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Regulation of Zygotic Transcription and Cell Cycle Checkpoints in Early Embryogenesis

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Abstract
For many organisms, the first goal of embryogenesis is to accumulate a large cell population to accommodate gastrulation. To achieve this quickly, embryos employ specialized cell cycles called cleavages that consist of continuous rounds of DNA replication and division. Cell proliferation occurs rapidly because cleavage cycles lack the gap phases and cell cycle checkpoints found in canonical cell cycles. Further, the genetic materials required to sustain cleavage cycles are preloaded during oogenesis, aiding efficient cell cycle progression. After a constant, organism-specific number of cleavages, many metazoan embryos undergo the mid-blastula transition (MBT), which initiates extensive cell cycle remodeling. Cell cycles lengthen, gap phases appear and checkpoint function is acquired. At the same time, the nearly quiescent zygotic genome is activated and transcriptional activity dramatically increases. This dissertation describes how these simultaneous MBT events are regulated. Chapter 2 addresses how zygotic transcription and cell cycle remodeling are coordinated. By artificially slowing cleavage cycles in zebrafish embryos, I demonstrate that increases in transcriptional activity are independent of cell cycle elongation and embryo age. I conclude that zygotic transcription is regulated by the nuclear-to-cytoplasmic (N:C) ratio, which increases after each round of replication in cleavage-stage embryos. Chapter 2 also shows the mechanisms governing DNA damage checkpoint acquisition at the MBT. DNA damage checkpoint acquisition does not require zygotic transcription. Instead, using immunostaining to examine checkpoint signaling, I show that cleavage-stage embryos cannot activate the checkpoint protein Chk1 kinase after damage induction. I conclude that the lack of Chk1 activity prior to the MBT limits DNA damage checkpoint function during cleavage cycles. Chapter 3 investigates how the spindle assembly checkpoint (SAC) is acquired at the MBT. I show that SAC acquisition is independent of the N:C ratio and other MBT events like cell cycle elongation and zygotic transcription. I conclude that SAC acquisition is age-dependent, and relies on a timer mechanism to regulate maternally-supplied SAC components. The studies reported in this dissertation demonstrate the various mechanisms embryos use to orchestrate simultaneous MBT events.

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REGULATION OF ZYGOTIC TRANSCRIPTION AND CELL CYCLE CHECKPOINTS IN EARLY EMBRYOGENESIS

DEDICATION

Dedicated to Walker: for everything

ACKNOWLEDGMENT

No man is an island, and I am no special case. I've been extraordinary lucky throughout my life, and my achievements to date would not have been possible without the small village of people that helped—and sometimes dragged—me through every step.

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ABSTRACT

REGULATION OF ZYGOTIC TRANSCRIPTION AND CELL CYCLE CHECKPOINTS IN EARLY EMBRYOGENESIS

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Michael Lampson

For many organisms, the first goal of embryogenesis is to accumulate a large cell population to accommodate gastrulation. To achieve this quickly, embryos employ specialized cell cycles called cleavages that consist of continuous rounds of DNA replication and division. Cell proliferation occurs rapidly because cleavage cycles lack the gap phases and cell cycle checkpoints found in canonical cell cycles. Further, the genetic materials required to sustain cleavage cycles are preloaded during oogenesis, aiding efficient cell cycle progression. After a constant, organism-specific number of cleavages, many metazoan embryos undergo the mid-blastula transition (MBT), which initiates extensive cell cycle remodeling. Cell cycles lengthen, gap phases appear and checkpoint function is acquired. At the same time, the nearly quiescent zygotic genome is activated and transcriptional activity dramatically increases. This dissertation describes how these simultaneous MBT events are regulated. Chapter 2 addresses how zygotic transcription and cell cycle remodeling are coordinated. By artificially slowing cleavage cycles in zebrafish embryos, I demonstrate that increases in transcriptional activity are independent of cell cycle elongation and embryo age. I conclude that zygotic transcription is regulated by the nuclear-to-cytoplasmic (N:C) ratio, which increases after

each round of replication in cleavage-stage embryos. Chapter 2 also shows the mechanisms governing DNA damage checkpoint acquisition at the MBT. DNA damage checkpoint acquisition does not require zygotic transcription. Instead, using immunostaining to examine checkpoint signaling, I show that cleavage-stage embryos cannot activate the checkpoint protein Chk1 kinase after damage induction. I conclude that the lack of Chk1 activity prior to the MBT limits DNA damage checkpoint function during cleavage cycles. Chapter 3 investigates how the spindle assembly checkpoint (SAC) is acquired at the MBT. I show that SAC acquisition is independent of the N:C ratio and other MBT events like cell cycle elongation and zygotic transcription. I conclude that SAC acquisition is age-dependent, and relies on a timer mechanism to regulate maternally-supplied SAC components. The studies reported in this dissertation demonstrate the various mechanisms embryos use to orchestrate simultaneous MBT events.

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Introduction: an overview of cell cycle regulation and transcriptional activity during early embryogenesis

INTRODUCTION

Following fertilization, most metazoans undergo a unique period of development characterized by rapid, synchronous cleavage divisions. Despite having an intact zygotic genome, development at this stage relies almost entirely on maternally supplied mRNAs that are loaded during oogenesis. The early cell cycles following fertilization are vastly different from canonical cell cycles. Embryonic cell cycles lack the G1 and G2 gap phases, when somatic cells typically grow in size. Instead, cleavage cycles subdivide the generous cytoplasm leading to increasing nuclear to cytoplasmic ratios with each division. Additionally, cleavage embryos lack functional cell cycle checkpoints that are essential to maintain genomic integrity in nearly all non-pathogenic cells. It is not until the mid-blastula transition (MBT) that zygotic transcription increases dramatically and cell cycles are remodeled into canonical cell cycles.

The purpose of this chapter is to review the unique features of cleavage-stage embryos of oviparous organisms like fish, frogs and flies where embryogenesis occurs outside the mother. This chapter will discuss the specialized embryonic cell cycles immediately following fertilization and the molecular mechanisms that regulate them. Further, this chapter will review how cleavage cycles are remodeled at the MBT. Finally, this chapter will examine transcription in early embryogenesis and discuss the models of how the zygotic transcription increases at the MBT.

PART I: CELL CYCLE CONTROL IN EARLY EMBRYOGENESIS

Unique features of embryonic cleavage cycles

Most actively proliferating cells progress through four distinct phases: the first gap phase (G1), DNA replication (S-phase), a second gap phase (G2) and mitosis (M). However, observation of early development in fish and frogs revealed that newly fertilized embryos have highly specialized cell cycles that differ significantly from their canonical counterparts (Oppenheimer, 1936; Graham and Morgan, 1966). Also called cleavages, these embryonic cell cycles are extremely short and lack the gap phases when cells typically grow (Fig 1.1A). These cleavage cycles are reductive divisions that subdivide a constant volume of cytoplasm with each division and increase the nuclear to cytoplasmic (N:C) ratio with each round of replication (Fig 1.1B). Rapid cleavage cycles, which establish a large cell population necessary for gastrulation, continue until the embryo undergoes the mid-blastula transition (MBT).

Many, if not most metazoans undergo a cleavage stage, though cell cycle timing varies slightly. In zebrafish embryos, the first cell division is initiated approximately 40 minutes after fertilization, followed by nine cell cycles, each lasting approximately 15 minutes. Once the embryo has reached 1000 blastomeres, embryos initiate the MBT and cell cycle remodeling commences (Kane and Kimmel, 1993). Amphibian embryonic cycles, also easily visualized, have similar dynamics though cleavage cycles are longer. In *Xenopus laevis*, the first cleavage occurs about 90 minutes after fertilization and is followed by 11 additional 30 minute cleavage cycles, after which the MBT is triggered (Wu and Gerhart, 1980; Newport and Kirschner, 1982a).

Invertebrate systems are under the same pressure to accumulate cells before gastrulation, but do so in a slightly different way. The *Drosophila* embryo is a multi-nuclear single-celled embryo, also known as a syncytial blastoderm. Nuclei still undergo replication and mitotic cycles in the rapid and synchronous manner observed in their vertebrate counterparts, but do so without cytokinesis (Sullivan and Theurkauf, 1995). After eight 8-minute S-M cycles, nuclei migrate to the periphery of the syncytium such that nuclei are adjacent to the plasma membrane in the 9th cycle (Foe and Alberts, 1983). Between the 9th and 13th mitoses, cell cycles progressively elongate from 9 to 21 minutes (Edgar et al., 1986). The 14^{th} cell cycle marks the end of cleavage divisions in flies, when cell cycles lengthen to 90 minutes or more. Cellularization, the process that creates cell membranes around each nucleus, also occurs at this time.

Molecular basis of rapid cleavages

Cleavage cycles employ many of the same regulators found in canonical cell cycles. However, early embryos have adapted their functions significantly in order to drive rapid cell proliferation. Central to cleavage cycles is the specialized regulation of Cyclin-dependent kinase (Cdk) activity, which differs significantly from canonical cell cycles. The following section will describe these differences in Cdk regulation in cleavage cycles, including cyclin availability, synthesis and proteolysis, and Cdk phosphorylation.

Cdks are a family of serine-threonine kinases that regulate cell cycle transitions by targeting hundreds of substrates to promote cell cycle progression (Ubersax et al., 2003). Conserved from yeast to mammals, there are multiple Cdk proteins present in cells and their activity relies heavily on association with specific cyclin proteins. While Cdk

protein levels remain relatively constant for the duration of the cell cycle, Cdk activity is highly oscillatory and relies on several independent mechanisms to ensure stringent control. Though largely dependent on its temporal association with cyclin proteins, Cdk activity is also significantly influenced by phosphorylation state. Furthermore, subcellular localization and degradation of cyclin and cyclin/Cdk complexes adds an additional level of regulation.

Since their initial characterization, many cyclins and Cdks have been identified in canonical cells. Cyclin A/Cdk2, cyclin D/Cdk4, cyclin D/Cdk6 and cyclin E/Cdk2 regulate cell cycle progression and replication during G1 and S phases. Cyclin B/Cdk1, on the other hand, is important for progression from G2 to M (Murray, 2004; Evans et al., 1983). Meanwhile, embryonic cleavage cycles contain only three cyclins, A,B, and E and two Cdks, Cdk1 and Cdk2 (Hartley et al., 1996). Cdk2 binds to cyclins A and E to mediate DNA replication and centrosome duplication while Cdk1 binds to cyclins A and B to drive mitotic progression (Murray and Kirschner, 1989; Rempel et al., 1995; Strausfeld et al., 1996). The following section will describe how early embryos regulate Cdk activity in order to generate rapid cleavage divisions.

