metaphase arrest at fertilization, producing a second polar body. The fertilized egg then degrades mitotic cyclins and enters interphase of the first mitotic cell cycle, initiating the first embryonic cell division (Murray and Hunt 1993). After the first embryonic division, which takes 90 minutes, the 2nd – 12th cleavages are rapid and synchronous, completing a cycle of DNA synthesis and mitosis every 25-30 minutes (Newport and Kirschner 1982a). The twelfth division marks a point known as the midblastula transition (MBT). At the MBT, the embryo reaches a critical nuclear-to-cytoplasmic ratio, which is thought to be sensed by the depletion of some cytoplasmic factor (Hara et al, 1980) that is no longer able to interact stoichiometrically with the exponentially increasing DNA (Newport and Kirschner 1982a). The MBT corresponds with the acquisition of gap

phases and active checkpoints, degradation of maternal mRNAs, initiation of both embryonic transcription, and cell motility (Newport and Kirschner 1982a).

Not all events marking the MBT are dependent upon the nuclear-to-cytoplasmic ratio. Events not dependent upon the nuclear-to-cytoplasmic ratio appear to be dependent upon a timing mechanism activated at fertilization. Cyclin E protein accumulates during Meiosis II and the first mitotic cycle, and the level of cyclin E protein remains constant until the twelfth division (Rempel et al. 1995; Hartley et al. 1996). Instead of varying protein level, cyclin E associated kinase activity is thought to be regulated by tyrosine phosphorylation of cyclin E/Cdk2 (Kappas et al. 2000). Cyclin E/Cdk2 activity peaks at both S and M-phase, suggesting a role in embryo DNA synthesis and mitosis, although it is predominantly active during DNA synthesis (Guadagno and Newport 1996).

At the MBT, cyclin E degradation is one event that is independent of cell cycle progression, new protein synthesis or the nuclear-to-cytoplasmic ratio (Howe and Newport 1996). However, the timing of cyclin E degradation is linked to the activity of Cdk2 (Hartley et al. 1997; Ciliberto et al, 2003). Inhibition of cyclin E/Cdk2 activity with a truncated form of Xic1 (Δ34-Xic1), a specific cyclin E/Cdk2 inhibitor, results in a slowing of the cell cycle by approximately 25% (Hartley et al. 1997), suggesting that timing of the cell cycle is dependent upon cyclin E/Cdk2 activity, and that the developmental timer regulating pre-MBT cell divisions is terminated at the MBT due to degradation of maternal cyclin E (Hartley et al. 1997). Further, when cyclin E/Cdk2 activity is inhibited, the initiation of zygotic transcription is delayed (Hartley et al. 1997), suggesting that some MBT remodeling events are dependent upon the developmental cyclin E degradation timer.

In contrast to the constant cyclin E protein levels that drive DNA synthesis during early *Xenopus* embryo development, the synthesis and accumulation of cyclin A1 and B protein carefully controls entry into mitosis as these cyclins form complexes with Cdc2 (Murray and Kirschner 1989). Exit from mitosis is driven by the degradation of cyclins A and B (Murray and Kirschner 1989; Hartley et al. 1996). Cyclin A1 is a mitotic cyclin synthesized from maternally provided mRNAs. Cyclin A1 only associates with Cdc2 to assist in driving mitosis and is present only before the MBT after which both protein and mRNA disappear (Howe et al. 1995). While in the somatic cell cycle cyclin A forms a dimer with both Cdc2 and Cdk2, cyclin A1 only forms a dimer with Cdc2. A second isoform, cyclin A2, is present in very low levels as a maternally provided mRNA, but is heavily synthesized during the MBT to take over as the zygotic cyclin A after gastrulation. Cyclin A2 associates with both Cdc2 and Cdk2 driving mitosis and DNA synthesis, respectively, more like the mammalian somatic cell cycle (Howe et al. 1995).

The main driving force of embryonic mitosis is the cyclin B/Cdc2 complex, also denoted as maturation promoting factor, MPF (Masui and Market, 1971; (Dunphy et al. 1988); (Gautier et al. 1990). MPF was discovered when a small amount of cytoplasm from progesterone-treated oocytes was transferred to immature oocytes. When the immature oocytes were injected with a small amount of cytoplasm from mature oocytes, the immature oocytes matured and entered meiosis I, indicating that some protein or complex of proteins in the cytoplasm of a mature oocyte can drive an oocyte to enter M-phase (Masui and Market, 1971). That protein complex was termed MPF, for maturation promoting factor, and was later discovered to be a cyclin, cyclin B, in complex with a Cdk, Cdc2 (Lohka et al. 1988; Gautier et al. 1990).

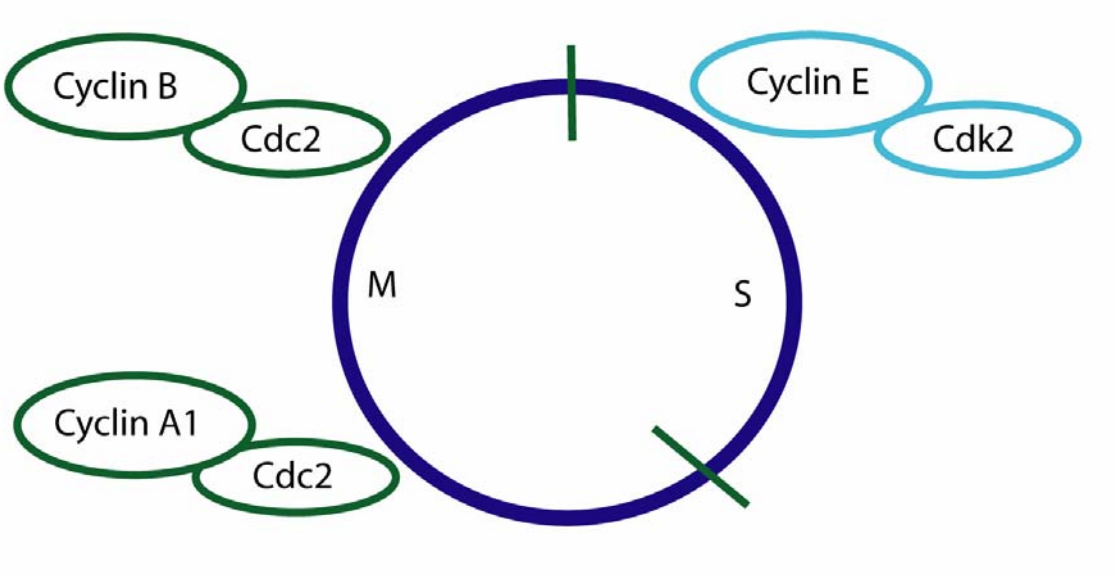

Figure 3. The embryonic cell cycle. Without gap phases, the embryonic cell cycle is a simpler context for cell cycle study. The rapid DNA synthesis phases are regulated by constant levels of cyclin E and phosphorylation of Cdk2. Mitosis is regulated by cycling levels of cyclins B and A1 in complex with Cdc2.

MPF activity is regulated by synthesis and degradation of cyclin B as well as by phosphorylation of tyrosine 15 as detailed in previous sections (The Somatic Cell Cycle). MPF activity is low in the immature oocyte but peaks to lead to meiosis I. Activity then falls off to increase again and induce meiosis II. The oocyte remains arrested with high levels of MPF until fertilization. Fertilization releases the arrest, with a quick decline in MPF activity. The cyclic changes in MPF activity correspond to the rapidly alternating S/M divisions of the early embryonic cell cycle (Gerhart et al. 1984). Further, these punctuated changes in activity are independent of DNA synthesis and spindle assembly. Gerhart (1984) found that cytosolic factors alone activate and inactivate MPF and drive the cell into and out of mitosis.

1.6 The Chk1 Kinase during *Xenopus laevis* Development

It is the rapid, synchronous cell cycle divisions oscillating between DNA synthesis and mitosis and the absence of cell cycle checkpoints that characterize the development of the early *Xenopus* embryo and make the system of interest to cell cycle biologists. The early embryo will continue cell divisions regardless of blocks to DNA replication (Kimelman et al. 1987; Newport and Dasso 1989; Clute and Masui 1997) or damage to the genome (Anderson et al. 1997; Hensey and Gautier 1997). Cell cycle checkpoints are thought to be acquired at the MBT (Newport and Dasso 1989; Clute and Masui 1997; Kappas et al. 2000), enabling the embryo to arrest its cell cycles in cases of DNA damage or blocks to DNA replication. Interestingly, much of the information available on *Xenopus* cell cycle checkpoints has been discovered in cell-free extracts derived from unfertilized *Xenopus* eggs. These cell-free extracts are used to simulate simple somatic-like cell cycle events. Addition of Ca^{2+} to the extract activates the extract to drive sperm nuclei added to the extract to undergo DNA synthesis and mitosis (Murray 1991). While blocks to DNA replication and damaged DNA are unable to activate a checkpoint pathway in an intact embryo, egg extracts supplemented with sufficiently damaged or unreplicated sperm nuclei arrest with low Cdc2 activity that is insufficient to induce mitosis (Dasso and Newport 1990; Kumagai et al. 1998). Studies in *Xenopus* egg extracts suggest that checkpoint signaling is present and functional but not operational in the early *Xenopus* embryo.

Experiments with *Xenopus* egg extracts have shown that *Xenopus* Chk1 is phosphorylated in response to unreplicated DNA; this phosphorylated form of Chk1 is required for the DNA replication checkpoint and is able to phosphorylate Cdc25C in response to a replication block (Kumagai et al. 1998). Phosphorylation of Chk1 also

occurs in response to a DNA replication block in the early embryo, but this event does not occur until shortly after the MBT is initiated at stage 8 (5 hrs pf; (Kappas et al. 2000). This phosphorylation event also occurs in response to DNA damage induced by IR, again not until after the MBT (Kappas et al. 2000). So, while the data from *Xenopus* egg extracts clearly shows that the Chk1 pathway is present and can be artificially stimulated in response to unreplicated DNA, in the embryo, the pathway is not operational until the MBT.

Even though Chk1 is not activated in response to a DNA replication block until the MBT, over expression of Chk1 through injection of mRNA encoding Chk1 at the single-cell stage will arrest the early embryonic cell cycles at the 2-or 4-cell stage, well before the MBT, suggesting an override of the mechanisms that keep the checkpoint from initiating before the MBT (Kappas et al. 2000). In addition, the over expression of Chk1 in early embryos initiates inhibitory phosphorylation of both embryonic Cdks, Cdk2 and Cdc2, on tyrosine 15 earlier than sibling controls injected with luciferase mRNA, suggesting that over expression of Chk1 arrests the early cell cycles through Cdk inhibition (Kappas et al. 2000). When Chk1 function is compromised through injection of an mRNA encoding a catalytically inactive "dominant-negative" form of Chk1, Cdc25A is not phosphorylated and targeted for degradation, and the resulting embryos do not gastrulate but demonstrate distinctive death morphology (Shimuta et al. 2002). Healthy *Xenopus* embryos transiently activate Chk1 at the MBT, and this activation is required in order for Chk1 to phosphorylate Cdc25A and target it for degradation (Shimuta et al. 2002). Further, Chk1 phosphorylates Cdc25A on the C-terminal domain at threonine 504 (Uto et al. 2004). This phosphorylation event inhibits Cdc25A binding to its cyclin/Cdk substrates, is required for the DNA replication checkpoint, and does not target Cdc25A for degradation, suggesting that Chk1

modulates cyclin/Cdk activity in *Xenopus* embryos through inhibiting Cdc25A binding to its substrates (Uto et al. 2004). If Cdc25A activity is not held in check at the MBT, the point where Chk1 is transiently activated, the whole embryo undergoes a dramatic form of cell death that has been characterized as apoptotic in nature (Sible et al. 1997; Shimuta et al. 2002). The mechanisms of this death are not understood, but may be related to changes in regulation of replication origin firing during the MBT.

In early *Xenopus* embryos, replication origins are randomly positioned (Hyrien and Mechali 1992), and no special DNA sequence is required for replication of DNA molecules (Harland and Laskey 1980). This random firing is thought to occur in order for the embryo to alternate quickly between DNA synthesis and mitosis. Two of the hallmarks of the MBT, occurring at cycle 12, are the slowing of DNA replication(Frederick and Andrews 1994) and the activation of cell cycle checkpoints (Kappas et al, 2000). Inhibition of Chk1 results in additional rounds of DNA synthesis during the MBT (Carter and Sible 2003). The transient activation of Chk1 during the MBT (Shimuta et al. 2002), as well as the additional rapid rounds of DNA replication observed when Chk1 activity is inhibited suggests that Chk1 is responsible for slowing down DNA replication in the early embryo by regulating the activity of Cdc25A, since Cdc25A is a target of Chk1 (Sagata 2002). Cdc25A is also responsible for the activation of key S-phase cyclin/Cdk complexes, particularly cyclin E/Cdk2, and the resulting overactive Cdc25A increases the activity of the cyclin E/Cdk2 complex in loading Cdc45 onto the origin site allowing for excess DNA replication (Shechter et al. 2004). Therefore, the dramatic apoptosis-like cell death observed when Cdc25A activity is left unchecked may be the result of continued promiscuous origin firing after the MBT.

1.7 Apoptosis and Development

Apoptosis is a suicide program initiated by the cell in instances of cellular distress (Orrenius et al. 2003). Apoptosis is of interest because it is an example of death that is active, requires ATP, and is genetically controlled, the result of evolutionarily conserved pathways involving complex cell signaling systems (Orrenius et al. 2003). Apoptosis is a recycling approach to cell death, where all cellular materials are reabsorbed by the organism. One of the first situations in which this was described was the absorption of the tadpole tail during frog development (Kerr et al. 1974).

The high degree of condensation and subsequent fragmentation of the genome during apoptosis is a prime example of the conservative nature of the process. During apoptosis, the cell undergoes increased nuclear and cytoplasmic condensation in comparison to the condensation observed during mitosis. The condensation of the cellular compartments leads to the "blebbing" of the plasma membrane (Orrenius et al. 2003). The blebbing leads to the formation membrane enclosed fragments, called apoptotic bodies, which are then engulfed by neighboring phagocytic cells, recycling the cellular materials (Jesenberger and Jentsch 2002). Apoptosis is marked by its lack of an inflammatory response, in opposition to necrosis, which is characterized by irreversible swelling of the cytoplasm, cell lysis, and release of noxious cellular components (Orrenius et al. 2003).

Due to the lack of an inflammatory response, apoptosis is of great significance to the organism during development. During metazoan development, excess cells are produced followed by an apoptotic culling during later stages of development, usually for proper organ formation (Meier et al. 2000). For instance, during mammalian development, the Mullerian duct develops into the uterus and oviduct in females, but must be removed by apoptosis in males (Meier et al. 2000). In contrast, the

Wolffian duct is the precursor for the male reproductive organs and is deleted by apoptosis in females (Meier et al. 2000). During development, cells that are in excess fail to receive the appropriate trophic signals for survival and, consequently, activate an innate self-destruct mechanism (Meier et al. 2000; Yuan and Yanker 2000). For example, during neuronal development, immature neurons are deleted in large numbers (Yuan and Yanker 2000). Continued neuronal survival requires a positive survival signal; therefore, trophic factor deprivation activates a cellular suicide program in vertebrate neurons (Yuan and Yanker 2000).

The early cell cycles of the *Xenopus laevis* embryo provide an interesting developmental context in which to study apoptosis. The embryo can be treated with aphidicolin to inhibit DNA replication or IR in order to damage the genome, and embryonic cells will continue to divide until the MBT (Newport and Dasso 1989; Anderson et al. 1997; Clute and Masui 1997; Hensey and Gautier 1997; Stack and Newport 1997; Kappas et al. 2000; Carter and Sible 2003) at which time, the embryo undergoes apoptosis (Sible et al. 1997). Further, embryonic cells with compromised protein synthesis (Hensey and Gautier 1997; Sible et al. 1997; Stack and Newport 1997), altered methylation (Kai et al. 2000; Stancheva et al. 2001) or blocked transcription (Hensey and Gautier 1997; Sible et al. 1997; Stack and Newport 1997) before the MBT will initiate the apoptosis within a very short period of time after the MBT (Sible et al. 1997). After the MBT, the embryo seems to have acquired active checkpoints and in the case of genomic assault, the cells are able to arrest, making the embryos more resistant to apoptosis (Anderson et al. 1997; Hensey and Gautier 1997).

1.8 Cyclin/Cdks during Apoptosis

While apoptosis has been studied for over twenty years in a variety of contexts, the mechanisms governing initiation, regulation and execution of apoptosis are still not clearly understood (Wyllie and Golstein 2001). Cells carefully balance the controls regulating proliferation, and those pathways regulating cell division are, in some instances, subverted for the execution of apoptosis (Evan et al. 1995). In many instances, the same genes that are involved in regulating the cell cycle regulate apoptosis (Nagano et al. 2000).

Mitosis and apoptosis display some very similar morphological features: cells lose substrate attachment, become rounded, shrink, condense their chromatin and display membrane blebbing (Pucci et al. 2000). Further, both processes must phosphorylate and disassemble the nuclear lamins in order to dissociate the nucleus. The similarities between mitosis and apoptosis are partly due to Cdk activity during both processes (Shi et al. 1994). For example, Cdc2 forms complexes with both cyclins A and B to control cell entry into mitosis. These complexes initiate dissolution of the nuclear membrane and promote chromatin condensation, events also marking apoptosis (Shi et al. 1994). Further, premature Cdc2 activation may be a general mechanism by which cells induced to undergo apoptosis initiate the disruption of the nucleus (Shi et al. 1994).

Activation of Cdk2 is also a hallmark of apoptosis in some cell types. Mouse mesangial cells show increased Cdk2 activity when treated with UV to induce apoptosis that is distinct from Cdk2 activity during DNA synthesis (Hiromura et al. 2002). When Cdk2 activity is inhibited with roscovitine, an inhibitor of Cdk1 and Cdk2, or with a dominant negative form of Cdk2, UV-induced apoptosis is abrogated (Hiromura et al. 2002). In rat cardiomyocytes, hypoxia-induced apoptosis also results in an increase in cyclin A/Cdk2 associated activity (Adachi et al. 2001).

As with UV-induced apoptosis, hypoxia-induced apoptosis can be blocked by expression of a dominant-negative form of Cdk2 (Adachi et al. 2001). Alternatively, infection of rat cardiomyocytes with cyclin A adenovirus results in the appearance of DNA fragmentation ladders characteristic of apoptotic cells (Adachi et al. 2001). Bax and Bcl-2, upstream regulators of caspase activity (Harvey et al. 1998), can also regulate Cdk2 activation (Gil-Gomez et al. 1998). Bax-accelerated apoptosis results in premature Cdk2 activation, while blocking apoptosis through over expression of Bcl-2 delays the appearance of Cdk2 activation (Gil-Gomez et al. 1998).

Treatment of SK-HEP-1 cells with Ginsenoside Rh2 (G-Rh2), an agent that inhibits cell growth and induces apoptosis in a variety of cancer cell types (Jin et al. 2000), results in proteolytic activation of caspase-3 and cleavage of Cip/Kip family member, p21 (Jin et al. 2000). The result of p21 cleavage is the abolition of cyclin A binding and increased cyclin A-associated activity that can be inhibited by the Cdk2 inhibitor, olomucine (Jin et al. 2000). Therefore, p21 is cleaved so that it will no longer be able to inhibit cyclin A/Cdk2 activity. Similarly, treatment of HeLa cells with staurosporine to induce apoptosis results in an increase in caspase-dependent Cdk activity (Harvey et al. 2000). The resulting caspase-dependent Cdk activity is abrogated in HeLa cell extracts where Cdk2 has been depleted (Harvey et al. 2000). Expression of DN-Cdk2 inhibits chromatin condensation in cells treated with TNF-α or staurosporine (Harvey et al. 2000). Over expression of p21 and p27 also inhibited chromatin condensation as a result of treatment with staurosporine or TNF-α (Harvey et al. 2000). However, while DN-Cdk2 expression abrogates the nuclear morphological events associated with staurosporine induced apoptosis, it does not inhibit caspase activity or caspase dependent cleavage of p21 (Harvey et al. 2000).

The data suggest that during apoptosis in mammalian somatic cells, cyclin A/Cdk2 activity is dependent on active caspases. Harvey et al (2000) showed that they could block certain physical events marking apoptosis, mainly chromatin condensation, while not blocking caspase activity. Further, over expression of cyclin A by infection with an adenovirus results in the appearance of DNA fragmentation ladders (Adachi et al. 2001), indicating characteristic apoptotic DNA fragmentation. Taken together, the data indicate that cyclin A/Cdk2 activity has a consistent role in the initiation of the DNA fragmentation that is a signature event during apoptosis downstream of caspase activity.

1.9 Cyclin A1/Cdk2 during Apoptosis in Early *Xenopus laevis* Embryos

In contrast to mammalian somatic cells, the *Xenopus* embryo expresses an early developmental cyclin A, cyclin A1, as well as a somatic-type cyclin A, cyclin A2. Cyclin A2 is much closer in sequence homology to mammalian somatic cyclin A than the early embryonic cyclin A1 (Howe et al. 1995). Both forms of cyclin A are cleaved when *Xenopus* embryos are treated with hydroxyurea (HU) to inhibit zygotic transcription and induce apoptosis (Stack and Newport 1997). Both cleavage forms of cyclin A1 and cyclin A2 remain with the addition of inhibitors of the proteosome or ubiquitination indicating that the appearance of these cleaved forms is not regulated by the proteosome (Stack and Newport 1997). However, the appearance of the cyclin A2 cleavage product can be abrogated by the addition of caspase-3 inhibitors, and the cyclin A2 protease is activated by the addition of cytochrome c to extracts of embryos treated with HU, indicating that the cleavage of cyclin A2 is mediated by caspase-3 (Stack and Newport 1997). Cyclin A2 is also cleaved when *Xenopus* embryos are treated with

IR to induce apoptosis, and this cleavage event is abrogated by specific inhibition of caspase-3 (Finkielstein et al. 2002). Further, addition of recombinant cleaved cyclin A2 to *Xenopus* cell-free extracts results in the appearance of apoptotic-like nuclei, independent of caspase activity, suggesting that caspases cleave cyclin A2 to drive the nuclear changes associated with apoptosis (Finkielstein et al. 2002).

Cleavage of a protein by caspases usually results in inactivation of that protein (Rich et al. 2000). Therefore, cleavage of *Xenopus* cyclin A2 by caspase-3 during apoptosis could indicate that the cyclin is being removed from the cell cycle in order to keep it from wasting cellular energy by continuing to replicate a genome damaged beyond repair. However, cleavage of cyclin A2 by caspase-3 removes the destruction box required for ubiquitination of cyclin A2, so that cyclin A2 persists during IR-induced apoptosis (Finkielstein et al. 2002). When cleaved cyclin A2 is immunoprecipitated for Cdk2 binding, it was determined that the cleaved cyclin A2 is still capable of forming a complex with Cdk2 (Finkielstein et al. 2002). Further, the cleaved cyclin A2/Cdk2 complex is more active than its non-cleaved counterpart due to inability to bind the *Xenopus* p27 inhibitor, Xic1, and cleaved cyclin A2 expands its substrate specificity to include histone H2B as well as histone H1 (Finkielstein et al. 2002), suggesting that the cleavage of cyclin A2 during IR-induced apoptosis is necessary for the additional chromatin condensation and DNA fragmentation observed during apoptosis. The idea that cyclin A2 cleavage mediates apoptotic DNA fragmentation is further supported by the addition of recombinant cleaved cyclin A2 to *Xenopus* cell-free extracts, which resulted in the appearance of apoptotic-like nuclei independent of caspase activity (Finkielstein et al. 2002). Therefore, like the mammalian cyclin A/Cdk2 complex, *Xenopus* cyclin A2/Cdk2 is an important regulator for the morphological events that characterize apoptosis.

1.10 Relevance

The early cell cycles of *Xenopus laevis* development are unique for a variety of reasons. The system undergoes a critical period of developmental change, the midblastula transition (MBT). Cell checkpoint molecules, like Chk1, are capable of being activated in response to DNA assaults (Kappas et al. 2000), and maternal transcripts are degraded and their zygotic counterparts are transcribed to replace them, as is the case with cyclins A1 and A2 (Howe et al. 1995). These molecular changes are important to enable the cell to make the decision between arrest and apoptosis, and manipulations of these molecules during the MBT can provide a wealth of information on the molecular switches required for the cell to make the decision between arrest and apoptosis.

The delay of the early *Xenopus* embryo in initiating apoptosis until a specific developmental time period provides a window of opportunity to study the molecules required for the regulation of apoptosis. As stated at the beginning of this section, the mechanisms regulating apoptosis in general are not well understood, but studies suggest that some of the molecules regulating the cell cycle are involved in the regulation of apoptosis. These studies examine the role of two cell cycle molecules, Chk1 and Cyclin A1, and their involvement in the regulation of apoptosis during the early cell cycles of the *Xenopus laevis* embryos. Chapter 3 describes the effect of inhibiting the function of Chk1 before the MBT on the post-MBT embryo. Chapter 4 examines the role of cyclin A1 during the initiation of apoptosis by a block to DNA replication. The fourth chapter also examines the sufficiency and requirement for cyclin A1 in the initiation of apoptosis at the MBT.

2 Materials and Methods

2.1 Maintenance and Manipulation of the Embryos

All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Tech. Eggs from wild-type *Xenopus laevis* (Xenopus Express) were fertilized *in vitro*, dejellied in 2% cysteine, 0.1X MMR (0.5 mM HEPES (pH 7.5), 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 according to Nieuwkoop and Faber (1975). For some experiments, embryos were microinjected at the single-cell stage with 18 ng DN-Chk1 mRNA, 18 ng luciferase mRNA, 1.4 $X10^{-4}$ pmol activity/min recombinant cyclin A1/Cdk2 or buffer and then maintained in 0.1X MMR containing 5% Ficoll. To block transcription, embryos were microinjected at the single cell stage with 50 ng α-amanitin (Sigma), while control embryos were injected with an equal volume of H_2O. To block DNA replication, embryos were incubated at the indicated times in 0.1X MMR containing 100μg/ml aphidicolin (Calbiochem) and 1% DMSO. Control embryos were maintained in 0.1X MMR, 1% DMSO. Embryos were photographed using an Olympus SZX12 stereo microscope equipped with an Olympus DP10 digital camera. For all experiments, the beginning of the MBT (Stage 8) occurred between 5 and 6 hr post-fertilization.

2.2 Expression of DN-Chk1 in *Xenopus laevis* Embryos

Generation of plasmids encoding FLAG-tagged, catalytically inactive, dominant-negative Chk1 (DN-Chk1 with asparagine 135 mutationally altered to alanine) and luciferase was described previously (Kappas et al. 2000). In these experiments, capped mRNAs were synthesized using the SP6 mMessage mMachine *in vitro* transcription kit

(Ambion) and unless otherwise indicated, 20 ng mRNA was injected into one-cell embryos.

2.3 Western Analysis of FLAG, Chk1, Cyclin A1, and Cyclin A2

Embryos were collected at the times indicated, snap frozen on dry ice, and homogenized in EB buffer (20 mM HEPES, pH 7.5, 80 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 1mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 1 mM microcystin). Lysates were stored in 5X gel loading buffer (1X = 0.6 mM Tris base, 2% glycerol, 3% SDS, 0.002% bromophenol blue) containing 25% β-mercaptoethanol, heated at 95ºC for 2 min, and resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and each protein was detected by its specific antibody. Immunoblotting of exogenous FLAG-tagged luciferase and DN-Chk1 was performed as described previously using a mouse monoclonal antibody against the FLAG epitope (Kappas et al. 2000). Immunoblotting of endogenous and exogenous Chk1 was performed with a rabbit polyclonal antibody against Chk1, as described (Kappas et al. 2000). Immunoblotting of Cyclin A1, cyclin A2, and Cdk2 were performed with mouse monoclonal antibodies generously provided by Tim Hunt and Julian Gannon (Cancer Research, UK). Immunoblotting of Ser-14 phosphorylatd Histone H2B was performed using a rabbit monoclonal antibody against the Ser-14 phosphorylated species of Histone H2B purchased from Upstate Biotechnology. Immunoreactivity was detected with a horseradish peroxidase-conjugated secondary antibody raised in donkey or goat (Jackson Labs) and the ECL plus (Amersham) chemiluminescence detection kit.

2.4 Assessment of Nuclear Morphology

Embryos were collected at the indicated times, fixed in 4% paraformaldehyde, dehydrated through an ethanol series, cleared in Citrisolv (Fisher) embedded in paraffin, sectioned 7 μm thick, deparaffinized by clearing with Citrisolv, rehydrated and stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma). Sections were viewed and photographed on an Olympus AX70 fluorescence microscope equipped with a Color View 12 digital camera.