Phospho-regulation of Cdk1

During canonical cell cycles, Cdk1 activity is regulated by phosphorylation on key residues. Cyclin binding requires phosphorylation of a threonine adjacent to the active site (Ducommun et al., 1991). Additionally, the Wee1 and Myt1 kinases inhibit Cdk1 and Cdk2 by adding the inhibitory phosphate to threonine 14 and tyrosine 15. These inhibitory phosphorylations maintain cyclin-bound Cdk in an inactive state until mitosis,

when they are removed by Cdc25 phosphatases (Krek and Nigg, 1991; Atherton-Fessler et al., 1994).

In *Xenopus* embryos, Tyr15 phosphorylation on Cdk1 and Cdk2 appears in each pre-MBT cell cycle, caused by Wee1 kinase, which appears shortly after fertilization (Murakami and Vande Woude, 1998; Kim et al., 1999). However, this inhibitory phosphorylation of Cdks occurs at relatively low levels (Kim et al., 1999). Furthermore, although Cdc25A protein is not detected in *Xenopus* oocytes, maternally deposited *cdc25* mRNA is translated upon fertilization and Cdc25 protein steadily increases during the cleavage stages (Sha et al., 2003; Pomerening et al., 2003; Kim et al., 1999; Bouldin and Kimelman, 2014; Yang and Ferrell, 2013). The low level of Cdk1 inhibitory phosphorylation in cleavage-stage embryos keeps Cdk1 in a 'primed' state, ready for activation upon cyclin binding.

Regulation of mitotic cyclin protein levels

Unlike canonical cell cycles, phospho-regulation of Cdk1 activity plays a minor role during early embryogenesis. Instead, Cdk regulation in cleavage-stage vertebrate embryos is predominately regulated by cyclin synthesis and degradation. In *Xenopus*, protein levels of cyclins A and B oscillate once per cell cycle, with a nearly identical pattern of expression. Cdk1 activity closely parallels cyclin expression and also oscillates with each cell cycle (Hartley et al., 1996). In canonical cell cycles, cyclin protein expression occurs via stage-specific transcription (Pines and Hunter, 1989). In contrast, cyclin transcripts are preloaded maternally in embryos during oogenesis and cyclin protein accumulation is post-transcriptionally regulated. Cell cycle phase-specific

translation of cyclins relies mainly on the adenylation of mRNAs, which changes in a cell cycle-dependent manner (Groisman et al., 2002).

Like in canonical cell cycles, cyclin protein levels are also closely regulated by protein degradation. Mitotic exit is regulated by cyclin B degradation which is mediated by the highly conserved E3 ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C) (Skaar and Pagano, 2009). In cleavage-stage *Xenopus* embryos, APC/C activity is inhibited by XErp1/Emi2 during mitosis. However, when Cdk1 becomes maximally activated, it antagonizes XErp1/Emi2 function leading to APC/C activation (Tischer et al., 2012). Subsequently, activated APC/C polyubiquinates cyclin B, tagging it for proteasomal degradation. Once cyclin B levels are sufficiently lowered, Cdk1 activity diminishes and cells exit mitosis (King et al., 1996). This negative feedback loop between Cdk1, APC/C activation and cyclin degradation encourages the rapid cyclin oscillations observed in cleavage-stage embryos.

Cyclin B synthesis can also be mediated by Cdk1 activity in an adenylation-independent manner. In *Xenopus*, cyclin B protein increases in interphase but plateaus before mitotic entry (Pomerening et al., 2005). This could be a result of either proteasomal degradation of cyclin B or the inhibition of synthesis. To distinguish between these two possibilities, cycling *Xenopus* extracts were treated with a proteasome inhibitor. Cyclin B levels still plateaued at mitosis, demonstrating that cyclin levels at mitotic entry are determined translation rates rather than degradation. Inhibiting Cdk1 leads to increased cyclin B protein levels while prematurely activating Cdk1 decreases cyclin B accumulation. These results demonstrate that Cdk1 participates in a negative-feedback loop that attenuates the production of cyclins before mitosis. Limiting

cyclin production increases the efficiency and sensitivity of the Cdk1-APC/C negative feedback loop by decreasing the burden of cyclin B degradation at anaphase (Kang and Pomerening, 2012).

Cyclin/Cdk activity is also well characterized in *Drosophila.* In contrast to vertebrate embryos, global cyclin levels do not seem oscillate and Cdk1 activity is constitutively high. Consistently, inhibitory phosphorylation of Cdk1 is not detected until nuclear division 10 of *Drosophila* pre-MBT embryos, leaving most syncytial divisions devoid of phospho-regulation (Edgar et al., 1994; Stumpff et al., 2004). However, blocking cyclin B degradation leads to mitotic arrest in syncytial embryos, demonstrating that degradation of the protein is still necessary for mitotic exit (Su et al., 1998).

Subcellular localization of cyclin/Cdks likely explains the confounding observations in the early *Drosophila* embryo. In canonical cells, cyclin B accumulates cytoplasmically during interphase, localizing to centrosomes. At the onset of mitosis, it accumulates on mitotic spindles and chromosomes and facilitates many mitotic events. As metaphase ends, cyclin B is first degraded from the spindle poles, then cytoplasmically (Pines and Hunter, 1991). *Drosophila* syncytial embryos have the same localization pattern of cyclin B during mitosis. At the end of metaphase, spindle-associated cyclin B is degraded in a centrosome-dependent manner (Huang and Raff, 1999; Wakefield et al., 2000). However, the cytoplasmic pool remains intact, demonstrating that localized degradation of cyclin B dictates the regulation of Cdk1 activity in early *Drosophila* embryos.

Regulation of cyclin synthesis in flies also differs from their vertebrate counterparts. Though mRNA polyadenylation also plays a role in regulation cyclin B

synthesis in *Drosophila* syncytial embryos, the PAN GU kinase complex predominantly regulates cyclin translation in flies. PAN GU antagonizes the activity of the protein PUMILIO, which binds to cyclin B mRNA and inhibits translation (Lee and Orr-Weaver, 2003; Vardy and Orr-Weaver, 2007).

Regulation of cyclin E/Cdk2

In somatic cells, cyclin E/Cdk2 activity mediates the transition from G1 to S (Elledge et al., 1992). Similarly, cyclin E/Cdk2 regulates the progression of S-phase in cleavage-stage embryos. Inhibition of CyclinE/Cdk2 activity moderately increases cell cycle lengths in pre-MBT *Xenopus* embryos, showing that the activity of Cdk2 also contributes to rapid cell cycle progression (Hartley et al., 1996, 1997). Though cyclins A and B protein levels oscillate during the cleavage cycles, cyclin E protein levels steadily increase following fertilization (Hartley et al., 1996). Despite this, cyclin E/Cdk2 activity oscillates twice per cleavage cycle independently of protein synthesis. While more studies are required to elucidate the regulation of Cdk2 oscillations in cleavage-stage embryos, mathematical modeling argues that phosphorylation state rather than cyclin accumulation regulates Cdk2 pre-MBT activity (Ciliberto et al., 2003). Indeed, Cdk2 activity is regulated by inhibitory phosphorylation by the Wee1 kinase in *Xenopus* egg extracts and embryos (D'Angiolella et al., 2001; Wroble et al., 2007).

Influence of replication on cleavage cycles

Although accumulation and degradation of cyclins is certainly important for mitotic entry, knockdown of two *Drosophila* mitotic cyclins and a gene reduction of the third via

RNA interference (RNAi) did not prolong interphase but rather lead to a partial activation of mitotic events. These data suggest that accumulation of cyclin alone is not enough to mediate all aspects of rapid cell cycle progression (McCleland and O'Farrell, 2008; McCleland et al., 2009a).

A second mechanism for maintaining short cell cycles could rely on DNA replication itself. Replication occupies the majority of interphase during cleavage divisions and proceeds quickly due to the close proximity of origins of replication (Harland and Laskey, 1980; Spradling, 1999). To test whether replication could directly time embryonic nuclear cycles, McCleland and colleagues inhibited replication in syncytial embryos by injecting Geminin, which blocks the licensing of origins (McGarry and Kirschner, 1998; Quinn et al., 2001). Geminin abolished S-phase and lead to premature mitotic entry, demonstrating that replication defines interphase length in cleavage-stage embryos (McCleland et al., 2009b). This idea was corroborated in *Xenopus*, where replication factors were recently identified that can directly modify cleavage cycle lengths. Highly expressed during the cleavage stages, Cut5, Treslin, Drf5 and RecQ4 levels decrease significantly at the MBT, coinciding with cell cycle elongation. Importantly, overexpression of these factors abolished cell cycle lengthening at the MBT (Collart et al., 2013).

In conclusion, pre-MBT cells are preloaded with many of the same cell cycle regulators as seen in most somatic cells. However, it is their specialized regulation that leads to rapid cell proliferation. Another component of canonical cell cycles is checkpoints, which also regulate Cdk activity to mediate cell cycle progression, though

this is not well understood during the cleavage stages. Chapter 2 and 3 of this dissertation investigates this topic.

Cell cycle checkpoints in early embryogenesis

Cell cycle checkpoints are essential for maintaining the integrity of the genome. The DNA damage checkpoint detects DNA damage and stalled replication, inducing cell cycle arrest during interphase. Meanwhile, the spindle assembly checkpoint (SAC) causes metaphase arrest when kinetochore-microtubules are unattached during mitosis. Despite their presence in almost all nonpathogenic somatic cells, cleavage-stage embryos forgo checkpoint function in their commitment to rapid cell proliferation. Little is known about why checkpoints do not function during the cleavage divisions and how checkpoints are acquired at the MBT. The following section will review our current knowledge of SAC and DNA damage checkpoint function during prior to the MBT.

The DNA damage checkpoint function in cleavage-stage embryos

In somatic cells, DNA damage causes the activation of two phosphoinositide 3-kinase-related protein kinases (PIKKs): ataxia-telangiectasia mutated (ATM) and ATM and RAD3-related (ATR). ATM and ATR are very similar and share many of the same substrates. However, one or the other is preferentially activated depending on the type of damage incurred. DNA double-strand breaks (DSBs), which can be caused by ionizing radiation (IR), are sensed by the Mre11-Rad51-Nbs1 (MRN) complex, which localizes to DNA DSBs and activates ATM. While ATM is activated by DSBs, ATR activation is triggered by single-strand DNA (ssDNA) and junctions of ssDNA and double-stranded

DNA (dsDNA), either as a result of replicative stress or DSB end processing. ssDNA is coated by Replication protein A (RPA), which recruits the ATM-ATRIP heterodimer to DNA lesions, leading to ATR activation (Fig 1.2). Meanwhile, ssDNA-dsDNA junctions activate ATR via the regulators Rad17-RFC and Rad9-Rad1-Hus1 (9-1-1). In the presence of ssDNA-dsDNA junctions, the Rad17-RFC complex recruits the 9-1-1 complex onto DNA (Cimprich and Cortez, 2008; Flynn and Zou, 2011). In human cells, full activation of ATR also requires DNA topoisomerase II binding protein 1 (TOPBP1). TOPBP1 has functions in initiation of DNA replication, but is also recruited to ssDNA-dsDNA by the 9-1-1 complex via its interaction with Rad9. TOPBP1 stimulates ATR activity (Flynn and Zou, 2011).