2.5 Whole-Mount TUNEL Assay

Albino embryos were injected with 18 ng DN-Chk1 or luciferase mRNA, or treated with 100 μg/ml aphidicolin and collected when development appeared abnormal and controls were gastrulating. Embryos were fixed 1 – 2 hr in MEMFA (100 mM MOPS, pH 7.2, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), dehydrated in ethanol, and stored in ethonol at -20°C overnight. Embryos were then rehydrated through an ethanol series, washed sequentially in PBS, PTween (PBS containing 0.2% Tween 20), PBS, and TdT buffer (Invitrogen). Embryos were then incubated overnight at room temperature in TdT buffer containing 150 U/ml terminal deoxynucleotidyl transferase (TdT; Invitrogen) and 0.5 mM digoxygenin-dUTP (Boehringer Mannheim). The next day, embryos were washed twice for 1 hr at 65°C with PBS containing 1 mM EDTA, four times for 1 hr with PBS at room temperature, once for 15 min with PBT (PBS containing 2 mg/ml BSA and 0.1% Triton X-100), and once for 1 hr or more with PBT containing 20% normal goat serum. Embryos were then incubated overnight at 4°C in PBT containing 20% normal goat and alkaline phosphatase-conjugated anti-digoxygenin Fab fragments (1:2000; Boehringer Mannheim). Embryos were washed for 24 hr with multiple changes of

PBT and then briefly with alkaline phosphatase buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 5 mM levamisol; Sigma). The chromagenic reaction was performed in alkaline phosphatase buffer containing 0.33 mg/ml NBT and 0.175 mg/ml BCIP (Roche). When reactions reached the desired intensity, embryos were photographed using an Olympus SZX12 stereo microscope equipped with an Olympus DP10 digital camera.

2.6 Assays for Caspase Activity

Embryos were assayed for caspase activity by the cleavage of recombinant poly ADP-ribose polymerase (PARP) substrate, as modified from the protocol of Hensey and Gautier (1997). Three embryos were collected at the times indicated, snap frozen on dry ice, and homogenized in caspase extraction buffer (CEB: 80 mM β-glycerophosphate, 15 mM MgCl2, 20 mM EGTA, 10 mM DTT). Lysates were incubated with 5 ng/μl recombinant human PARP (Alexis Biochemicals) at 30°C for 15 – 30 min, then resolved on 7.5% Anderson SDS-polyacrylamide gels (Kappas et al, 2000), and transferred to nitrocellulose membranes. Western analysis of PARP was performed by hybridizing membranes with anti-PARP antibody (Alexis Biochemicals) diluted 1:5000 in 10% nonfat dry milk in PBS. Immunoreactivity of proteins was visualized using horseradish peroxidase-conjugated secondary antibody (Jackson), and chemiluminescence from the secondary antibody was detected with West Pico (Pierce).

2.7 Incorporation of ^{3}H-thymidine into DNA

To measure the effect of DN-Chk1 on DNA replication, embryos were injected at the one-cell stage as described above with 18 ng mRNA

encoding luciferase or DN-Chk1. At 70 min post fertilization, embryos were transferred to Danilchik's medium (Peng, 1991) containing 5% Ficoll and 700 μCi/ml ^{3}H-thymidine (ICN, 68 Ci/mmol). At 4 hr pf, embryos were transferred to 0.1X MMR. Ten embryos were collected at the time points indicated and snap frozen. Embryos were homogenized in 500 μl lysis buffer (1% SDS, 10 mM Tris, pH 7.0, and 10mM EDTA, 50 μg/ml RNase; Roche) and incubated at 37°C for 2 hrs. After 2 hrs, 10 μg porteinase K was added, and the lysate was incubated at 37°C for another 3 hrs. The lysate was phenol-extracted twice and then chlorophorm/phenol extracted twice to remove excess phenol. DNA was precipitated with 100% ethanol at -80°C for 30 minutes, washed with 70% ethanol, and resuspended in Tris/EDTA, pH 7.0. The DNA was assayed for incorporation of ^{3}H into DNA, as described previously (Newport and Kirshner, 1982; Sible et al, 1997).

2.8 Northern Analysis of GS17 Expression

Northern analysis of GS17 expression was performed essentially as described (Sible et al, 1997). Total RNA was isolated from embryos with TriReagent (Molecular Reseach Center). A total of 20 μg of each RNA was resolved by denaturing gel electrophoresis, transferred to a 0.2 μm Nytran membrane with a TurboBlotter apparatus (Schleicher & Schuell) and then cross-linked to the membrane with a Stratagene UV cross-linker. A probe was generated by a random priming reaction (Roche) using [α^{32}P]dCTP and the GS17 gene (Krieg and Melton,1985) as template.

2.9 Immunoprecipitation

Immunoprecipitation of cyclin-associated kinase activity was performed essentially as described previously (Anderson et al, 1997). Embryos collected at the indicated times were homogenized in EB (10 μl of EB per embryo), centrifuged, and the supernatant was pre-cleared with 50% protein G-Sephrose beads in EB at 4°C for 1 h. The pre-cleared supernatant was transferred to a clean tube and incubated overnight on ice with 4 μl crude cyclin A1 or cyclin E antiserum. After incubation, 25 μl of 50% protein G-Sepharose beads in EB were added to the supernatant and incubated on a rocking nutator at 4C for 1 h. The lysate was removed and the beads were washed twice with low salt buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 100 mM NaCl), twice with high salt buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 1 M NaCl) and used for kinase assays (described below).

2.10 Kinase Assays

Immunopreciptiation of cyclin A1-associated kinase activity or cyclin E-associated kinase activity was performed as described above. The resulting immunoprecipitates were washed twice in kinase buffer (20 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol), and then incubated 20 min at 37°C with 25 μl kinase buffer containing 0.2 mg/ml bovine serum albumin, 0.5mg/ml histone H1, 200 μM [γ^{32}P]ATP [4 μCi/reaction]. The reaction was stopped by the addition of 25 μl 5X gel loading buffer containing 25% β-mercaptoethanol and heated at 95°C for 2 min. Samples were loaded on a 12.5% Lamelli acrylamide gel and electrophoresed at 200V, 0.35 A for 4 h. Gels were dyed with Coomassie Blue for 1 h, and destained overnight in destain solution (45% methanol, 10% glacial acetic acid). Incorporation of

[γ^{32}P]ATP was determined by Cerenkov scintillation counting the excised histone H1 band.

2.11 Inhibition of Cyclin A1 and Cyclin A2 Expression

Embryos were injected with 100 ng cyclin A1 (AATTTAGG-TCAACTGCTACAACCAG) and/or cyclin A2 (TCTGACCATCTGCTGCGG-GATGAGC) morpholinos (GeneTools) synthesized to bind to the mRNA head at the single cell stage. Embryos were then treated at stage 8 (5 hr pf) with 100 μg/ml aphidicolin or 40 Gy γ-irradiation (IR). Induction of apoptosis was determined by gross embryo morphology and induction of caspase activity (described above).

3 Loss of Chk1 Function Results in Apoptosis in Early *Xenopus Laevis* Embryos

3.1 Abstract

Prior to the midblastula transition (MBT), *Xenopus laevis* embryos do not engage cell cycle checkpoints, although over expression of the kinase Chk1 arrests cell divisions. At the MBT, Chk1 transiently activates and promotes cell cycle lengthening. In this study, endogenous Chk1 was inhibited by the expression of a dominant negative Chk1 (DN-Chk1). Development appeared normal until the early gastrula stage, when cells lost attachments and chromatin condensed. TUNEL and caspase assays indicated these embryos died by apoptosis during gastrulation. Embryos with unreplicated DNA also died by apoptosis. Embryos expressing DN-Chk1 proceeded through additional rapid rounds of DNA replication but initiated zygotic transcription on schedule. Therefore, Chk1 is essential in the early *Xenopus* embryo for cell cycle remodeling and for survival after the MBT.

3.2 Introduction

Early embryonic cell cycles of *Xenopus laevis* provide rare examples of non-pathological cell divisions that lack cell cycle checkpoints. Early embryos divide rapidly and synchronously, from fertilization until the 12th cell cycle, where dramatic developmental changes mark a point called the midblastula transition (MBT). Prior to the MBT, cell divisions are rapid and synchronous (Newport and Kirschner 1982a), driven by oscillations in cyclin-dependent kinase (Cdk) activity due to the synthesis and degradation of mitotic cyclins (Murray and Kirschner 1989; Hartley et al. 1996). The MBT also marks the initiation of zygotic transcription and cell motility (Newport and Kirschner 1982b). In addition, the MBT delineates a period of extensive

cell cycle remodeling (Frederick and Andrews 1994) characterized by cell cycle lengthening and the loss of synchronous divisions (Newport and Kirschner 1982a). At the molecular level, maternally provided cyclin mRNAs are degraded (Harley et al. 1996; Howe et al. 1995; Howe and Newport 1996; (Rempel et al. 1995), and their zygotic counterparts are synthesized (Howe et al. 1995).

Since pre-MBT *Xenopus* embryos lack cell cycle checkpoints, when treated with aphidicolin, an inhibitor of DNA polymerase α, these embryos do not arrest cleavage cell divisions, but instead continue development with unreplicated DNA (Newport and Dasso, 1989). However, these embryos fail to gastrulate and reportedly die by apoptosis after the MBT (Hensey and Gautier 1997). As embryos reach the MBT, checkpoint pathways become partially functional, and embryos are now able to arrest the cell cycle in response to treatment with aphidicolin (Kimelman et al. 1987; Clute and Masui 1997; Hartley et al. 1997). Embryos treated with aphidicolin after the MBT arrest development and do not die by apoptosis (Hensey and Gautier 1997), suggesting acquired resistance to apoptosis after the MBT.

In addition to the initiation of zygotic transcription and checkpoint pathways, the MBT also marks a period when embryos become susceptible to apoptosis. Embryonic cells with DNA damage (Anderson et al. 1997; Hensey and Gautier 1997), compromised protein synthesis (Hensey and Gautier 1997; Sible et al. 1997; Stack and Newport 1997), altered methylation (Kai et al. 2000; Stancheva et al. 2001) or blocked transcription (Hensey and Gautier 1997; Sible et al. 1997; Stack and Newport 1997) prior to the MBT engage a maternally regulated default program of apoptosis (Sible et al. 1997) by the early gastrula transition (EGT; Stack and Newport 1997). These results suggest that survival beyond the MBT depends upon embryonic synthesis of an anti-apoptotic factor. However, no such factor has been identified, although it is

possible the anti-apoptotic factor is a molecule involved in checkpoint regulation. After the MBT, embryos become resistant to apoptosis (Anderson et al. 1997; Hensey and Gautier 1997), indicating that sufficient anti-apoptotic factor has accumulated.

One checkpoint kinase that has been extensively studied in this laboratory as well as others is *Xenopus* Chk1. This kinase functions within a biochemical pathway that inhibits cyclin-Cdk complexes. During somatic cell cycles and in frog egg extracts, the G2/M cell cycle checkpoint is engaged in response to unreplicated DNA. When DNA replication is blocked, ATR kinase phosphorylates and activates Chk1 (Guo et al. 2000; Hekmat-Nejad et al. 2000) which, in turn, phosphorylates Cdc25C phosphatase, facilitating its binding to the 14-3-3 cytoskeletal protein and sequestration in the cytoplasm (Kumagai et al. 1998). Thus, Cdc25C cannot enter the nucleus to remove inhibitory phosphates from threonine 14 and tyrosine 15 of Cdc2, the mitotic Cdk (Kumagai et al. 1998). Chk1 also phosphorylates and activates the opposing kinase Wee1 (Lee et al. 2001). Inhibition of Cdc25C and activation of Wee1 by Chk1 renders Cdc2 inactive, preventing cell cycle progression into mitosis.

In the *Xenopus* embryo, the Chk1 pathway is intact but not functional before the MBT. Chk1 protein level remains relatively constant from egg through tadpole stage (Kappas et al. 2000; (Nakajo et al. 1999), and over expression of Chk1 induces tyrosine phosphorylation of Cdc2 and Cdk2, causing cell cycle arrest (Kappas et al. 2000). However, Chk1 does not become activated by phosphorylation in response to a DNA replication block until the embryo reaches an approximate MBT DNA content (Kappas et al. 2000). Shimuta et al. (2002) demonstrated that Chk1 is transiently activated at the MBT and that Chk1 function is required for the timely degradation of Cdc25A as well as the lengthening of embryonic cell cycles. The following

experiments examine the developmental fate of embryos lacking Chk1 with respect to several events of the MBT: initiation of apoptosis, the onset of zygotic transcription and remodeling of the cell cycle.

3.3 Results

3.3.1 Embryos expressing DN-XChk1 develop normally until the early gastrula transition.

To determine whether Chk1 is required for early development in the *Xenopus* embryo, single-cell embryos were microinjected with 18 ng of RNA encoding a catalytically inactive, dominant-negative form of Chk1 (DN-Chk1). The dominant-negative construction was generated by mutating the codon for asparagine 135 to that for alanine. Control embryos were injected with equal amounts of FLAG-tagged luciferase mRNA. The resulting embryos were staged according to (Nieuwkoop and Faber 1975). Luciferase- and DN-Chk1-expressing embryos developed at the same rate and with no obvious differences or indication of altered timing of cell divisions until after the MBT, about 5 hours post fertilization (hr pf). At the EGT (9 hr pf), embryos expressing DN-Chk1 still appeared relatively normal (Figure 4A, middle), but soon began to lose attachments and burst through the blastocoel (Figure 4A bottom). As the control embryos completed gastrulation, >90% of the embryos expressing DN-Chk1 had detached cells filling the cavity of the vitelline membrane (Figure 4B; Shimuta et al. 2002). Control embryos developed normally despite expression of a similar level of FLAG-tagged protein (Figure 4C). The level of exogenous DN-Chk1 was several fold higher than the level of endogenous Chk1 (Figure 4D), consistent with a dominant-negative effect.