One of the earliest consequences of DNA damage is the Ser139 phosphorylation of the histone variant H2AX (γH2AX) by ATM and ATR. The formation of so-called γH2AX foci creates an important docking site surrounding the DSB that many DNA damage proteins are recruited to, promoting DNA repair and checkpoint signal amplification (Sirbu and Cortez, 2013). γH2AX is an important read-out for DNA damage checkpoint initialization and the successful sensing of DNA damage (Dickey et al., 2009).

Additionally, ATM and ATR also activate the serine/threonine kinases Chk1 and Chk2, which play a central role in mediating cell cycle arrest (Bartek and Lukas, 2003). Activated ATM recruited to DSBs by the MRN complex enables ATM to phosphorylate multiple local substrates, including Chk2. Meanwhile, the activation of Chk1 by ATR requires the adaptor protein Claspin, which normally associates with active replication forks. After damage, Claspin is phosphorylated by ATR, enabling it to bind to Chk1,

serving as a scaffolding protein for ATR-Chk1 interaction.

Once activated, Chk1 and Chk2 have largely redundant functions and phosphorylate many proteins to achieve their ultimate goal of inhibiting the activity of Cdks. For example, Chk1 and Chk2 phosphorylate Cdc25 phosphatases, targeting them for degradation. At the same time, Chk1 can also activate the Wee1 kinase via phosphorylation (Patil et al., 2013).

Post-MBT embryos with canonical cell cycles have a robust DNA damage response and induce cell cycle arrest efficiently after DNA damage (Hensey and Gautier, 1997; Maller et al., 2001). However, when *Xenopus* pre-MBT embryos or egg extracts are treated with IR, cleavage cycles continue without arrest or even cell cycle delay (Hensey and Gautier, 1997). Furthermore, replication stalls or stress also do not limit mitotic entry in pre-MBT embryos (Kimelman et al., 1987; Newport and Dasso, 1989; Anderson et al., 1997; Clute and Masui, 1997; Hensey and Gautier, 1997; Kappas et al., 2000). Aphidicolin, a potent inhibitor of DNA polymerases, leads to replication stalls that trigger S-phase arrest in somatic cells and post-MBT embryos (Dasso and Newport, 1990). However, *Drosophila* embryos, *Xenopus* embryos, and *Xenopus* egg extracts all continue cycling after treatment with replication inhibitors or in mutant embryos that have improper DNA replication (Kimelman et al., 1987; Dasso and Newport, 1990; Freeman et al., 1986; Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). Similarly, zebrafish embryos exposed to replication inhibitors like aphidicolin, camptothecin or etoposide fail to arrest prior to the MBT (Ikegami et al, 1997a).

Rapid checkpoint activation and DNA repair to explain the lack of cell cycle arrest after damage seems implausible, as irradiated embryos have high levels of DNA

fragmentation (Anderson et al., 1997; Hensey and Gautier, 1997; Finkielstein et al., 2001). Instead, it is much more likely that checkpoint signaling is defective. For example, DNA damage could not be properly sensed, leading to failure in the initiation of the checkpoint signaing pathway. For example, it is possible that the MRN complex cannot efficiently localize to DSBs during the cleavage stages, and thus ATM cannot be properly activated. Alternatively, cleavage-stage embryos could lack the ability to detect ssDNA and ssDNA-dsDNA juntions: RPA, TOPBP1, Rad17-RFC and the 9-1-1 complex may not be functional prior to the MBT, rendering ATR activation impossible. Alternatively, activation of Chk1 by ATR may be inefficient if adaptor proteins like Claspin cannot facilitate ATR-Chk1 interactions. Furthermore, the checkpoint could be successfully initiated but ultimately inefficient in inhibiting Cdk activity. Despite our detailed knowledge of checkpoint signaling in many types of eukaryotic cells, checkpoint signaling is not well understood in cleavage-stage embryos. Chapter 2 of this dissertation investigates this question.

Spindle assembly checkpoint function in cleavage-stage embryos

In most cells, the spindle assembly checkpoint (SAC) delays mitotic exit until all kinetochores are successfully attached to microtubules in order to prevent chromosome missegregation. The SAC is active for a large proportion of mitosis because it is stimulated by unattached kinetochores that are present as the chromosomes gradually align and sister kinetochores become bi-oriented. Single unattached kinetochores from a sister kinetochore pair (known as a monotelic attachment) recruits SAC proteins. Monotelic attachments lead to the formation of the mitotic checkpoint complex (MCC),

composed of the SAC proteins MAD2, BUBR1/Mad3, BUB3 and CDC20 (Lara-Gonzalez et al., 2012). Several of these proteins were first identified in yeast screens where mutations in these genes enabled cells to bypass mitotic arrest after disruption of the mitotic spindle by treatment with spindle poisons like nocodazole (Hoyt et al., 1991; Li and Murray, 1991).

SAC activity and formation of the MCC inhibits mitotic exit/anaphase onset by targeting Cdc20. Cdc20 is a co-activator of the Anaphase-promoting complex/cyclosome (APC/C), an ubiquitin ligase that polyubiquinates cyclin B and securin, leading to their destruction which is required for anaphase onset (Amon et al., 1994; Pines, 2011). By sequestering Cdc20 from the APC/C, the MCC prevents APC/C ubiquitin ligase activity, preventing mitotic exit. The SAC remains active until all kinetochore-microtubule (KT-MT) attachments are made, when SAC proteins are depleted from kinetochores and MCC disassembly frees Cdc20 to activate APC/C (Lara-Gonzalez et al., 2012).

Despite robust function after the cleavage stage, the SAC is not observed in pre-MBT embryos. Nuclei in *Xenopus* cleavage-stage embryos or egg extracts treated with spindle poisons have a dramatically different morphology than their post-MBT counterparts. There is significant formation of irregularly shaped, fragmented micronuclei, which are suspected to arise from inappropriate anaphase onset (Clute and Masui, 1992; Newport and Kirschner, 1984). Furthermore, time spent in mitosis does not change in control versus nocodazole-treated pre-MBT embryos (Ikegami et al., 1997b; Clute and Masui, 1992).

DNA damage or spindle stress sustained prior to the MBT result in embryonic lethality. Pre-MBT *Xenopus* embryos treated with IR accumulate dense, small nuclei that

are typical of apoptosis beginning at the onset of the MBT (Anderson et al., 1997). Using nuclei morphology and TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) to detect apoptosis, researchers demonstrated that embryos turn on a maternally-supplied apoptotic program at the MBT, but not before (Anderson et al., 1997; Hensey and Gautier, 1997; Stack and Newport, 1997; Sible et al., 1997).

Though the lack of DNA damage checkpoint and SAC function has been observed in fish, flies and frogs, little is known about why they do not function during the cleavage stages. Importantly, it is not well understood to what degree checkpoint signaling pathways are intact prior to the MBT. Chapters 2 and 3 of this dissertation investigate both SAC and DNA damage checkpoint function in cleavage-stage embryos.

Cell cycle remodeling at the MBT

After completion of a constant, organism-specific number of cleavage divisions, the embryo undergoes the MBT. One hallmark of the MBT is cell cycle remodeling, in which cells elongate their cell cycles, add gap phases and gain functional cell cycle checkpoints. This section will review our current understanding of cell cycle elongation and checkpoint acquisition during this important developmental transition.

Timing the onset of cell cycle remodeling

How embryos time the onset of cell cycle remodeling has been the subject of intense experimentation for many decades. Initially, it was observed that the cell cycle remodeling always occurs after a fixed, specific number of cleavages for any given animal on a precise time schedule. This led to the hypothesis that a mechanism that can

measure the number of cell divisions or elapsed time post fertilization triggers the MBT. However, partial embryo ligature experiments in *Xenopus* demonstrated that MBT onset is not directly caused by either parameter. Using the Spemann method (De Robertis, 2006), in which a single strand of hair is tied around the embryo, *Xenopus* embryos were partially constricted at the single-cell stage to trap the nucleus on one side of the embryo. This effectively halved the cytoplasmic volume carrying the nucleus. The section with the nucleus cleaved 11 times before cell cycles became asynchronous. In some cases, a daughter nuclei migrated through the narrow channel of the constriction (usually after two cleavages) to the cytoplasmic side that originally had no nucleus. This nucleus cleaved 11 more times before becoming asynchronous, despite having already undergone several mitoses prior to migration (Newport and Kirschner, 1982a).

These landmark observations were essential for shifting the focus to the N:C ratio as a key regulator of MBT onset. Indeed, addition of extra DNA in cleavage-stage embryos via polyspermic eggs or exogenous DNA to mimic post-MBT N:C ratios also caused premature MBT events (Newport and Kirschner, 1982a; b; Dasso and Newport, 1990). Furthermore, haploid *Drosophila* embryos undergo one extra syncytial division, presumably because the N:C ratio associated with MBT is achieved one cell cycle later than in diploid embryos (Edgar et al., 1986; Di Talia et al., 2013). Taken together, work in *Xenopus* and *Drosophila* demonstrated that MBT onset is not necessarily triggered by time post fertilization, the number of divisions or due to a progressive change in chromatin state with each cell cycle, as others had suggested (Satoh and Ikegami, 1981). Rather, the MBT is initiated when embryos reach a threshold N:C ratio resulting from the rounds of replication without cell growth.

How does the N:C ratio control the onset of MBT? Newport and Kirschner proposed that a cytoplasmic factor could inhibit the onset of MBT during the cleavage cycles until it can be titrated out by a threshold amount of DNA. This creates a tempting scenario in which a master regulator could coordinate the major MBT events like ZGA, cell cycle remodeling and cell motility. Alas, no holy grail, master regulator has been identified. However, several candidate factors that could act as the titration-sensitive switch for MBT onset will be discussed in the following sections.

Molecular mechanisms of cell cycle elongation

The goal of cell cycle elongation at the MBT is achieved by the restraint and modification of cyclin/Cdk activities found in the cleavage stage. To achieve this, embryos use several mechanisms to downregulate Cdc25 phosphatase, which allows Cdk1 to accumulate inhibitory phosphorylation and become inactivated after the last cleavage-stage mitosis. At the first asynchronous cycle after the MBT, lowered Cdk1 activity results in an extended replication (Farrell et al., 2012). After replication is complete, cells must wait until zygotic Cdc25 can be synthesized to restore Cdk1 activity for mitotic entry. In effect, these delays represent cell cycle elongation via extension of S-phase and the acquisition of G2 phase.

Regulation of Cdc25 at the MBT has been studied extensively in flies. *Drosophila* embryos express two maternally supplied Cdc25 homologs, String and Twine (Edgar et al., 1994). By altering the number of maternal copies of these Cdc25 homologs, Edgar and colleagues showed that mutant embryos with increased maternal supplies of Cdc25 have one extra rapid, synchronous mitotic cycle. Conversely, mutant embryos with

reduced maternal Cdc25 elongate cell cycles prematurely. These findings provided strong evidence that maternally loaded Cdc25 phosphatases are dosage-sensitive regulators that determine when cell cycles elongate (Edgar and Datar, 1996). Recent studies extend these initial observations of mRNAs and examined the stability of String and Twine proteins. These studies show that Twine protein is rapidly degraded at the MBT and is responsible for cell cycle elongation (Di Talia et al., 2013; Farrell and O'Farrell, 2013).

Cell cycle elongation via zygotic transcription

Cdc25 stability at the MBT is sensitive to the N:C ratio, as Twine protein in haploid embryos is degraded one cell cycle later in haploids compared to diploids (Farrell and O'Farrell; 2013). It is thought that Cdc25 degradation is a result of N:C ratio-regulated transcription of specific genes that may control Cdc25 protein dynamics. Embryos injected with α-amanitin, an RNA Polymerase II inhibitor, do not activate the zygotic genome at the MBT. These embryos undergo an extra round of rapid division and have extended Twine stabilization (Farrell and O'Farrell, 2013). Furthermore, a mutant for RNA polymerase II *RPII215*, which prematurely activates zygotic transcription, decreases the number of nuclear divisions before cellularization. This data independently corroborates that zygotic transcription affects the timing of cell cycle remodeling at the MBT (Sung et al., 2013).

The genes transcribed at the MBT that regulate Cdc25 destruction are just beginning to be elucidated. One candidate zygotic gene that regulates Cdc25 is *tribbles,* which mediates Cdc25 destruction via proteolysis (Mata et al., 2000). Precocious *tribbles* expression via mRNA injections can arrest embryos in cycle 13 and is a result of a

significant reduction in Twine levels (Grosshans and Wieschaus, 2000; Farrell and O'Farrell, 2013). Additionally, RNA sequencing of staged embryos revealed that *tribbles* expression increases dramatically at the MBT. Importantly, gene expression profiling of haploid embryos demonstrated that *tribbles* expression is sensitive to the N:C ratio (Lott et al., 2011; Lu et al., 2009). The Cdk1 inhibitor (CKI) *fruhstart* is another zygotic gene important for cell cycle elongation at the MBT. Also sensitive to the N:C ratio, *fruhstart* appears immediately after the last cleavage division at the beginning of the 14th cell cycle (Lu et al., 2009). Moreover, when precociously expressed via mRNA injection, *fruhstart* can lead to cell cycle arrest during cleavage divisions (Grosshans et al., 2003). To inhibit Cdk1, Fruhstart binds tightly to mitotic cyclins, sequestering them from Cdk1 (Gawliński et al., 2007).

Work in zebrafish has added valuable insight on the influence of zygotic genome activation on cell cycle remodeling in vertebrate systems. Similar to the results in *Drosophila*, inhibiting zygotic transcription in zebrafish embryos hinders the acquisition of G1 phase at the MBT, which can typically be detected at the 11th cell division. However, the regulation of cell cycle lengthening and zygotic transcription seems more complex, since acquisition of G2 is independent of zygotic transcription (Nogare et al., 2009).

Developmental use of the DNA damage checkpoint for cell cycle elongation

Proteins involved in the cellular response to DNA damage or replication stress also contribute to cell cycle remodeling at the MBT. The DNA damage checkpoint is a signaling pathway that arrests cell cycles after DNA damage or stalled replication in

order to repair DNA or mediate apoptosis. Most checkpoint proteins are dispensable in somatic cells; while mutations and aneuploidy arise, knocking out individual components rarely leads to inviability. However, several checkpoint proteins are essential for viability in a variety of model systems, demonstrating that some checkpoint proteins also have non-checkpoint related cell cycle functions and possibly a role in early development (Brown and Baltimore, 2000; Liu et al., 2000)

The Chk1 serine/threonine kinase is an important DNA damage checkpoint component that causes cell cycle arrest by negatively regulating Cdc25. Active Chk1 attenuates Cdc25 phosphatase activity by targeting it for proteasomal degradation via phosphorylation (Lukas and Bartek, 2009). In *Xenopus*, Chk1 is activated transiently at the MBT (Shimuta et al., 2002). Furthermore, exogenous expression of wildtype Chk1 in *Xenopus* embryos induces a dose-dependent delay of cleavage cycles and increased inhibitory phosphorylation of Cdk1 (Kappas et al., 2000). Moreover, dominant-negative forms of Chk1 injected into embryos stabilizes Cdc25A protein while wild-type Chk1 overexpression leads to precocious Cdc25A destruction (Petrus et al., 2004; Shimuta et al., 2002). Additionally, Chk1 can also regulate the interaction between Cdc25 and Cdk1: phosphorylation of Cdc25 by Chk1 inhibits it from interacting with cyclin/Cdk complexes (Uto et al., 2004; Petrus et al., 2004).

The replication checkpoint is also used as a means to elongate cell cycles in flies. Chk1 (known as *grapes* in *Drosophila*) mutant embryos have unusually fast syncytial mitoses, undergo extra cleavage divisions, and die at gastrulation(Sibon et al., 1997, 1999). While MBT degradation of Twine protein is not substantially disturbed in *grapes* mutant embryos, String, the other *Drosophila* Cdc25 homolog, is degraded in a Chk1

dependent manner. In control embryos, String protein degradation happens gradually and starts before the MBT (Farrell and O'Farrell, 2013; DiTalia et al., 2013). However, String protein is stabilized during the cleavage divisions in *grapes* mutants (Sibon et al., 1997; Su et al., 1999). In addition to regulating String protein levels, Chk1 can also prevent nuclear accumulation of cyclin B, which prevents its interaction with Cdk1 in the nucleus (Royou et al., 2008). These data demonstrate that Chk1 is a potent regulator of cell cycle lengths during early embryogenesis and is necessary for early embryonic development.

While the molecular mechanisms of Chk1 activation at the MBT have yet to be fully elucidated, several hypotheses exist and all stem from the influence of the N:C ratio (Edgar and O'Farrell, 1989; Edgar et al., 1994; Sibon et al., 1999). In one model, a maternally-loaded replication factor is titrated by increasing chromatin concentrations in cleavage-stage embryos. Eventually, limiting amounts of this replication factor lead to delays in replication. While this is not DNA damage *per se,* it may effectively recognized as replication stress: ssDNA and ssDNA-dsDNA-binding proteins like RPA, Rad17-RPC and 9-1-1 may have an opportunity to bind, leading to Chk1 is activated.

Supporting this possibility, a recent study identified four specific replication factors that could regulate cell cycle length during *Xenopus* cleavage divisions (Collart et al., 2013). In an initially confounding result, overexpression of the replication factors Cut5, Treslin, RecQ4 and Drf1 led to increased replication origin firing and premature Chk1 activation. However, this was caused by premature nucleotide depletion due to the increased replicative activity, and this activation of Chk1 was greatly reduced if nucleotides were co-injected with the replication factors. In the unperturbed cleavage-stage environment where these replication factors are abundant but there is no danger of

premature nucleotide depletion, the rapid origin firing on DNA may not allow binding of ATR and Chk1 activating factors. However, when Cut5, Treslin, RecQ4 and Drf1 are gradually reduced at the MBT, replication could slow and permit ATR-Chk1 activation..

Another mechanism has been suggested to explain Chk1 activity at the MBT. Chk1 activation in somatic cells and *Xenopus* egg extracts is heavily dependent on the adaptor protein Claspin, which recruits Chk1 to ATR for phosphorylation (Chini and Chen, 2003; Kumagai and Dunphy, 2000, 2003). Claspin is typically phosphorylated by ATR, but *Xenopus* embryos phosphorylate Claspin even in the absence of ATR activity (Gotoh et al., 2011). Despite this, phosphorylation is still responsive to the N:C ratio, as addition of sperm nuclei to increase N:C ratios to MBT levels resulted in Claspin and Chk1 phosphorylation, suggesting that the N:C ratio activates Chk1 via a mechanism that is independent of a *bona fide* replication checkpoint response. Gotoh and colleagues suggest that the increasing N:C ratio triggers two independent events. In one, maternally supplied replication factors are titrated, slowing replication and activating the checkpoint. Additionally, the increasing DNA also titrates out a kinase inhibitor that mediates Claspin phosphorylation (Gotoh et al., 2011).

Checkpoint acquisition at the MBT

One longstanding question in early embryogenesis is how embryos acquire fully functional checkpoints as a part of cell cycle remodeling at the MBT. The molecular mechanisms that underlie checkpoint acquisition during early embryogenesis are poorly understood. The following section will review what is known about DNA damage and SAC acquisition at the MBT, with an emphasis on the role of the N:C ratio.

DNA damage checkpoint acquisition

The first hints towards elucidating checkpoint regulation came from studies using *Xenopus* egg extracts. As mentioned above, addition of DNA replication inhibitors, DNA damaging agents or spindle poisons had no effect on cell cycle progression in control extracts (Dasso and Newport, 1990; Kumagai et al., 1998). However, when additional DNA was supplied via the addition of sperm chromatin, the extracts became sensitive to replication stress and DNA damage and arrested their cell cycles, indicative of restored checkpoint function.

Several models have been proposed to explain the influence of the N:C ratio on DNA damage checkpoint acquisition. One hypothesis suggests that low N:C ratios in pre-MBT embryos cannot efficiently amplify the DNA damage signaling response for full checkpoint function. Conn and colleagues investigated this by injecting embryos with varying amounts of double-stranded DNA which mimic DNA double-strand breaks (DSBs). This activated a robust, dose-dependent, precocious DNA damage response that caused in activation of Chk1, inhibitory phosphorylation Cdk1 and subsequent cell cycle delay. Further, the group showed that activation of the checkpoint occurs at a critical N:C ratio rather than a critical concentration of DNA DSBs (Conn et al., 2004; Peng et al., 2008).