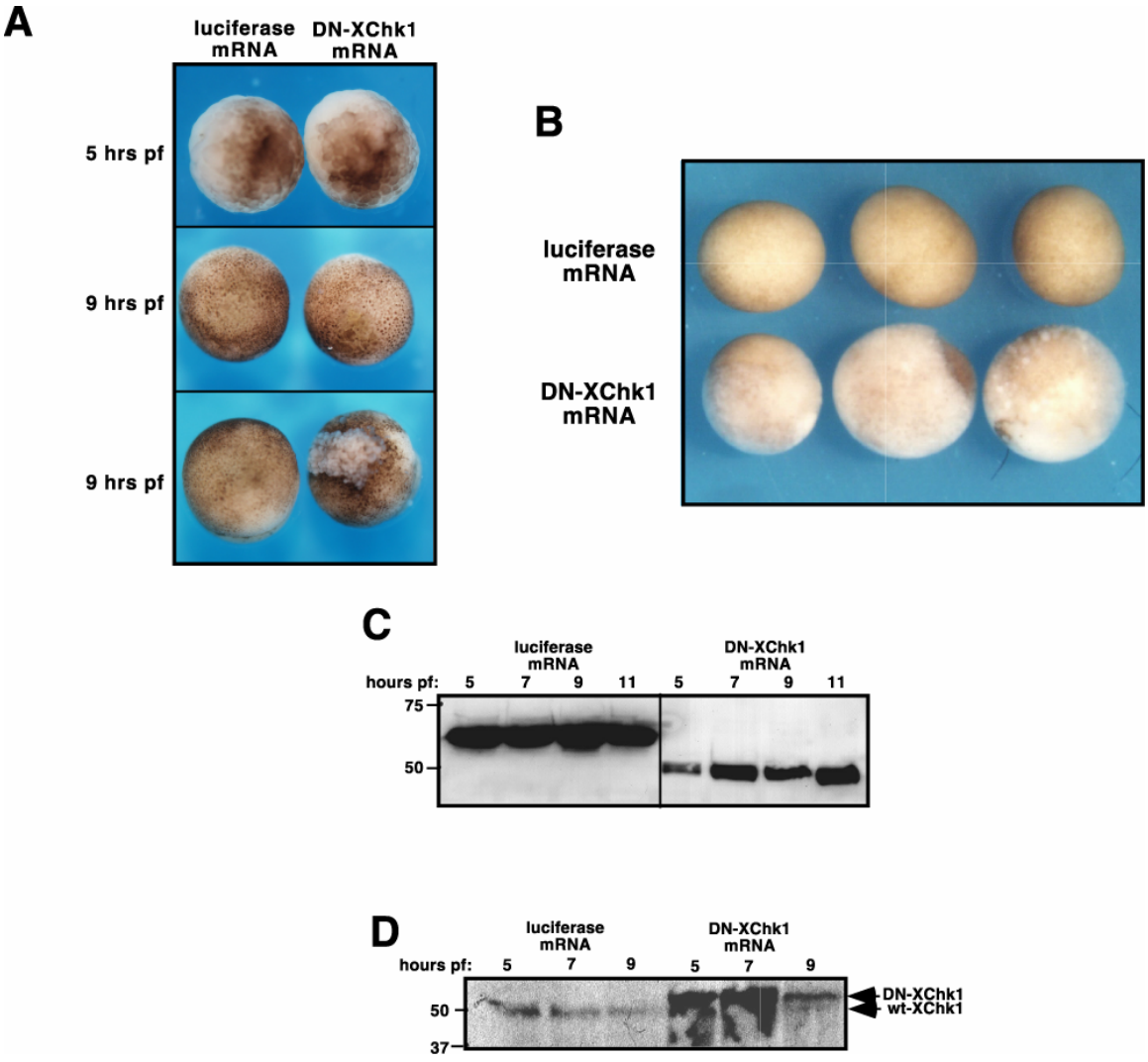

Figure 4. Embryos expressing DN-Chk1 develop abnormally after the MBT. Embryos were injected with 18ng luciferase or DN-Chk1 mRNA at the single cell stage and photographed at various stages of development. A) Embryo morphology at 5 hr pf (post-fertilization, top) and 9hr pf (middle, bottom). B) Embryos were photographed when embryos expressing luciferase began to gastrulate (>12 hr pf). C) Embryos were processed for immunoblotting of the FLAG epitope. D) Embryos were processed for immunoblotting of Chk1. Positions of endogenous (wt) and exogenous (DN) Chk1 are indicated. The molecular weight markers (in kDa) are indicated (Carter and Sible 2003).

Although gross morphology and rate of cell division of embryos injected with DN-Chk1 appear normal prior to the EGT (Figure 5; Shimuta et al. 2002), it is possible that nuclear events (DNA replication and mitosis) are perturbed, since embryos continue cleavage divisions before the MBT even in the presence of damaged (Anderson et al. 1997)

or unreplicated (Newport and Dasso 1989) DNA. To determine whether nuclear morphology was altered in the embryos expressing DN-Chk1, nuclear morphology of embryos injected with luciferase or DN-Chk1 mRNA at the single-cell stage was examined by fluorescence microscopy of DAPI stained, paraffin-embedded embryos sectioned and mounted on microscope slides. Control and DN-XChk1 expressing embryos were fixed at 6 and 12 hr pf (Figure 5). As a positive control for altered nuclear morphology, sibling embryos were treated with aphidicolin, which is known to perturb DNA replication, at 3 hr pf and fixed at the same time points as the luciferase and DN-Chk1 injected embryos. Luciferase mRNA injected embryos collected at the MBT (6 hr pf) demonstrated mostly interphase nuclei with occasional mitotic figures (Figure 5A, arrow). The nuclei in embryos expressing DN-Chk1 appeared normal, indistinguishable in morphology and density from nuclei in embryos expressing luciferase at 6 hr pf. In contrast, embryos incubated in aphidicolin and collected at 6 hr pf possessed many abnormal nuclei with lacy chromatin (Figure 5A, asterisks), particularly in cells on the surface of the embryo, which had the greatest access to aphidicolin. Therefore, aphidicolin, but not DN-Chk1, induces nuclear abnormalities by the MBT detectable by light microscopy of sectioned embryos. If DN-Chk1 does induce nuclear abnormalities, then these occur below the threshold of detection of this assay and are morphologically distinct from the damage induced by aphidicolin.

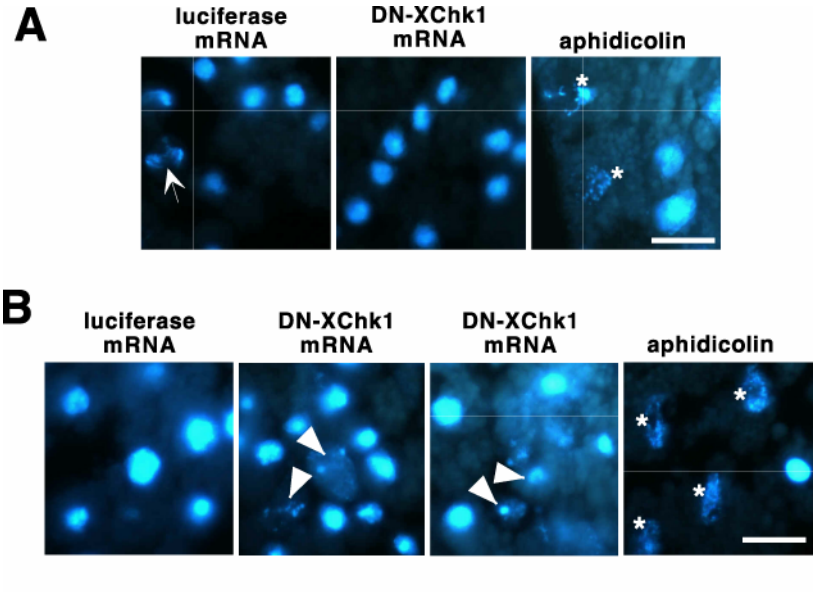

Figure 5. DN-Chk1 induces chromatin condensation after the MBT. Embryos were microinjected with 18 ng luciferase or DN-XChk1mRNA at the one-cell stage or treated with aphidicolin at 3 hr pf. Embryos were fixed at 6 (A) or >12 hr pf, sectioned, stained with DAPI, and photographed under fluorescence microscopy. Arrow = mitotic figure, asterisks = nuclei with lacy chromatin, arrowhead = apoptotic bodies with condensed chromatin, scale bar – 2.5μm (Carter and Sible 2003).

Embryos were also fixed at >12 hr pf, after controls had gastrulated and embryos treated with aphidicolin or expressing DN-Chk1 appeared abnormal (Figure 5B). At this time, DN-Chk1-expressing embryos contained fragmented nuclei with foci of condensed chromatin (arrowheads). Aphidicolin-treated embryos possessed lacy, abnormal nuclei similar to embryos collected at 6 hr pf, with further penetration into the embryo.

3.3.2 Embryos expressing DN-Chk1 die by apoptosis before completing gastrulation.

Embryos expressing DN-Chk1 demonstrated loss of cellular attachments (Figure 4) and condensation of chromatin (Figure 5) at the EGT, morphologic changes detected in embryos that undergo apoptosis

in response to damaged DNA (Anderson et al. 1997; Hensey and Gautier 1997) blocked transcription (Hensey and Gautier 1997; Sible et al. 1997) or inhibited protein synthesis (Hensey and Gautier 1997; Sible et al. 1997; Stack and Newport 1997). To determine whether loss of Chk1 function also results in apoptosis, albino embryos were injected with DN-XChk1 and luciferase mRNA and fixed when the controls began to gastrulate. The embryos were then labeled by whole-mount TUNEL assay to detect the double-stranded DNA breaks characteristic of apoptotic cells. In embryos collected when controls were gastrulating, there were very few TUNEL –positive cells in all of the embryos expressing luciferase, whereas 80-100% of the embryos expressing DN-Chk1 were TUNEL-positive for DNA fragmentation (Figure 6A). In particular, cells that had lost attachments (Figure 6A) demonstrated strong positive TUNEL reactions.

To verify apoptosis by a biochemical assay, cytosolic extracts of luciferase (control) and DN-XChk1-expressing embryos were assayed for caspase activity by incubation with recombinant human PARP protein, a known substrate for caspase-3 (Potten and Wilson 2004). PARP cleavage was determined by Western analysis with anti-PARP antibody. DN-Chk1-expressing embryos, but not luciferase-expressing embryos, were positive for PARP cleavage, indicative of caspase activity (Figure 6B). Caspase activity was first detected between 9 and 11 hr pf, correlating with gastrulation in control embryos. Therefore, inhibition of endogenous *Xenopus* Chk1 is sufficient to induce a program of apoptosis after the MBT in *Xenopus* embryos.

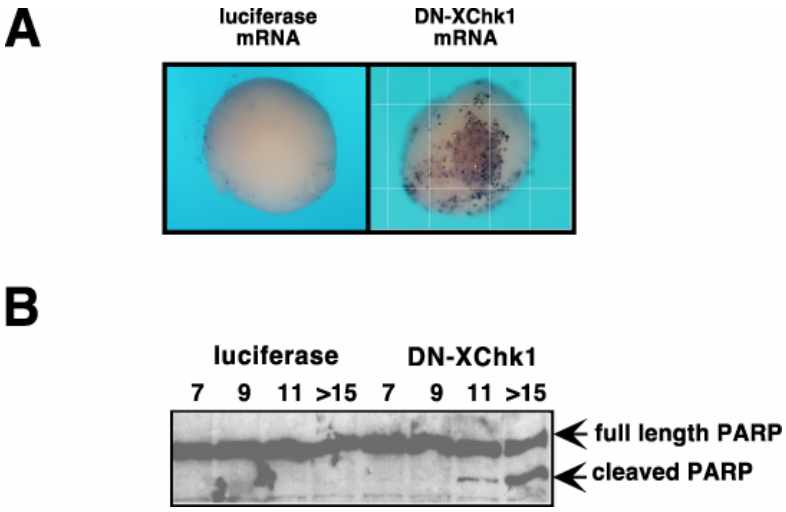

Figure 6. Inhibition of endogenous Chk1 results in apoptosis at the MBT. Embryos were injected with 18 ng luciferase or DN-XChk1 mRNA at the single-cell stage. A) Albino embryos were fixed and processed for TUNEL assays when morphology of embryos expressing DN-XChk1 appeared abnormal (approximately 12 hr pf). A positive TUNEL reaction is indicated by purple staining of nuclei. In a typical experiment, 0- 20% of luciferase expressing embryos and 80 – 100% DN-Chk1 expressing embryos scored TUNEL-positive. B) Embryos injected with luciferase or DN-XChk1 mRNA were assayed for caspase activity by the cleavage of the exogenous substrate, PARP. Times (hr pf) are indicated (Carter and Sible 2003).

3.3.3 A block to DNA replication also induces apoptosis

Because of the severe nuclear abnormalities (Figure 5) detected in aphidicolin-treated, we also tested aphidicolin treated embryos for apoptosis by TUNEL and caspase assays. Embryos treated with aphidicolin at the MBT (Figure 7) or anytime before the MBT (data not shown; Hartley 1997) develop with a slight delay in interdivision time, but otherwise appear normal through the MBT (Figure 7A). However, like embryos expressing DN-Chk1, aphidicolin-treated embryos lost cellular attachments when controls began gastrulating. These embryos demonstrated an increase in TUNEL-positive cells (Figure 7B), although the signal was generally lower than in DN-Chk1 embryos, probably due to reduced DNA content. Strong caspase activity was also detected in aphidicolin-treated embryos (Figure 7C). Therefore, a block to DNA

replication before or during the MBT also induces apoptosis at the EGT, in agreement with reports of Hensey and Gautier (1997).

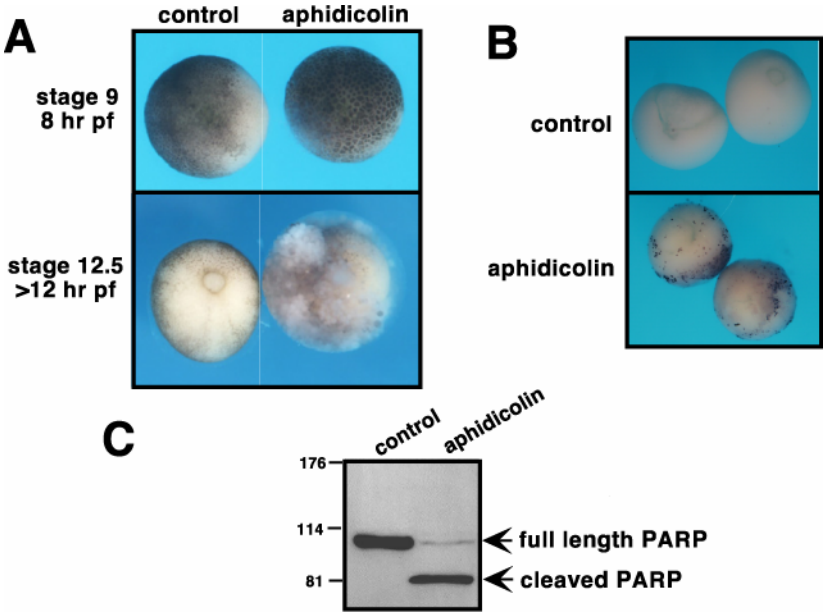

Figure 7. A block to DNA replication at the MBT induces apoptosis after the MBT in *Xenopus* embryos. A) Embryos were incubated in 100μg/ml aphidicolin during the MBT (6 hr pf), then were photographed when controls reached stage 9 (8 hr pf) and stage 12.5 (>12 hr pf). In aphidicolin treated embryos, cell cycle arrest was observed at 8 hr and embryos were dying by 12 hr. (B) Albino embryos were treated with aphidicolin at the MBT (5.5 hr pf) and fixed and processed along with sibling controls for TUNEL assays when morphology appeared abnormal (>12 hr pf). C) Control embryos and embryos treated with aphidicolin at the MBT (6 hr pf) were snap-frozen and assayed for caspase activity when controls gastrulated (approximately 12 hr pf). The migration of molecular weight standards (in kDa) is indicated on the left (Carter and Sible 2003).