In addition to corroborating the N:C ratio model for checkpoint acquisition, these data also provide some insight into the molecular mechanisms for the requirement for a threshold N:C ratio. Based on the data above, Maller's group hypothesized that the undamaged DNA serves to amplify DNA damage signaling and promote full checkpoint activation. Indeed, Peng and colleagues demonstrated that the endogenous, undamaged

chromatin changes significantly after DNA damage when checkpoint activation is induced in egg extracts with the addition of sperm chromatin: ATM was recruited to the chromatin and phosphorylated the histone variant H2AX, an early substrate for ATM. These results suggest that damaged DNA triggers changes on adjacent and *in trans* undamaged chromatin, and that this signaling is essential to checkpoint function. Based on this, the Maller group proposes a model in which threshold, MBT N:C ratio levels of DNA function as a platform to promote checkpoint signaling, leading to checkpoint acquisition at the MBT (Peng et al., 2007), though this idea has some caveats: it is unclear why this signaling would be deficient in cleavage-stage embryos and why it would change at a threshold N:C ratio, as the amount of DNA stays constant on a per-cell basis. Chapter 2 of this dissertation investigates damage signaling on chromatin before and after the MBT.

SAC acquisition at the MBT

SAC acquisition in early embryogenesis has also been investigated, though not extensively. Clute and Masui examined SAC acquisition by creating 'mini-embryos' with a reduced cytoplasmic volume. Using another modified version of the Spemann method, a loop of baby's hair was placed around the animal pole of a newly fertilized Xenopus embryo to constrict a portion of the nucleus-containing cytoplasm, effectively increasing the N:C ratio. Mini-embryos were produced that had a cytoplasmic volume of about 1/8-1/12 the size of a normal embryo. This mini-zygote continued to cycle like their unperturbed counterparts, but with a much higher N:C ratio (Clute and Masui, 1995). The cells cycles of these mini-zygotes become asynchronous two cycles early, at cleavage 10.

At this point, the N:C ratio of the mini-embryos corresponds to the N:C ratio of unperturbed embryos at cleavage 12, when the MBT occurs. This suggests that cell cycle remodeling in terms of elongation is controlled by the N:C ratio. Surprisingly, however, mitotic delay after treatment with nocodazole occurred at the same time as in control embryos, despite the disparity in N:C ratio and precocious cell cycle elongation in the mini-zygotes. The group also employed a modified Spemann method to reduce the N:C ratio compared to controls. When treated with nocodazole, these embryos also acquired SAC function at the same time as control embryos.

These studies suggest that the SAC in *Xenopus* is acquired at an absolute time, regardless of N:C ratio (Clute and Masui, 1995). However, they contradict earlier findings in egg extracts, which can activate a SAC and arrest nuclei in metaphase if enough sperm chromatin is added (Minshull et al., 1994), suggesting that SAC acquisition is coupled to the N:C ratio. As the case with the damage checkpoint, SAC signaling may not be efficient at the lower N:C ratios during cleavage stages. This is because full activation of the SAC relies on the generation of MCCs at kinetochores which are then distributed throughout the cytoplasm to inhibit APC/C or SAC inhibitors. However, cleavage stage embryos may not be able to generate enough MCCs to overcome the relatively large cytoplasmic volume characteristic to pre-MBT embryos. Instead, a threshold concentration of DNA (and also kinetochores) may be required to generate enough active MCCs (Fig 1.3). Alternatively, the threshold N:C ratio could trigger zygotic transcription, introducing the possibility that SAC components are not maternally supplied. Chapter 3 of this dissertation further investigates the influence of the N:C ratio, transcription, and developmental age on SAC acquisition at the MBT.

PART 2: TRANSCRIPTION DURING EARLY EMBRYOGENESIS

Cleavage-stage transcription

Transcriptional activity in early development was first measured in *Xenopus laevis* embryos by adding radiolabelled uridine (rUTP) to newly fertilized embryos and monitoring its incorporation into transcripts (Bachvarova et al. 1965). Although there is abundant transcriptional activity during oogenesis, newly synthesized RNA could not be detected in embryos until after the 12th cleavage cycle when the embryo undergoes the MBT (Newport & Kirschner 1982a; Newport & Kirschner 1982b; Bachvarova et al. 1965). Similar patterns of transcriptional silence and activation were also observed in zebrafish and *Drosophila*, after the 10th and 13th cleavages, respectively (Kane and Kimmel, 1993; Edgar et al., 1986; Edgar and Schubiger, 1986).

Based on these studies, cleavage-stage embryos were initially thought to be transcriptionally incompetent until the MBT. Confusingly, however, when a plasmid encoding a yeast leucine tRNA gene was injected into pre-MBT embryos along with rUTP, transcripts of the gene were detected well before the MBT, suggesting that cleavage-stage embryos have functional transcriptional machinery (Newport and Kirschner, 1982b). This apparent inconsistency was remedied when better detection techniques revealed that low levels of zygotic transcripts are indeed present during the cleavage stages (Yang, 2002; Shiokawa et al., 1994). With the advent of *in situ* hybridization and microarray analyses, a significant number of specific zygotic transcripts were identified in pre-MBT embryos of *Xenopus*, *Drosophila* and zebrafish (Pritchard and Schubiger, 1996; De Renzis et al., 2007; Lund et al., 2009; Tan et al.,

2012; Blythe et al., 2010; Leung et al., 2003; ten Bosch et al., 2006; Skirkanich et al., 2011; Collart et al., 2014; Porcher et al., 2010).

The developmental roles of these early zygotic products are just emerging, and the *Xenopus* system has offered valuable insight into the regulation and developmental function of a small set of pre-MBT zygotic transcripts. For example, pre-MBT transcription of the nodal genes *Xnr5* and *Xnr6* occurs up to 6 cleavage divisions prior to the MBT (Yang, 2002). The pre-MBT expression of these genes is essential for key features of development like mesendoderm induction, activation of the Nodal pathway, and morphogenesis (Skirkanich et al., 2011). Additionally, the microRNA mir-427 is also expressed prior to the MBT in *Xenopus* (Lund et al., 2009). miR-427 is an important mediator of maternal mRNA degradation, and is important in dorsal-ventral axis formation (Rosa et al., 2009). These data shed light on the emerging role of pre-MBT gene expression in development, and more specific examples are sure to follow as transcript detection methods become increasingly sensitive.

Large-scale genome activation at the MBT

Large-scale activation of the zygotic genome is another signature of the MBT. Zygotic transcription during this period is particularly important because gastrulation requires the expression of many new genes. Additionally, the maternally supplied mRNAs supporting development have been subjected to regulated destabilization and destruction. Indeed, ZGA at the MBT leads to the transcription of at least 15% of the zygotic genome in *Drosophila*, and 12% in zebrafish (Lécuyer et al., 2007; Andersen et al., 2012).

Using high-throughput gene expression analysis, two major waves of ZGA have been identified in *Drosophila* embryos (De Renzis et al., 2007; Lu et al., 2009; Tadros and Lipshitz, 2009). The first significant wave of zygotic transcripts appears at around the 8th nuclear division. This is followed by a second, much larger wave of zygotic transcripts that appear at nuclear division 14, concurrent with other MBT events (De Renzis et al. 2007). *In situ* hybridization analyses in zebrafish have revealed similar patterns of zygotic gene expression. A minor group of genes are expressed at the 6th cleavage stage, while 3400 zygotic genes were expressed at the MBT and beyond, 64% of which were expressed at the onset of the MBT. These data demonstrate that zygotic genome activation also occurs in minor and major waves in zebrafish (Mathavan et al., 2005). The following section will review what is known about zygotic transcriptional activity during embryogenesis and the proposed models for ZGA.

The N:C ratio

Because of the significant role it in regulating MBT onset, it is no surprise that the N:C ratio is also thought to govern ZGA. This model was first posed upon the discovering that transcription could occur prematurely in polyspermic *Xenopus* embryos or when exogenous DNA is added to pre-MBT embryos to mimic MBT DNA content (Newport and Kirschner, 1982b, 1984). This point is also made in a mutant zebrafish line that lacks the ability to perform chromosome segregation but continues replications and cleavage as usual. These embryos accumulate cells with high N:C ratios that have premature RNA polymerase II Ser2/5 phosphorylation, a marker for transcriptional activity (Dekens, 2003). These observations led to the hypothesis that the cytoplasm contains a

transcriptional repressor that can be titrated out by DNA with each successive replication cycle.

Several candidate repressor proteins have been identified that regulate transcription of specific genes, though no overall repressor for the entire zygotic genome has been found. Proteins that regulate hypermethylation-associated gene silencing have been proposed as possible regulators of ZGA. During the cleavage cycles, DNA is hypermethylated, and loss of methylation at specific gene promoters in *Xenopus* coincides with their expression at the MBT (Stancheva and Meehan, 2000). The DNA methyltransferase xDnmt1 and the methylated-DNA binding protein Kaiso have both been implicated in the global repression of gene transcription before the MBT in vertebrate embryos (Ruzov et al., 2009, 2004; Stancheva and Meehan, 2000). When these factors are depleted via injection of morpholinos that inhibit their translation, precocious gene activity is seen with radiolabelled UTP incorporation (Ruzov et al., 2004).

Work in *Drosophila* has identified one additional representative example of a regulator of transcription that could be titrated by the N:C ratio. The maternally supplied transcription factor, *tramtrak* (*ttk*) acts as a transcriptional repressor when bound to the promoter and enhancer regions of specific genes (Brown et al., 1991). *ttk* was initially discovered by analyzing the expression of the segmentation gene *fushi tarazu* (*ftz*). Expression of a mutant *ftz* with point mutations that eliminate *ttk* binding resulted in premature expression of the gene as early as nuclear division 3, well before the expression of endogenous *ftz* at nuclear division 9 (Brown et al., 1991). Similarly, embryos *from ttk*+/- mothers that resulted in reduced Ttk protein levels also showed precocious expression of *ftz*. Conversely, increasing the dose of Ttk protein leads to

delayed expression of *ftz* (Pritchard and Schubiger, 1996). Although these experiments have not shown a direct link between the N:C ratio and ZGA, their mechanism of action, dose-dependent activity and timing of expression make them attractive candidates for a titratable factor.

A developmental timer

A second model proposes that zygotic transcription is dictated by maternal age via a timer initiated during egg activation or fertilization. This model posits that the embryo requires an absolute time in which to accumulate components of the transcriptional machinery. Alternatively, embryos could require an absolute time to undo silencing of the zygotic genome discussed above. Indeed, certain MBT events appear to be triggered by embryonic age. For example, work in Xenopus has demonstrated that the destruction of Cyclins A and E1 are independent of the N:C ratio (Howe and Newport, 1995, 1996). When Howe and colleagues decreased DNA synthesis with hydroxyurea, a drug that depletes deoxyribonucleotides, the timing of the degradation of Cyclins A and E did not change compared to control embryos. Degradation of these cyclins at stage 10.5 also was not affected by inhibiting cell divisions or transcription, providing further evidence for a maternally supplied, age-dependent destruction program.

One possible candidate protein that may need time to accumulate to activate the zygotic genome is Zelda, a transcription factor identified in *Drosophila* (Liang et al., 2008). *Zld* mutant embryos do not cellularize and fail to activate many zygotic genes. Alternatively, ZGA could require the downregulation of a maternally supplied transcriptional inhibitor. Smaug, an RNA-binding protein that recruits a deadenylase

complex to RNA, is responsible for destruction of nearly 2/3 of maternal mRNAs at the MBT (Semotok et al., 2005; Tadros et al., 2007). Smaug protein is low in oocytes, but starts accumulating after fertilization with peaks in gene expression starting from nuclear division 10 (Benoit et al., 2009). Accumulation of Smaug may be required for the destruction of an as yet-undiscovered maternally-supplied transcriptional repressor. Importantly, Smaug mutant embryos are highly defective in ZGA. Using microarray-based gene expression analysis, Benoit and colleagues determined that Smaug was responsible for the MBT upregulation of 85% of zygotic genes they queried (Benoit et al., 2009). Strikingly, when wildtype *smaug* mRNA was injected into one end of mutant Smg embryos, a gradient of rescue measured by cellularization, cell cycle remodeling and transcriptional activity was observed. Based on the above observations, Benoit and colleagues proposed a simple model for ZGA, where Smaug protein accumulation drives a maternal clock that leads to the degradation of maternal mRNAs that code for transcriptional repressors (Benoit et al., 2009). Indeed, Smaug has been shown to trigger the degradation of *ttk* (Tadros et al., 2007a), the transcription repressor protein described above.

Cell cycle elongation/transcript abortion

A third model for ZGA suggests that transcription is coupled to cell cycle remodeling at the MBT. This model focuses on the unique challenges that gene expression machinery faces during early embryogenesis, as the production of RNAs is limited by the time required for transcription. S-phase is possibly incompatible with transcription because of the frequent occupancy of replication factors on DNA (Wolffe and Brown, 1986), while

multiple mechanisms during mitosis could also limit transcriptional activity (Shermoen and Farrell, 1991; Hartl et al., 1993). Therefore, it is possible that the alternating rapid progression between S and M phases in pre-MBT embryos does not allow for transcription, either because of the transcriptional-limiting capacity of replication or transcript abortion by rapid entry into mitosis.

Evidence that longer cell cycles are required for transcription comes from experiments where cell cycles were precociously lengthened prior to the usual time of ZGA onset. When *Drosophila* embryos are arrested at nuclear division 12 by the addition of cycloheximide, an inhibitor of protein synthesis that prevents re-accumulation of cyclins, labeled RNA was detected precociously, at cycle 12 instead of cycle 14 as in control embryos (Edgar and Schubiger, 1986). Similar results were seen in *Xenopus* in a comparable experiment also using cycloheximide to arrest pre-MBT cleavage cycles, although induction of transcription was less dramatic in prematurely elongated cleavage cycles (Kimelman et al., 1987). The recent studies by Collart and colleagues corroborate the hypothesis that the brief, replication-dense cleavage S-phases cannot support transcription, since overexpression of replication factors lead to extra cleavage divisions and defective ZGA (discussed above) (Collart et al., 2013).

The inability for transcription to occur during mitosis has also been demonstrated during early embryogenesis. Shermoen and O'Farrell tracked transcript formation in *Drosophila* using in situ hybridization for RNA polymerase as it travelled along a gene during active transcription, finding that this hybridization disappeared during mitosis (Shermoen and Farrell, 1991). Additionally, *in vitro* work using *Xenopus* egg extracts showed that transcription of RNA polymerase III genes is inhibited during mitosis. This

group proposed a mechanism by which a mitosis-specific kinase could phosphorylate and inhibit a component of the transcriptional machinery (Hartl et al., 1993).

In addition to supporting the hypothesis that mitosis is incompatible with transcription, the above findings also suggest that short transcripts are more likely than long transcripts to be transcribed prior to the MBT, as transcription of a short gene could be completed before mitotic entry. To this end, tracking long and short genes in Drosophila reveals that nascent transcripts of longer genes are aborted upon mitotic entry (Rothe et al., 1992; McHale et al., 2011; McKnight and Miller, 1976). Indeed, analysis of the few genes expressed before the MBT in Drosophila and zebrafish show that they are mostly short genes that lack introns (De Renzis et al., 2007; Heyn et al., 2014).

The Chromatin Landscape

DNA sequence and chromatin state are well-established regulators of gene expression in somatic cells and has been proposed to regulate the transcriptional activity during early embryogenesis. The dramatic chromatin remodeling that occurs once oocytes mature and become transcriptionally silent suggests that chromatin state regulates transcriptional activity. At fertilization, nucleosomes rearrange and form more regular arrays that are in a condensed, inactive state (Landsberger and Wolffe, 1997). However, at the onset of the MBT, the chromatin is remodeled, concomitant with ZGA.

DNA methylation occurs on CpG dinucleotides, known as CpG islands and recruits methyl-CpG-binding proteins (MBPs) that repress transcription (Klose and Bird, 2006). Data from *Xenopus* shows a 40% reduction in DNA methylation between pre- and post-MBT embryos, suggesting a generalized regulatory mechanism for ZGA onset

(Stancheva and Meehan, 2000; Stancheva et al., 2002). However, work in zebrafish using ChIP coupled to high-density microarray hybridization shows that the methylation state of promoters and genes does not change significantly (Andersen et al., 2012). Despite this, the methylation marks are still important, as proteins like xDnmt1 and Kaiso (discussed above) bind specifically to methylated regions and have been demonstrated to repress transcription. These data suggest that DNA methylation per se may not have a direct role in regulating ZGA timing, rather, it may have an indirect 'priming' role through interactions with other transcriptional activator proteins (Østrup et al. 2013).

It is well known that histone methylation also plays a large part in transcriptional regulation, although its use in the developmental transcriptional program is complex. In most cells, the addition of lysine 4 trimethylation on Histone H3 (H3K4me3) marks the transcription start site of genes, and correlates with transcriptional activity, while methylation at H3K27me3 or H3K9me3 is associated with transcriptional repression (Berger, 2007). These chromatin marks have recently been characterized in zebrafish embryos. Lindeman and colleagues used chromatin immunoprecipitation (ChIP) to profile histone methylation on promoters before and during ZGA. They found that the number of promoters with histone methylation were frequent in pre-MBT embryos and increased dramatically at the MBT (Lindeman et al., 2011; Østrup et al., 2013).

Finally, histones themselves may play a role in transcriptional activation at the MBT. Mice and Xenopus have an embryo-specific Histone H1 variant, and recent work in Drosophila has identified a cleavage stage-specific variant of Histone H1 protein, dBigH1 (Pérez-Montero et al., 2013). In Drosophila, the embryonic H1 variant is replaced with somatic histone H1 at the MBT. Perez-Montero specifically showed that

dBigH1 protein decreases dramatically during cellularization and is replaced by somatic H1. The group also demonstrated that homozygous loss of dBigH1 causes precocious RNA polymerase II elongation, suggesting that the presence of dBigH1 during cleavage stages is sufficient to prevent ZGA. Future investigations on the post-translational modifications and regulation of dBigH1 and other embryonic Histone H1 variants should offer valuable insight on the unique properties that allow for transcription repression.

Though the types of regulation of ZGA summarized above differ significantly in their mechanism of action, it is apparent that they are not mutually exclusive and likely account for inconsistencies found across different model systems. Chapter 2 investigates the possible regulators of transcriptional activity during the cleavage stages and through the MBT.

Figure 1.1

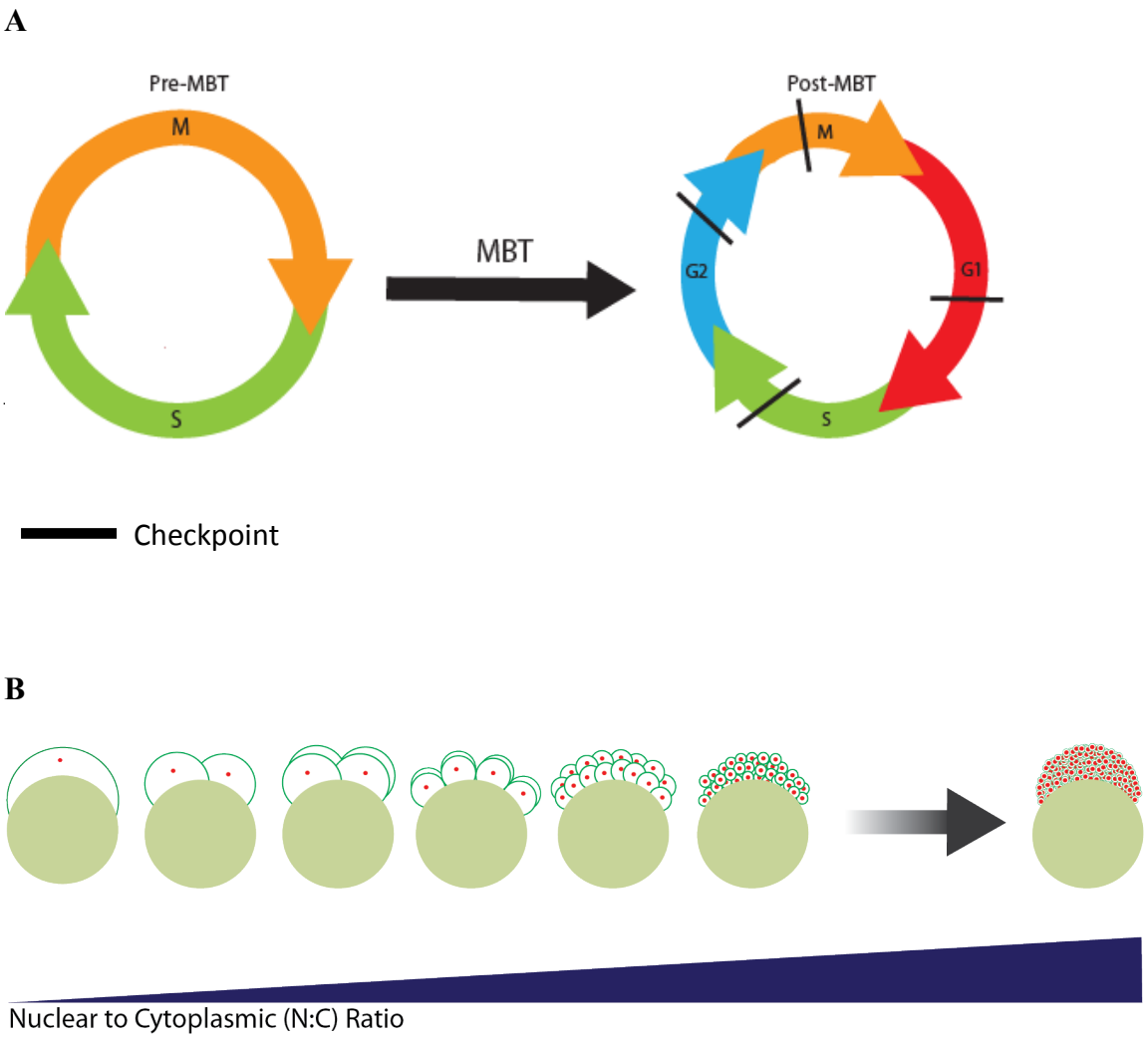

A
Pre-MBT
M
S
MBT
Post-MBT
M
G1
S
G2
Checkpoint
B
Nuclear to Cytoplasmic (N:C) Ratio

Figure 1.1 Embryonic cell cycles before and after the mid-blastula transition

(A) During the cleavage cycles prior to the MBT, embryos have simplified cell cycles consisting of continuous rounds of replication and mitosis. Cell cycles are rapid and do not have checkpoints. At the MBT, the specialized embryonic cleavage cycles are remodeled into canonical cell cycles resembling those observed in most nonpathogenic, somatic cells. G1 and G2 phases are added to cell cycles and checkpoint become functional. (B) Reductive cleavage divisions in zebrafish embryos lead to increasing N:C ratios throughout the cleavage stage.