3.3.4 Embryos expressing DN-Chk1 replicate DNA normally until the MBT then undergo several additional rounds of DNA replication.

Expression of DN-Chk1 and treatment with aphidicolin both induced apoptosis with similar timing and gross morphologic changes (compare Figures 4 and 6 to Figure 7). To examine the possibility that like aphidicolin, loss of Chk1 function likewise perturbed DNA replication prior to the MBT, embryos injected with luciferase or DN-Chk1 mRNA

were loaded with ^{3}H-thymidine from 70 minutes to 4 hours pf. Genomic DNA was isolated from the samples, and the amount of ^{3}H-thymidine incorporation was measured (Figure 8). Both control and experimental embryos entered the MBT at 5 hr pf with the same amount of ^{3}H-thymidine incorporation. Beginning at 5 hr pf, control embryos doubled their incorporation of ^{3}H-thymidine 60 minutes later (6 hr pf), evidence of lengthening cell cycles at the MBT, consistent with previous reports (Newport and Kirschner 1982a; Newport and Kirschner 1982b). In contrast, beginning at the MBT (5 hr pf), DN-Chk1-expressing embryos doubled the incorporation of ^{3}H-thymidine at 5.5 hr pf and then again at 6 hr pf, followed by a 60 minute doubling (7 hr pf). These results suggest two additional rapid rounds of rapid DNA replication in DN-Chk1-expressing embryos, similar to the additional rapid syncytial cell cycles in *Drosophila* embryos lacking the Chk1 homolog, Grp (Fogerty et al. 1997; Sibon et al. 1997). It is also possible that increased ^{3}H-thymidine incorporation is due to incorporation into DNA by a mechanism other than replication (i.e. repair). However, Shimuta et al. (2002) observed an increased content of genomic DNA at 9 hr pf in embryos expressing DN-Chk1, supporting additional rapid rounds of DNA replication.

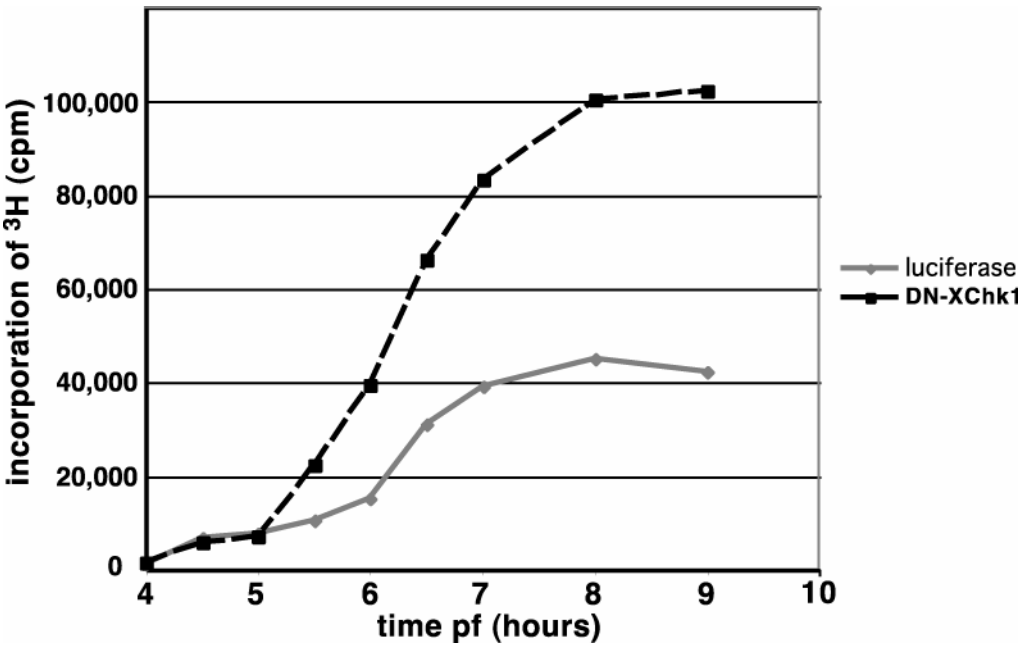

Figure 8. Expression of DN-Chk1 results in elevated incorporation of ^{3}H-thymidine after the MBT. Embryos were injected with 18 ng luciferase or DN-XChk1 mRNA at the single cell stage, incubated in medium containing ^{3}H-thymidine from 70 min – 4 hr pf, collected at the times indicated and assayed for the incorporation of ^{3}H into DNA. This data, from a single experiment, is representative of multiple experiments (Carter and Sible 2003).

3.3.5 Loss of Chk1 function does not delay the onset of zygotic transcription

Like Grp in *Drosophila* (Fogarty et al. 1997; Sibon et al. 1997), Chk1 function is required for cell cycle lengthening (Shimuta et al. 2002). Loss of Grp also impairs the expression of zygotic genes (Sibon et al. 1997). Further, like the loss of Chk1, inhibition of zygotic transcription induces apoptosis in *Xenopus* embryos as determined by assays for DNA fragmentation (Hensey and Gautier 1997; Sible et al. 1997) and caspase activity (Figure 9A). To determine whether Chk1 was also required for the initiation of zygotic transcription, DN-XChk1- and luciferase-expressing embryos were collected at time points spanning the MBT. The mRNA was extracted and analyzed by Northern Blot for expression of GS-17, an early zygotic gene that is first expressed during the MBT and is

used as a marker for the initiation of zygotic transcription (Krieg and Melton 1985). Northern analysis indicated that GS-17 was transcribed beginning 7 hr pf in both luciferase and DN-Chk1-expressing embryos (Figure 9B), and there was no difference between initiation of transcription in DN-XChk1-expressing embryos and initiation of transcription in control embryos (Figure 9B). Therefore, XChk1 is not required for the proper timing of zygotic transcription initiation as indicated by transcription of GS-17 mRNA.

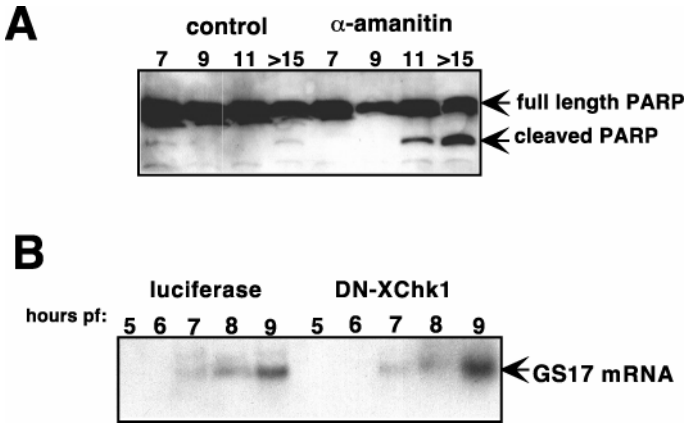

Figure 9. Expression of DN-Chk1 does not prevent the initiation of GS-17 transcription at the MBT. A) A block to zygotic transcription induces apoptosis. Embryos were injected with H_2O (control) or α–amanitin at the single-cell stage and assayed for caspase activity by cleavage of PARP substrate. B) Embryos expressing luciferase or DN-XChk1 were assayed for onset of zygotic transcription by Northern analysis of GS17 mRNA (Carter and Sible 2003).

3.4 Discussion

Apoptosis is frequently employed during embryonic development and by proliferating somatic tissues. *Xenopus laevis* embryos possess a specific developmental window after the MBT that may compensate for the lack of cell cycle checkpoints before the MBT. Embryonic cells with unreplicated or damaged DNA or that are compromised in their ability to transcribe the zygotic genome die by a maternally programmed default pathway of apoptosis during this window of time (Anderson et al. 1997; Hensey and Gautier 1997; Greenwood et al. 2001); (Shiokawa et al. 2000); (Sible et al. 1997; Stack and Newport 1997). Our studies indicate that this developmental program of apoptosis is also triggered when endogenous Chk1 function is inhibited (Figure 6) or when DNA replication is blocked at the MBT (Figure 7).

Chk1 function also correlates inversely with apoptosis in mammalian cells. Cultured Jurkat cells treated with the Chk1 inhibitor 7-hydroxystaurospurine (UCN-01) undergo apoptosis (Wang et al. 1995). Targeted disruption of the Chk1 gene by homologous recombination in mice results in apoptosis in blastocytes and embryonic death before day 3.5 of development (Liu et al. 2000; Takai et al. 2000). Mouse embryos lacking the ATR kinase, which functions upstream of Chk1, also demonstrate high levels of apoptosis early in development (Brown and Baltimore 2000). Therefore, the requirement for Chk1 function for survival of early embryos may be a universal feature of vertebrate development.

The onset of apoptosis in mice lacking ATR is preceded by chromosomal fragmentation, suggesting apoptosis may be triggered by the accumulation of damaged DNA (Brown and Baltimore 2000). However, we have not detected evidence of DNA damage in *Xenopus* embryos lacking Chk1 function prior to the time when they undergo

apoptosis (unpublished data). Our observations are in agreement with the fact that activated Chk1 is not normally detected until the MBT (Shimuta et al. 2002) and therefore, Chk1 is unlikely to be required for early cell cycles.

Apoptosis is also triggered in embryos treated prior to the MBT with aphidicolin to block DNA replication (Figure 7) or α-amanitin to block zygotic transcription (Figure 9A; (Hensey and Gautier 1997; Sible et al. 1997). However, embryos expressing DN-Chk1 appear to replicate DNA at a normal rate until the MBT (Figure 8; Shimuta et al. 2002) and initiate zygotic transcription on schedule (Figure 9). Therefore, all measurements of early development indicate that embryos reach the MBT without defect.

At the MBT, Chk1 is activated transiently (Shimuta et al. 2002), and loss of Chk1 perturbs several cell cycle remodeling events, including the degradation of maternal Cdc25A and tyrosine phosphorylation of Cdc2 (Shimuta et al. 2002). The predicted effect would be a delay in the lengthening of embryonic cell cycles as in *Drosophila* embryos lacking Grp (Fogarty et al, 1997; Sibon et al 1997). This prediction is supported by increased incorporation of ^{3}H-thymidine after the MBT in *Xenopus* embryos with impaired Chk1 function (Figure 8). Although formally possible that differences in ^{3}H-thymidine incorporation were due to differences in the endogenous thymidine pool, Shimuta et al. (2002) also present evidence for accumulation of additional DNA in embryos expressing DN-Chk1.

Why do *Xenopus* embryos lacking Chk1 die by apoptosis? One possibility is that the additional rapid rounds of DNA replication are not properly controlled, resulting in damaged or improperly replicated DNA, consistent with the accumulation of chromosomal damage in mouse embryos lacking ATR (Brown and Baltimore 2000). However, once *Xenopus* embryos pass the MBT, they become quite resistant to

apoptosis in response to DNA damage (Anderson et al. 1997; Hensey and Gautier 1997).

A second possibility is that Chk1 function is required more directly to block the apoptotic program that functions after the MBT (Hensey and Gautier 1997; Sible et al. 1997). Over expression of Chk1 in early embryos inhibits both Cdc2 and Cdk2 (Kappas et al. 2000). Cyclin A1/Cdk2, which is not normally present in early embryos, is associated with the onset of apoptosis in irradiated embryos, and exogenous cyclin A1/Cdk2 induces apoptotic-like changes in nuclear morphology in egg extracts (Anderson et al. 1997). Therefore, Chk1 function could be required at the MBT to prevent a default program of apoptosis that requires cyclin A1/Cdk2 (Sible et al. 1997). Furthermore, over expression of Cdc25A, which activates Cdk2, also induces death in embryos (Shimuta et al. 2002). In the next chapter, experiments were performed to determine the requirement of cyclin A1/Cdk2 for the initiation of apoptosis, as a candidate for the cyclin/Cdk complex regulated by Chk1 to inhibit apoptosis at the MBT.

4 Cyclin A1/Cdk2 Activity is Sufficient, but not Required, for Induction of Apoptosis in Early *Xenopus laevis* Embryos

4.1 Abstract

The processes of cell proliferation and cell death are intimately linked. In many situations, the molecules driving one process also catalyze the other. One example is the cyclin/Cdk, cyclin A/Cdk2. During the *Xenopus* early embryonic cell cycle, two distinct isoforms of cyclin A are expressed, at different times in development. Cyclin A1 is a maternally encoded isoform of cyclin A that is expressed from fertilization until a developmental phase called the midblastula transition (MBT), when maternally encoded proteins are degraded and replaced by their zygotic counterparts. Cyclin A2, which possesses a sequence homology closer to human somatic cyclin A than to *Xenopus* cyclin A1, is expressed after the MBT. Cyclin A1 only forms a dimer with Cdc2, the M-phase Cdk, while cyclin A2 pairs with Cdk2 and Cdc2, the Cdks responsible for S-phase and M-phase, respectively. Early *Xenopus* embryos treated with ionizing radiation (IR; to damage DNA) or aphidicolin (to block DNA replication) prior to the MBT, die before gastrulation from a developmentally regulated program of apoptosis. When the embryo dies in response to IR, cyclin A1 protein levels persist beyond the MBT and form a complex with Cdk2. To test the hypothesis that cyclin A1-associated activity is a common mediator of apoptosis, cyclin A1-associated kinase activity was measured during apoptosis induced by treatment of the embryo with aphidicolin and was found to be increased. To determine the sufficiency of cyclin A1/Cdk2 for apoptosis, a recombinant cyclin A1/Cdk2 protein complex was injected into single celled embryos. Recombinant cyclin A1/Cdk2 did induce apoptosis after the MBT. The converse experiment was performed with morpholinos to block cyclin A1 translation to determine whether cyclin A1 is required for

apoptosis in the early *Xenopus* embryo. Inhibition of cyclin A1 translation did not block the initiation of apoptosis after the MBT; therefore, cyclin A1 is not required for the initiation of apoptosis.

4.2 Introduction

Following fertilization, early *Xenopus laevis* development is characterized by rapidly alternating S (DNA synthesis) and M (mitosis) phases (Newport and Kirschner 1982a; Kimelman et al. 1987) controlled by the activity of cyclin/Cdk complexes. After the first embryonic division, which takes 90 minutes, the 2^{nd} – 12^{th} cleavages are rapid and synchronous, completing a cycle of DNA synthesis and mitosis every 30 minutes (Newport and Kirschner 1982a). The twelfth division marks a point known as the midblastula transition (MBT). At the MBT, the embryo reaches a critical nuclear-to-cytoplasmic ratio (Newport and Kirschner 1982a), which is sensed by the depletion of some maternal replication factor that is no longer able to interact stoichiometrically with the exponentially increasing DNA and is independent of the maternal timer (Hyrien et al. 1995). At the MBT, the embryo undergoes a period of developmental remodeling marked by the acquisition of gap phases, activation of checkpoints, degradation of maternal mRNAs, initiation of embryonic transcription, and motion of the cells (Newport and Kirschner 1982a). In addition, after the MBT, apoptotic programs can be initiated in response to DNA damage and blocks to DNA replication that have been incurred before the MBT (Anderson et al. 1997; Carter and Sible 2003). The same treatment after the MBT triggers cell cycle arrest instead of apoptosis (Hensey and Gautier 1997).