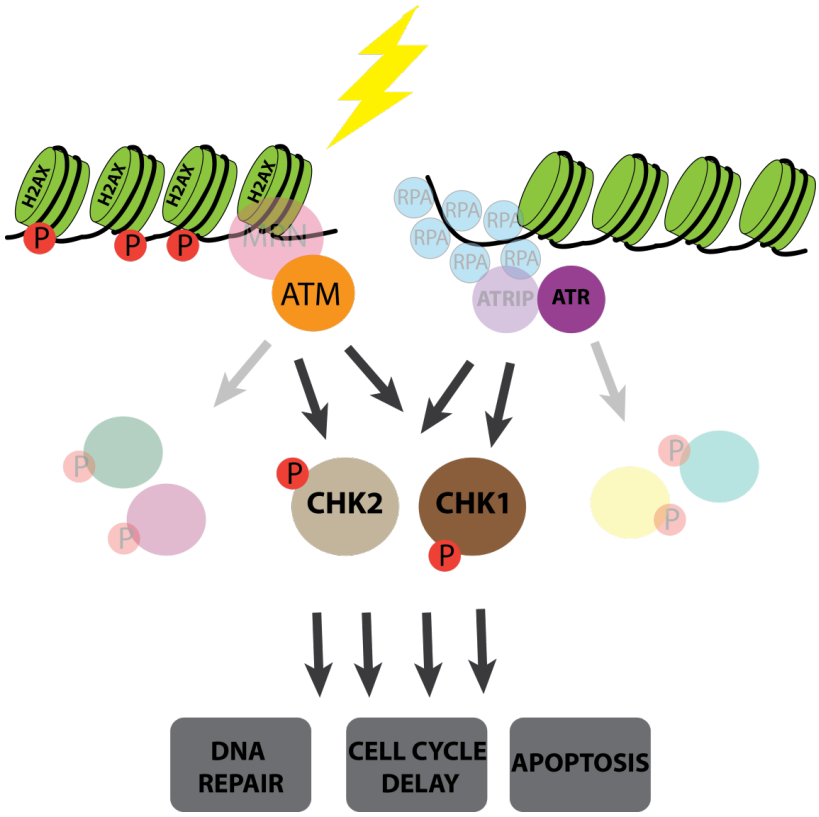


Figure 1.2 The DNA damage response Upon induction of DNA DSBs, the MRN complex senses the lesion and localizes to the site, where it initiates the DNA damage response by activating the master kinase ATM. Activated ATM phosphorylates a plethora of substrates, including the histone H2AX, which serves as a docking site for DNA repair proteins and amplifies checkpoint signaling. When ssDNA breaks occur after UV-irradation or replication fork collapse, RPA coats single-stranded DNA and recruits the ATRIP/ATR complex to the site, where it is activated. ATM and ATR also activate the mediator kinases Chk1 and Chk2, which play important roles in cell cycle arrest by promoting the degradation of Cdc25 phosphatases.

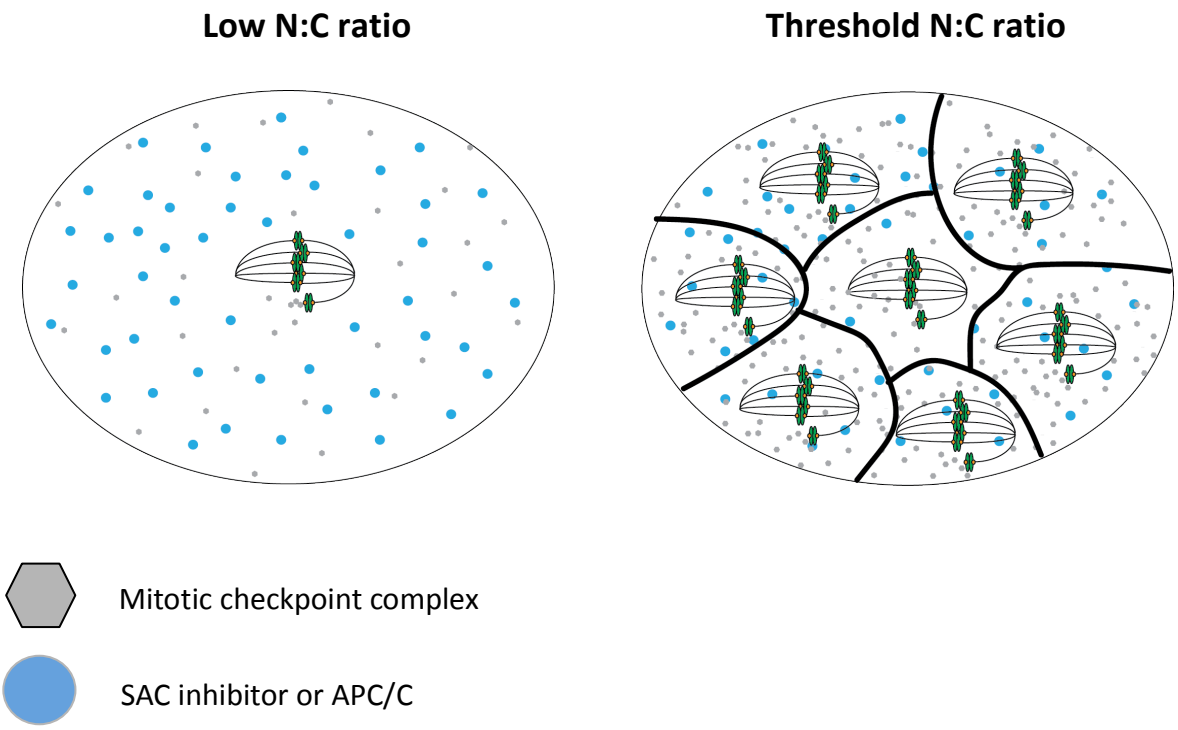


Figure 1.3 The N:C ratio model for SAC acquisition at the MBT During the cleavage stages, DNA concentration increases with each round of replication while the cytoplasmic volume remains the same. SAC function relies on the efficient generation and diffusion of the MCC from unattached kinetochores throughout the cytoplasm. In cells with a low N:C ratio (and hence lower numbers of kinetochores), the amount of active MCCs generated may not be sufficient to inhibit mitotic exit (left image). In contrast, embryos that have reached a threshold N:C ratio can generate more MCC units that can efficiently sustain SAC function and delay mitotic exit (right image)

CHAPTER 2

Regulation of zygotic genome activation and DNA damage checkpoint acquisition at the mid-blastula transition

This data in this chapter has been accepted for publication

ABSTRACT

Following fertilization, most metazoan embryos undergo rapid, transcriptionally silent cleavage divisions until the mid-blastula transition (MBT), when large-scale developmental changes occur, including zygotic genome activation (ZGA), cell cycle remodeling via lengthening, and checkpoint acquisition. Despite their concomitant appearance, whether these changes are co-regulated is unclear. Three models have been proposed to account for the timing of (ZGA). One model implicates a threshold nuclear to cytoplasmic (N:C) ratio, another stresses the importance of Chk1 activation and resulting cell cycle elongation, and the third model invokes a timer mechanism. We show that Chk1 activation before the MBT in zebrafish embryos elongates cleavage cycles and slows the increase in the N:C ratio. We find that cell cycle elongation and Chk1 activity do not lead to transcriptional activation. Rather, ZGA slows in parallel with the N:C ratio. We show further that the checkpoint program is maternally supplied and independent of zygotic transcription, and that Chk1 protein is present in pre-MBT zebrafish embryos. The Chk1 arm of the DNA damage response is not activated after damage, but the Chk2 arm functions properly. Our results are consistent with the N:C ratio model for ZGA. Moreover, the ability of precocious Chk1 activity to delay pre-MBT cell cycles indicates that Chk1 activity limits DNA damage checkpoint function during pre-MBT stages. We propose that Chk1 gain-of-function at the MBT underlies cell cycle remodeling, whereas zygotic genome activation is regulated independently by the N:C ratio.

INTRODUCTION

Immediately following fertilization, most metazoan embryos undergo synchronous cleavage divisions that lack gap phases and cell cycle checkpoints. During this period, most zygotic genes are transcriptionally silent; rather, embryos rely on maternally loaded mRNAs for development. These abbreviated cycles persist until the mid-blastula transition (MBT), when several large-scale changes occur together.

Zygotic genome activation (ZGA) is one hallmark of the MBT. With a few exceptions (Lu et al., 2009; De Renzis et al., 2007; Liang et al., 2008; Skirkanich et al., 2011; Blythe et al., 2010; Heyn et al., 2014; Lindeman et al., 2011) transcription of the vast majority of zygotic genes starts at the MBT. How the onset of ZGA is regulated is a long-standing question, and several models have been proposed. In one model, activation of zygotic transcription depends on a threshold nuclear to cytoplasmic (N:C) ratio that is achieved through the reductive cleavage divisions leading up to the MBT, when cells divide but do not grow (Fig 2.1A). Addition of exogenous DNA to increase the N:C ratio in pre-MBT *Xenopus* embryos can induce premature onset of zygotic transcription (Newport and Kirschner, 1982b). These findings suggest that transcription is regulated by an unknown cytoplasmic repressor that is titrated as the N:C ratio increases (Kimelman et al., 1987). A second model suggests that cell cycle elongation is required for ZGA (Kimelman et al., 1987), as rapid cell cycles may not support transcription, particularly of long genes (Fig 2.1B) (Shermoen and Farrell, 1991; Tadros and Lipshitz, 2009; McHale et al., 2011; Collart et al., 2013; Heyn et al., 2014). Thus, although it may not directly trigger ZGA, cell cycle elongation at the MBT may create a permissive environment for increased transcriptional activity. A third model postulates that a cell cycle-independent

timer governs ZGA, either through accumulation of transcription machinery components, loss of a transcriptional repressor, or degradation of maternal transcripts (Fig 2.1C) (Tadros et al., 2007; Tadros and Lipshitz, 2009; Lu et al., 2009).