Due to an incomplete checkpoint response in the pre-MBT *Xenopus* embryo (Kimelman et al. 1987; Newport and Dasso 1989; Anderson et al. 1997; Clute and Masui 1997; Kappas et al. 2000), there

is no initiation of cell cycle arrest in response to factors that damage DNA or block DNA replication in the pre-MBT embryo. Therefore, the embryo can be treated with aphidicolin to inhibit DNA replication or IR in order to damage the genome, and embryonic cells will continue to divide until the MBT (Newport and Dasso 1989; Anderson et al. 1997; Clute and Masui 1997; Hensey and Gautier 1997; Stack and Newport 1997; Carter and Sible 2003) at which time, the embryo undergoes a program of apoptosis (Sible et al. 1997). Further, embryonic cells with compromised protein synthesis (Hensey and Gautier 1997; Sible et al. 1997; Stack and Newport 1997) altered methylation (Kai et al. 2000; Stancheva et al. 2001) or blocked transcription (Hensey and Gautier 1997; Sible et al. 1997; Stack and Newport 1997) before the MBT will also initiate apoptosis (Sible et al. 1997). After the MBT, the embryo has acquired active checkpoints, is able to arrest the cell cycles, and initiates repair pathways, making the embryo more resistant to apoptosis (Anderson et al. 1997; Hensey and Gautier 1997). Therefore, the early development of the *Xenopus laevis* embryo provides an interesting context in which to study the how the cell decides between cell cycle arrest and apoptosis.

Another defining hallmark of the MBT is the degradation of maternal mRNA species and the transcription of the corresponding zygotic mRNAs (Newport and Kirshner, 1982), as is the case with cyclin A. Cyclin A1 is expressed from fertilization until the MBT, when maternally encoded proteins are degraded and replaced by zygotic proteins (Howe et al. 1995). Cyclin A2, with a sequence homology closer to human somatic cyclin A than to *Xenopus* cyclin A1, is expressed at very low levels before the MBT, with levels increasing greatly after the MBT (Howe et al. 1995; Strausfeld et al. 1996). Whereas cyclin A1 only pairs with Cdc2 to partly regulate mitosis, cyclin A2 pairs with both Cdk2 and Cdc2 to regulate DNA synthesis and mitosis, respectively (Minshull et al. 1990; Pagano et al. 1992; Strausfeld et al. 1996). Over

expression of cyclin A2 by injection of the complete cyclin A2 mRNA at the single cell stage has no effect on embryonic development until the early tail bud stage (Richard-Parpaillon et al. 2004), where it promotes over proliferation of cells, consistent with data in mammalian cell cultures (Resnitsky et al. 1995; Rosenberg et al. 1995) and *Drosophila* tissues (Sprenger et al. 1997).

Use of hydroxyurea and γ-irradiation (IR) to induce apoptosis both result in the cleavage of endogenous cyclin A2 at the N-terminus (Δcyclin A2). This cleavage event is catalyzed by caspase-3 (Stack and Newport 1997; Finkielstein et al. 2002). Addition of the recombinant Δcyclin A2 in complex with Cdk2 to *Xenopus* cell-free extracts results in the appearance of apoptotic-like nuclei, independent of caspase activity (Finkielstein et al. 2002). Cleavage of cyclin A2 by caspase-3 removes the destruction box required for ubiquitination of cyclin A2, so that Δcyclin A2 persists during IR-induced apoptosis (Finkielstein et al. 2002), therefore the changes in the function of cyclin A2 caused by the cleavage event are downstream of the caspase cascade. The Δcyclin A2/Cdk2 complex is more active than its full length counterpart due to inability to bind the *Xenopus* p27 inhibitor. In addition to histone H1, Δcyclin A2/Cdk2 acquires a new substrate specificity for histone H2B, a histone that only gets phosphorylated during apoptosis (Ajiro 2000; Finkielstein et al. 2002). The appearance of apoptotic-like nuclei and the change in substrate specificity when cyclin A2 is cleaved by caspase-3 suggests that caspases cleave cyclin A2 so that Δcyclin A2/Cdk2 can induce the nuclear condensation events associated with apoptosis (Harvey et al. 2000; Finkielstein et al. 2002).

In addition to cyclin A2 cleavage, when the embryo undergoes apotosis in response to IR, cyclin A1 protein levels persist and form a complex with Cdk2, a complex that only known to form when the embryo undergoes IR-induced apoptosis. Addition of recombinant cyclin

A1/Cdk2 protein to *Xenopus* cell-free extracts results in the appearance of condensed apoptotic-like nuclei (Anderson et al. 1997). The following experiments investigate whether cyclin A1-associated kinase activity functions as a common mediator of apoptosis, testing the sufficiency and requirement of cyclin A1 during apoptosis *in vivo*. The results support the idea that cyclin A1 is sufficient for the initiation of apoptosis initiated at the MBT, but not strictly required for all instances of apoptosis.

4.3 Results

4.3.1 Cyclin A1/Cdk2 activity increases during apoptosis induced by a block to DNA replication.

The pre-MBT *Xenopus* embryo can be treated with aphidicolin to inhibit DNA replication or treated with IR in order to damage the genome, and embryonic cells will continue to divide until the MBT (Newport and Dasso 1989; Anderson et al. 1997; Clute and Masui 1997; Hensey and Gautier 1997; Stack and Newport 1997; Kappas et al. 2000; Carter and Sible 2003) at which time, the embryo undergoes apoptosis (Sible et al. 1997). The mechanisms of this program are still unclear, but it has been suggested that cyclin A1 is a common mediator in the regulation of apoptosis (Anderson et al. 1997). In normal embryos, cyclin A1 protein levels begin to drop at stage 10.5, early gastrulation, disappearing completely by stage 11. When apoptosis is initiated in response to IR, which generates double-stranded breaks, cyclin A1 levels persist, staying relatively high until 16 hours post-fertilization, when sibling control embryos have reached stage 12 (late gastrulation) (Anderson et al. 1997). To determine whether apoptosis induced by agents other than IR has the same effect on cyclin A1 protein levels, pre-MBT embryos were treated with 100 μg/ml aphidicolin to block DNA replication and subsequently induce apoptosis. Cyclin A1 protein levels

were determined by Western bloting with an anti-cyclin A1 antibody. Figure 10A shows that the levels of cyclin A1 persist when apoptosis is initiated by a block to DNA replication, as observed when apoptosis is induced by double-stranded breaks. Since apoptosis induced by treatment with IR also induced cyclin A2 cleavage, cyclin A2 protein levels were examined by Western analysis to determine if a block to DNA replication would also induce cyclin A2 cleavage. Pre-MBT embryos were treated with 100 μg/ml aphidicolin to block DNA replication and subsequently induce apoptosis. At stage 10.5, as cyclin A2 levels began to increase in both control and aphidicolin-treated embryos, a smaller cyclin A2 fragment of 33 kDa began to appear in the aphidicolin-treated embryos (Figure 10B). This fragment increased in amount as the embryos underwent apoptosis, with the full size cyclin A2 protein levels diminishing during apoptosis. The cleavage of cyclin A2 correlated with the cleavage patterns observed in embryos undergoing apoptosis induced by IR treatment and treatment with hydroxyurea (HU; to inhibit nucleotide synthesis), and the fragment is the same size, 33 kDa, as the cleavage fragment observed in both instances (Stack and Newport 1997; Finkielstein et al. 2002). While not determined in embryos treated with aphidicolin, the cleavage of cyclin A2 during apoptosis induced by IR and HU has been demonstrated to be mediated by caspase-3 (Stack and Newport 1997; Finkielstein et al. 2002), and in the case of IR-induced apoptosis, has been shown to expand the substrate specificity of the cyclin A2/Cdk2 complex to include phosphorylation of H2B (Finkielstein et al. 2002).

Embryos treated before the MBT with IR (to induce double-stranded breaks) or α-amanitin (to inhibit zygotic transcription) demonstrate increased cyclin A1-associated kinase activity as the embryos undergo apoptosis (Anderson et al. 1997). To determine if the increase in cyclin A1-associated activity is common to other treatments

that induce apoptosis, embryos were treated with 100 μg/ml aphidicolin to inhibit DNA replication. As the embryos initiated apoptosis at stage 10.5, the levels of cyclin A1-associated kinase activity persisted until stage 12 (Figure 10C). Since cyclin E is the other cyclin that partners with and regulates Cdk2 activity, the same experiment was performed examining cyclin E-associated activity, to determine whether the increase in activity was unique to cyclin A1, or other cyclins. Embryos initiating apoptosis in response to a block to DNA replication with aphidicolin showed no appreciable difference in cyclin E associated activity compared to their gastrulating sibling controls (Figure 10D). The data suggest that both cyclin A1 and cyclin A2 have a common role in mediating apoptosis initiated in response to pre-MBT assaults on continued embryonic survival.

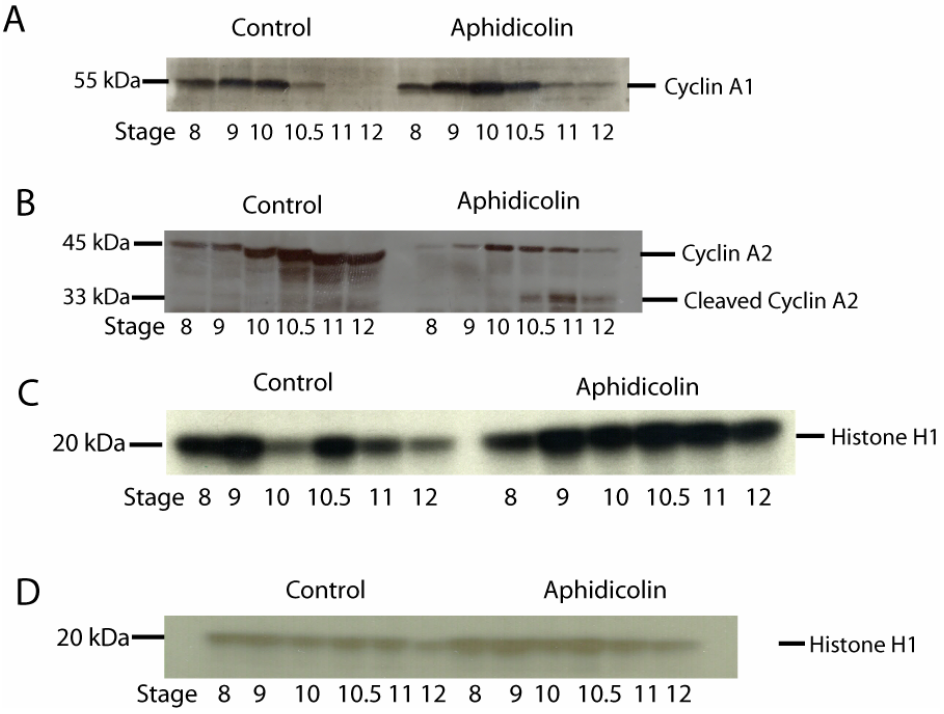

Figure 10. Analysis of A-type cyclins during apoptosis induced by a block to DNA replication. Embryos were treated with 100 μg/ml aphidicolin at Stage 8 (5 hr pf) and collected at the indicated developmental stages. Western analysis of cyclin A1 (A) and cyclin A2 (B). Cyclin A1-associated kinase activity (C) and Cyclin E-associated activity (D) were also determined.

4.3.2 Cyclin A1/Cdk2 is sufficient to induce apoptosis at the MBT.

Having shown that cyclin A1-associated activity is increased during aphidicolin-induced apoptosis, it was important to determine whether cyclin A1 was sufficient to induce apoptosis. To test this possibility, embryos were injected with 1.4 $X10^{-4}$ pmol activity/min cyclin A1/Cdk2 recombinant protein and observed until after the MBT. Cyclin A1/Cdk2 recombinant protein was used instead of cyclin A1/Cdc2 protein because results by Anderson et al. (1997) showed that during apoptosis induced by IR treatment cyclin A1 forms a rare complex with Cdk2, suggesting that induction of apoptosis is partly regulated by the cyclin A1/Cdk2 complex, not the cyclin A1/Cdc2 complex.

Before the MBT, the injected embryos developed at the same rate and with the same morphological characteristics as the control embryos (data not shown). After the MBT, the embryos began to exhibit the external characteristics of loose cellular attachments and fuzzy white ball appearance associated with apoptosis (Figure 11A). When examined for caspase activity, the embryos injected with the recombinant cyclin A1/Cdk2 demonstrated the ability to cleave exogenous PARP protein, a substrate of caspase-3, indicating active caspase-3 (Figure 11B). In addition to gross embryonic morphology, the embryos injected with cyclin A1/Cdk2 also demonstrated super-condensed nuclear morphology, indicating the chromatin condensation associated with apoptosis (Figure 12). Therefore, exogenous expression of cyclin A1/Cdk2 in the early developing embryo induces apoptosis at the MBT.

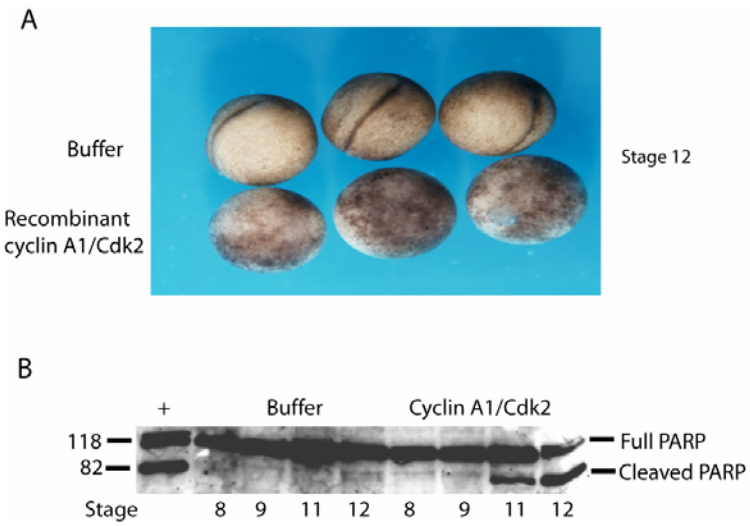

Figure 11. Exogenous cyclin A1/Cdk2 recombinant protein induces apoptotic embryo morphology and activates caspases. Embryos were injected with buffer or cyclin A1/Cdk2 recombinant protein at the single-cell stage. Control and cyclin A1/Cdk2 embryos at >15 hrs pf (A). Morphology of A1/Cdk2 embryos is consistent with apoptosis. Control and cyclin A1/Cdk2 embryos were collected at the indicated times and processed for active caspases as indicated by cleavage of PARP protein, a caspase-3 substrate (B).

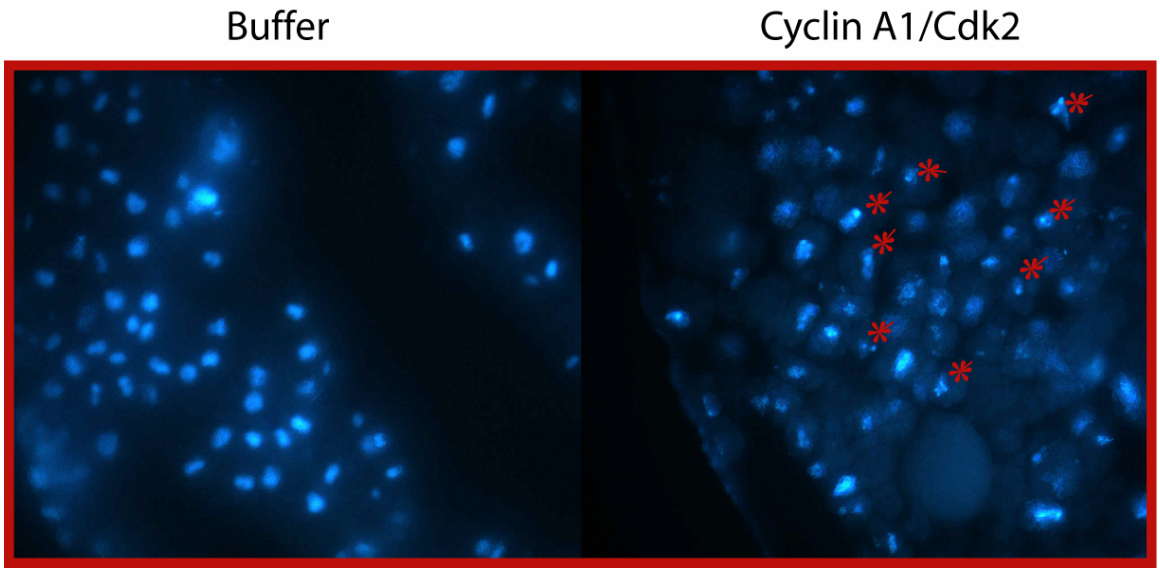

Figure 12. Expression of exogenous recombinant cyclin A1/Cdk2 results in apoptotic nuclear morphology. Single-cell embryos were injected with either buffer or 1.4 X10^{-4} pmol activity/min activity recombinant cyclin A1/Cdk2 protein and fixed in 4%-paraformaldehyde at developmental Stage 12. Sections were stained with 1μg/ml DAPI and photographed by an Olympus AX70 fluorescence microscope equipped with a Color View 12 digital camera. Super-condensed nuclei are indicated by the red asterisks.

4.3.3 Cyclin A1 is not required for apoptosis induced by IR or aphidicolin.

These studies have so far shown that cyclin A1-associated activity persists during apoptosis induced by aphidicolin and that cyclin A1/Cdk2 activity is sufficient to induce apoptosis in early *Xenopus laevis* embryos. To determine whether cyclin A1 and cyclin A2 are required for the induction of apoptosis, cyclin A1 and cyclin A2 expression was blocked by injection of morpholinos designed for each cyclin into the embryo to block cyclin A1 and cyclin A2 translation. Morpholinos block translation of the mRNA by binding to the head of the mRNA and blocking ribosomal binding (Heasman 2002). Injection of 100 ng cyclin A1 or cyclin A2 morpholino significantly decreased the amount of cyclin A1 and cyclin A2 translated in the embryo (Figure 13A), but did not perturb embryonic development upon entering the MBT (Figure 13B; left panel). Embryos were treated with 40 Gy IR or 100 μg/ml aphidicolin at stage 7 and observed until stage 12. Almost complete inhibition of cyclin A1 or cyclin A2 translation did not inhibit the development of untreated sibling controls, or the induction of apoptosis as measured by gross embryo morphology (Figure 13B; middle and right panels). Small amounts of cyclin A2 are present throughout embryonic development, and cyclin A1 levels persist as the embryo undergoes apoptosis (Howe et al. 1995; Strausfeld et al. 1996; Anderson et al. 1997). Thus, it is reasonable to suggest that when the translation of one cyclin A is inhibited, the other cyclin is able to compensate for the function of the missing cyclin. Therefore, a mix of morpholinos targeting both cyclins A1 and A2 was injected into embryos to test the requirement of both A-type cyclins for the induction of apoptosis (Figure 14). Embryos were injected at the single cell stage with 100 ng of cyclin A1 morpholino and 100 ng cyclin A2 morpholino, and treated with either 40 Gy IR or 100μg/ml aphidicolin. Again, inhibition of A-type cyclin expression upset neither

development of the embryo nor induction of apoptosis, leading to the conclusion that cyclin A1, while sufficient to induce apoptosis, is not required for the induction of apoptosis. Higher doses of morpholino, up to 300 ng, still did not perturb initiation of apoptosis (data not shown).

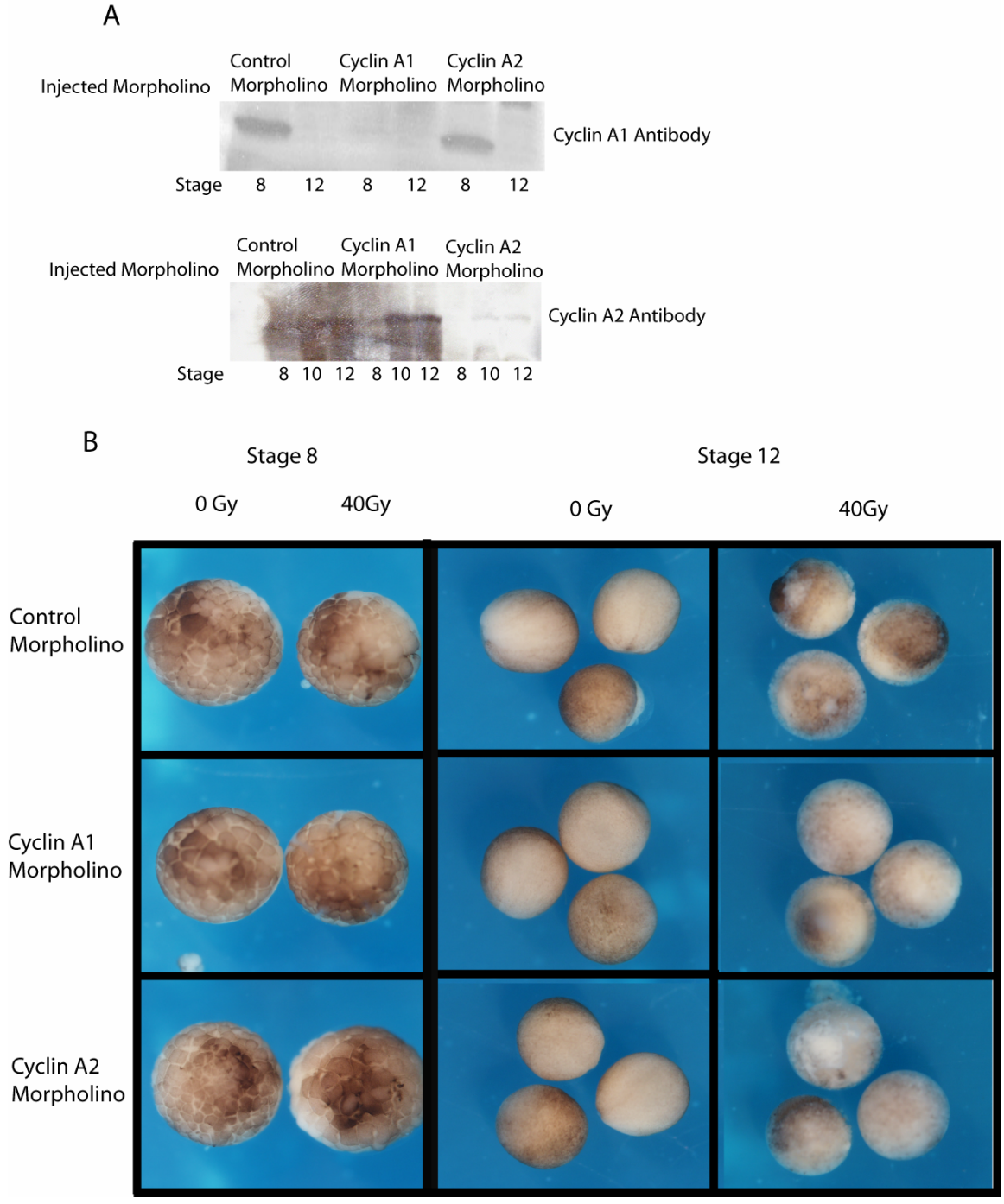

Figure 13. Injection of cyclin A1 of cyclin A2 specific morpholinos to inhibit translation does not inhibit apoptosis initiated by treatment with IR. Single-cell embryos were injected with 100ng Control, Cyclin A1 or Cyclin A2 morpholinos and cyclin A1 or cyclin A2 levels were examined by Western Blot (A). Embryos were photographed at Stage 8 (5 hr pf; B left panel) or Stage 12 (>15 hr pf; B middle and right panels).

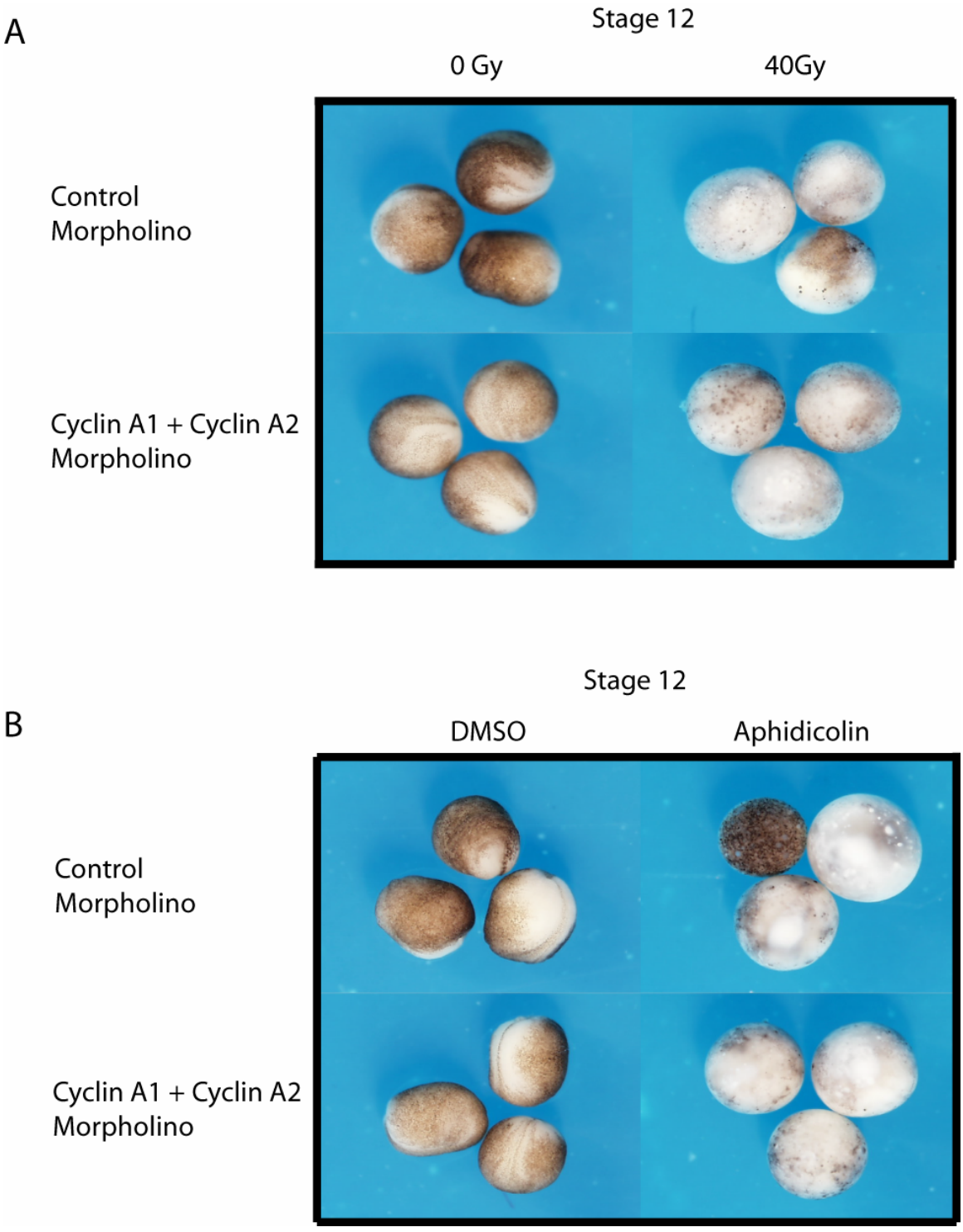

Figure 14. Inhibiting the expression of both cyclin A1 and cyclin A2 at the same time does not inhibit apoptosis. Embryos were injected with a mix of cyclin A1 and cyclin A2 morpholinos and treated with IR (A) or aphidicolin (B) and photographed at Stage 12 (>15 hr pf).

4.4 Discussion

When pre-MBT embryos are treated with aphidicolin to block DNA replication, cyclin A1 protein levels and cyclin A1-associated activity persist through the apoptotic response that is initiated at the MBT (Figure 10 A and C). The persistence of cyclin A1 protein levels and activity suggest that there is a regulatory role for cyclin A1-associated kinase activity during apoptosis initiated at the MBT in response to DNA replication blocks before the MBT. This persistence is in agreement with results obtained in embryos treated with IR or α-amanitin (Anderson et al. 1997) and supports the idea that cyclin A1-associated kinase activity is a common mediator in the initiation of apoptosis after the MBT in response to pre-MBT assaults on continued embryonic survival.

In addition to the results obtained with cyclin A1, cyclin A2 is cleaved in embryos treated with aphidicolin before the MBT. This cleavage event is presumably mediated by caspase-3 and produces a 33 kDa Δcyclin A2 fragment, which is the same size as the Δcyclin A2 fragment produced during apoptosis induced by treatment with IR (Finkielstein et al. 2002), suggesting that this cleavage event is also involved in the common regulation of apoptosis after the MBT. The behavior of both cyclin A1 and cyclin A2 during apoptosis in early *Xenopus laevis* embryos is summarized in Figure 15. In mammalian systems, $p21^{Cip1}$ is cleaved by caspase-3 to the abolish cyclin A binding and increase cyclin A/Cdk2 activity (Jin et al. 2000.). Increased regulation of Cyclin A/Cdk2 activity through caspase-mediated inactivation of $p21^{Cip1}$ is a hallmark of apoptosis induced by a variety of assaults in endothelial cells (Gervais et al. 1998; Levkau et al. 1998; Jin et al. 2000; Adachi et al. 2001). In the early *Xenopus* embryo, the cleaved cyclin A2/Cdk2 complex is more active than its complete counterpart due to inability to bind the *Xenopus* $p27^{Xic1}$ inhibitor, and cleaved cyclin A2 acquires a new substrate specificity for histone H2B

instead of histone H1 (Finkielstein et al. 2002). Addition of recombinant cleaved cyclin A2 to *Xenopus* cell-free extracts results in the appearance of apoptotic-like nuclei independent of caspase activity, suggesting that caspases cleave cyclin A2 to drive the nuclear changes associated with apoptosis (Finkielstein et al. 2002). In mammalian cells, caspase-dependent Cdk2 activity appears to be an important factor that is required for death associated chromatin condensation, cell shrinkage, and loss of cell adhesion (Harvey et al. 2000). The same may to be true for Δcyclin A2/Cdk2 activity in the early *Xenopus* embryo as a common mediator for apoptosis induced by a variety of challenges to the survival of the embryo.

Xenopus cyclin A2 is more closely related to human somatic cyclin A than it is to *Xenopus* cyclin A1 (Howe and Newport 1995). Therefore, the activity of cyclin A2 is likely to be more closely related to the activity of human somatic cyclin A than it is to the activity of *Xenopus* cyclin A1. The similarity of cyclin A2 to human somatic cyclin A is further evidenced by the fact that both cyclin A2 and human cyclin A are dependent upon caspase-3 in order to change their activity during apoptosis to heavily condense chromatin. This is not the case with cyclin A1. On the contrary, exogenous expression of recombinant cyclin A1/Cdk2 in a single-celled embryo initiates caspase-3 activity, while over expression of cyclin A2 mRNA has no apoptotic effect on *Xenopus* embryo development until well after the MBT, during neural groove formation (Richard-Parpaillon et al. 2004), suggesting that any activity cyclin A2 has during apoptosis in the early embryo is dependent upon caspase-3 activity, whereas cyclin A1-associated activity is upstream of caspase activity (Figure 11).

While cyclin A2 has been suggested as having a role in regulating chromatin condensation in early *Xenopus laevis* embryos undergoing apoptosis, less is known about the role of cyclin A1 during the initiation

of apoptosis. Cyclin A1 is not cleaved during apoptosis induced by a variety of methods (data not shown), but does persist beyond the MBT, when it should be degraded and replaced with cyclin A2. When recombinant cyclin A1/Cdk2 is added to *Xenopus* interphase egg extracts, the nuclei became super condensed in a manner reminiscent of apoptosis (Anderson et al. 1997); however, caspase activity was not measured. When exogenous cyclin A1/Cdk2 was injected into single-celled embryos, there was an apoptotic response after the MBT, suggesting that cyclin A1 could partner with Cdk2 in order to initiate the apoptotic response in early embryos. The mechanisms of involvement are unclear, but may be due to the role of cyclin As in general in regulating DNA synthesis.

In somatic cells, over expression of either cyclin A or cyclin E will drive cultured cells into the cell cycle by initiating S-phase. In *Xenopus* egg extracts, activation of DNA replication at S-phase is dependent upon Cdk2 activity (Hua et al. 1997) in complex with cyclin E. In somatic cells, Cdk2 activity is responsible for limiting replication to a single round through the phosphorylation of Cdc6 and geminin by cyclin A/Cdk2 (Coverley et al. 2002). However, it is cyclin A1/Cdc2 in *Xenopus* egg extracts that inhibits DNA replication by initiating mitosis. Addition of recombinant cyclin A1 to interphase extracts induces disassembly of the nuclear envelope and DNA condensation in to chromosome threads (Strausfeld et al. 1996), not S-phase events. It is possible that by injecting recombinant cyclin A1/Cdk2, apoptosis is not the direct result of the activity of cyclin/Cdk complex on any apoptosis targets, but the result of excess cyclin A/Cdk2 activity in directing origin firing during DNA synthesis. Future studies will examine the effect of expressing exogenous cyclin A1/Cdk2 in early embryos on DNA synthesis.

That the initiation of apoptosis when cyclin A1/Cdk2 is exogenously expressed is not due to a direct mechanism of cyclin

A1/Cdk2 on the mechanisms regulating apoptosis is further supported by the final experiments in this study. The injection of morpholinos that target the translation of cyclin A1 or cyclin A2 specifically did not abrogate the initiation of apoptosis induced by treatment with IR or aphidicolin, indicating that neither cyclin is required for the initiation of apoptosis in this system. Further experiments should attempt to eliminate Cdk2 from the process of apoptosis initiation in order to determine that initiation of apoptosis is not dependent upon that Cdk. These studies do show that there is a common role for cyclin A1 during apoptosis initiated at the MBT in the early *Xenopus laevis* embryo (Figure 10). However, that role is not required in order for apoptosis to occur, indicating that there is at least one protein, possibly cyclin E, that is able to compensate for the loss of cyclin A1.

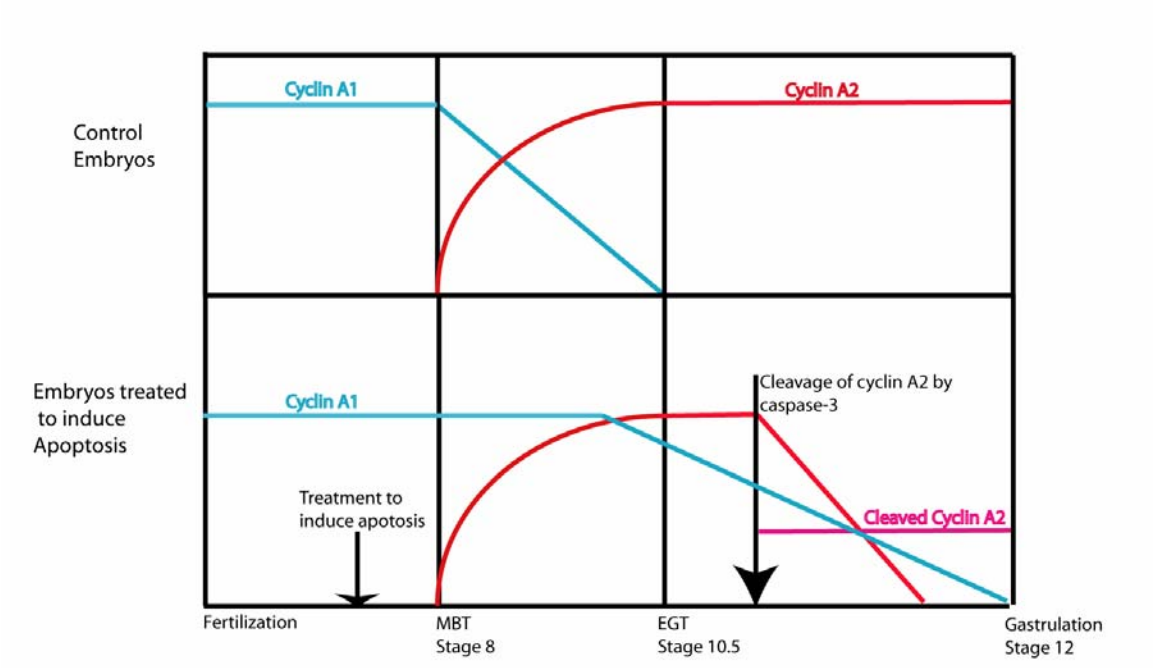

Figure 15. Summary of A-type cyclin behavior in early *Xenopus laevis* embryos. At fertilization, cyclin A1 levels are high and the cyclin only complexes with Cdc2 to regulate the events of mitosis. At the MBT, cyclin A1 levels begin to fall as the maternal mRNA is degraded, disappearing completely at the EGT. When apoptosis is induced in early embryos, cyclin A1 levels persist until gastrulation, and the cyclin forms a complex with Cdk2, a complex whose function is not understood. Cyclin A2 mRNA levels steadily increase at the MBT, due to initiation of zygotic transcription, and by the EGT, the only available cyclin is the zygotic cyclin A2. When apoptosis is initiated in an early embryo, cyclin A2 undergoes a caspase-3 mediated cleavage event, and this cleavage expands the substrate specificity of cyclin A2 to include histone H2B.

5 Concluding Remarks

This investigation has shown that the two cell cycle components studied, Chk1 and cyclin A1, are significant factors in the regulation of apoptosis during early development of *Xenopus laevis* embryos. Inhibition of Chk1 activity results in the initiation of apoptosis at the MBT and additional rapid rounds of DNA replication at the start of the MBT (Chapter 3). The initiation of apoptosis could be due to the increased amount of DNA resulting from these additional rounds of replication, or it could be the result of a second anti-apoptotic function of Chk1 that has yet to be established. The initiation of apoptosis in embryos without functional Chk1 in conjunction with the transient activation of Chk1 at the MBT (Shimuta et al. 2002) in healthy embryos indicates an essential function for Chk1 at the MBT in the remodeling of the cell cycle that occurs at the MBT. Since the transient activation of Chk1 results in the degradation of Cdc25A and non-degradable Cdc25A also initiates apoptosis at the MBT (Shimuta et al. 2002), it is logical to suggest that the apoptosis that results from the loss of Chk1 function is the result of unregulated cyclin/Cdk activity at the MBT; activity that is normally regulated by the degradation of Cdc25A.

Cdc25A activates cyclin E/Cdk2 to begin loading the pre-replication complex (Pre-RC) onto chromatin to initiate DNA synthesis (Sagata 2002). Degradation of Cdc25A resulting from Chk1 phosphorylation turns off Pre-RC loading and allows for replication to occur without re-replicating the genome (Shechter et al. 2004). From fertilization until the MBT, the embryo undergoes promiscuous firing of DNA replication origins in order for the replication of the genome to occur every 30 minutes during those early cell cycles (Harland and Laskey 1980; Hyrien and Mechali 1992). At the MBT, the slowing down of the cell cycles is partly the result of ordered and specific origin firing,

through increased regulation of cyclin E/Cdk2 (Shechter et al. 2004). Therefore, Chk1 may be responsible for that regulation of cyclin E/Cdk2 that slows down the firing of DNA replication origins at the MBT. In addition, loss of Chk1 function at the MBT may result in apoptosis because the replication of the genome is still regulated by the molecular machinery that regulates those rapid cell cycles instead of slowing at the MBT. The accumulation of excess DNA initiates those apoptotic pathways that are activated by the embryo in response to the inability to continue development past the MBT (Chapter 3).

The apoptotic event at the MBT is also somehow regulated by cyclin A1-associated activity. When the embryo initiates apoptosis, cyclin A1 protein levels persist, and cyclin A1-associated kinase activity increases greatly (Anderson et al. 1997; Chapter 4). In the case of IR, this associated activity has been shown to be the result of cyclin A1 forming a dimer with Cdk2, not the usual partner Cdc2 (Anderson et al. 1997). The same is probably true of apoptosis induced in aphidicolin treated embryos, but must be confirmed by experimentation. This unusual pairing may change the function of cyclin A1 from a mitotic cyclin to an S-phase cyclin, like the zygotic cyclin A2. Cyclin A2 is responsible for initiating DNA synthesis once cyclin E/Cdk2 has loaded the Pre-RC onto the chromatin (Coverley et al. 2002) and is also activated by Cdc25A (Uto et al. 2004). It is possible that by pairing with Cdk2, cyclin A1 gets directed towards that same function, instead of the normal mitotic function.

The protein levels of cyclin A1 and its associated kinase activity have not been determined for *Xenopus* embryos without Chk1 function. If in concurrence with the data from embryos treated with IR and aphidicolin, the persistence of cyclin A1 protein levels and associated kinase activity would suggest that the degradation of Cdc25A by Chk1 phosphorylation is partly responsible for the proper timing of cyclin A1

degradation at the MBT. Possibly the reason that cyclin A1 does not form a complex with Cdk2 in the early embryonic cell cycles is due only to the lack of free Cdk2, as Cdc2 is present in much greater abundance in the early embryo (Minshull et al. 1990), and the changes at the MBT, activation of Chk1 and degradation of Cdc25A, keep this complex from forming when cyclin E is degraded by the maternal timer (Hartley et al. 1997), releasing free Cdk2 into the cells. Without active Chk1, cyclin A1 is free to partner with Cdk2, activated by excess Cdc25A, and the resulting excess rounds of DNA synthesis trigger an apoptotic response from the embryo instead of continuing development with excess DNA.

The continued development of the *Xenopus* embryo after the MBT is dependent upon having active Chk1 and degraded Cdc25A (Chapter 3; Shimuta et al. 2002). In addition, continued development is dependent upon the controlled regulation of cyclin A1, and possibly the degradation of cyclin A1 mRNA at the MBT (Chapter 4). The MBT represents a developmental transition where the embryo makes the decision to sacrifice the speed of unregulated cell division cycles for the reliability of regulated cell division cycles. As such, it represents a non-pathological model for the study of those pathways regulating that decision. Because cancer cells make the opposite decision, the early *Xenopus laevis* system is also a model for studying the molecular switches involved in the loss of controlled cell divisions in a somatic cell. These studies have attempted to understand how the activities of Chk1 and cyclin A1/Cdk2 regulate the developmental changes observed at the MBT. These two molecules are important molecular switches in regulating the decisions described above. Through modification of the activities of these two cell cycle components in the early *Xenopus* embryo, these studies also attempt to further understand how a cell loses control over the pathways regulated by these two molecules to become cancerous.

Appendix I

Table of Abbreviations

Abbreviation	Description
MBT	midblastula transtion
EGT	early gastrula transition
Chk1	checkpoint kinase 1
IR	ionizing radiation
Cdk	cyclin-dependent kinase
Cdc	cell division cycle
UCN-01	7-hydroxystaurosporine
MAPK	mitogen activated protein kinase
ERK	extracellular signal regulated kinase
MEK	MAPK/ERK kinase
ATM	ataxia-telangietasia mutated
ATR	ATM and Rad3 related
RB	retinoblastoma (general protein family)
pRB	retinoblastoma (the specific protein)
TNF	tumor necrosis factor
SWI/SNF	chromatin remodeling factors
CAKs	cyclin activating kinases
CKIs	cyclin dependent kinase inhibitors
hr	hours
pf	post-fertilization
HU	hydroxyurea
H1	histone 1
H2B	histone 2b

Underlined letters comprise the abbreviation.

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