Massive cell cycle remodeling is a second hallmark of the MBT, as embryonic cleavage divisions transform into typical somatic cell cycles: losing cell division synchrony, elongating the cell cycle dramatically, and adding gap phases that are lacking during pre-MBT cell cycles. Both activation of Chk1, independent of damage (Kappas et al., 2000; Shimuta et al., 2002), and degradation of the Cdk1-activating phosphatase Cdc25 (Sung et al., 2013; Farrell and O'Farrell, 2013; Di Talia et al., 2013) contribute to these changes. Another major component of cell cycle remodeling at the MBT is acquisition of DNA damage checkpoints. Pre-MBT embryos neither delay their cell cycles nor initiate DNA repair and apoptotic pathways in response to DNA damage (Ikegami, R., Rivera-Bennetts, A., Brooker, D., and Yager, 1997; Hensey and Gautier, 1997). Mechanisms underlying this aspect of cell cycle remodeling at the MBT are poorly understood.

Despite the striking synchrony of ZGA and DNA damage checkpoint acquisition, how these events are coordinated is unclear. One model hypothesizes that checkpoint function is directly coupled to ZGA: pre-MBT checkpoint signaling pathways might lack essential components provided only by the zygotic genome. Adding exogenous DNA to pre-MBT *Xenopus* embryos leads to precocious checkpoint function (Conn et al., 2004), but this effect could be indirect if the N:C ratio controls zygotic transcription.

By experimentally manipulating Chk1 activity before the MBT in zebrafish embryos, we interrogate its role in cell cycle remodeling at the MBT and distinguish

between models for ZGA. We find that zygotic transcription increases in parallel with the N:C ratio and not in response to premature cell cycle elongation. We also show that the DNA damage checkpoint program is independent of zygotic transcription, and that checkpoint function is limited by the lack of Chk1 activity prior to the MBT. Overall, we conclude that while happening simultaneously, cell cycle remodeling and zygotic genome activation are regulated independently at the MBT.

RESULTS

Precocious cell cycle elongation prior to the MBT does not lead to zygotic genome activation

To determine whether cell cycle elongation leads to ZGA, we first tested whether premature Chk1 activity could lengthen pre-MBT cell cycles. We expressed exogenous wildtype GFP-tagged zebrafish Chk1 (Chk1-GFP) or a constitutively active, phosphomimetic form (Chk1-4E-GFP) (Katsuragi et al., 2004) in pre-MBT zebrafish embryos. Pre-MBT cell cycles progressively lengthened to an average of 21.0 min (Chk1-GFP) or 26.1 min (Chk1-4E-GFP) between the 6^{th} and 10^{th} cleavages, compared to 16 min in control embryos (Fig. 2.2). We also tested two Chk1 truncation mutants. ΔC-Chk1-GFP contains residues 1-99 of zebrafish Chk1, corresponding to the N-terminal kinase domain, which by itself is catalytically inactive (Kosoy and Connell, 2008; Nakajo et al., 1999; Caparelli and O'Connell, 2013; Oe et al., 2001). ΔN-Chk1-GFP contains residues 215-410 of zebrafish Chk1, which corresponds to the C-terminal regulatory domain (Oe et al., 2001; Nakajo et al., 1999). Neither truncation mutant affected cleavage cycle lengths, indicating that the cell cycle lengthening depends on Chk1 kinase activity (Fig 2.2B).

Cell cycle elongation caused by exogenous Chk1 allowed us to test models for how the timing of ZGA is controlled. If ZGA depends directly on cell cycle elongation, which occurs prematurely in Chk1-4E embryos, we expect transcription at a lower N:C ratio compared to controls. Similarly, if ZGA is controlled by a cell cycle-independent timing mechanism, we expect transcription at a lower N:C ratio when cell cycles are elongated in Chk1-4E embryos, compared to controls, because the N:C ratio increases more slowly. In contrast, if ZGA is controlled by the N:C ratio, we expect control and Chk1-4E embryos to exhibit a similar increase in transcriptional activity with respect to the N:C ratio.

To examine the relationship between N:C ratio and transcription, we measured both simultaneously in individual fixed embryos. As a surrogate for transcription, we monitored the phosphorylation of RNA polymerase II using a phospho-specific antibody against Rpb1 Ser2/5 (pRpb1), which is specific for phosphoepitopes present on active, elongating RNA polymerase II in pre-MBT zebrafish and *Xenopus* embryos (Dekens, 2003; Blythe et al., 2010; Nogare et al., 2009). This assay allows us to evaluate global RNA polymerase II activity in combination with N:C ratio on an individual cell basis.

To confirm that pRpb1 staining correlates with transcription, we examined expression of *lrat* and *apoeb*, two zygotically expressed genes that only appear after the MBT, at 3.75 HPF (Fig 2.3A) (O'Boyle et al., 2007). Injection with α-amanitin, an inhibitor of RNA polymerase II, abolished transcript expression at 3.75 HPF. Consistent with the transcript levels, pRpb1 staining is low at 2.5 HPF and high at 3.5 HPF in both control and Chk1-4E embryos (Fig 2.3B). We also tracked changes in the N:C ratio by measuring nuclei densities at 15-minute intervals between 2.25 and 3.5 HPF. The nuclei

density increased more slowly in Chk1-4E embryos compared to control embryos (Fig 2.3C), as expected for elongated cell cycles. We find that pRpb1 staining increases with nuclear density (and therefore, N:C ratio) in Chk1-4E embryos similarly as in control embryos (Fig 2.3D). In addition, pRpb1 staining did not increase prematurely at 2.5 HPF in Chk1-4E embryos, though cell cycles are elongated (Fig 2.3B). These data suggest that transcriptional activity increases throughout the cleavage stages and is coupled to the N:C ratio rather than cell cycle elongation, and is not controlled by a timing mechanism.

Acquisition of DNA damage checkpoints is independent of zygotic transcription

Chk1 is typically activated in response to DNA damage or replication stress, and phosphorylation of Chk1 substrates leads to cell cycle delay and DNA repair (Stracker et al., 2009). The ability of precocious Chk1 activity to lengthen pre-MBT cell cycles (Fig. 2.2) indicates that downstream checkpoint signaling is intact and suggests that the lack of Chk1 activity limits checkpoint function prior to the MBT. Alternatively, other components could be provided via zygotic transcription, which would coordinate checkpoint acquisition and ZGA.

To test whether checkpoint acquisition depends on zygotic transcription at the MBT, we inhibited transcription by injecting one-cell stage embryos with α-amanitin, an inhibitor of RNA polymerase II (Nogare et al., 2007; Meinecke, B. and Meinecke-Tillmann, 1993). To confirm that the treatment prevented activation of zygotic transcription, we stained for pRpb1 and monitored expression of the zygotic genes *nanor* and *lrat*. Post-MBT embryos treated with α-amanitin have decreased pRpb1 staining

compared to uninjected control embryos and lack *nanor* and *lrat*, indicating successful transcription inhibition (Fig. 2.4A,B).

To test for a DNA damage response in the absence of zygotic transcription, embryos were injected with α-amanitin and then treated with hydroxyurea (HU) to induce DNA damage. Hydroxyurea is a ribonucleotide reductase inhibitor which causes stalled replication forks that eventually lead to fork collapse and DNA single-strand breaks (Shechter et al., 2004). We verified the presence of DNA damage with HU treatment with the alkaline comet assay, which detects DNA double and single strand breaks as well as DNA-repair intermediates (Fig 2.7). After HU treatment, embryos were fixed and stained for phospho-Ser10 histone H3 (pH3), a well-established marker for mitosis (Paulson and Taylor, 1982), to track cell cycle arrest after DNA damage. Under normal conditions, post-MBT embryos treated with HU have a robust checkpoint response: the mitotic index decreases from ~20% to 5%, demonstrating successful cell cycle delay and inhibition of mitotic entry after sustaining DNA damage (Fig. 2.4C, left 4 panels). Inhibition of zygotic transcription did not affect the cell cycle response to HU, as the mitotic index was not affected by α-amanitin (Fig. 2.4C, right 4 panels). Our results demonstrate that pre-MBT embryos have a maternally supplied checkpoint program, as checkpoint acquisition does not depend on zygotic transcription.

Pre-MBT embryos have one of two DNA damage signaling pathways intact

Our results show that checkpoint signaling downstream of Chk1 is intact in pre-MBT embryos, since premature Chk1 activation leads to cell cycle delay, and further that all components of the checkpoint program are maternally provided. We next asked how

DNA damage signaling changes at the MBT. The cellular response to DNA damage can be divided into two main signaling cascades whose activation typically depends upon the type of damage sustained (Reinhardt and Yaffe, 2009). The ATR and Chk1 kinases are activated in the case of single-strand breaks caused by replication fork collapses, which can be induced by treatment with HU. ATR is activated after binding to sites of stalled or incomplete replication and phosphorylates downstream targets including the effector kinase Chk1. In the second pathway, double strand breaks caused by ionizing radiation (IR) lead to activation of ATM kinase, which phosphorylates the effector kinase Chk2. Both pathways converge on Cdc25, a positive regulator of cell cycle progression that is inhibited by phosphorylation by Chk1 or Chk2 via degradation (Bouldin and Kimelman, 2014).

One of the earliest cellular responses to DNA damage is phosphorylation of the histone variant H2AX, which creates foci at sites of DNA damage that act as docking sites for additional DNA damage response proteins (Paull et al., 2000). ATM phosphorylates H2AX, although ATR activation can also lead to phosphorylated H2AX (γH2AX), either directly as a substrate of ATR (Ward and Chen, 2001), or indirectly via activation of ATM by ATR (Stiff et al., 2006). Given its essential role in DNA damage response initiation, we monitored γH2AX by immunofluorescence in pre- and post-MBT embryos in response to either HU or IR, using a phospho-specific antibody. After the MBT, treatment with either HU or IR induced DNA damage (Fig. 2.7, see fragmented nuclei in IR treated embryos, Fig 2.5A) and a dramatic increase in γH2AX, as expected in embryos with fully functional damage checkpoints (Fig. 2.5A, B right panels). γH2AX levels also increased in response to IR in pre-MBT embryos, but HU had no effect, even

when treated up to 2.5 hours (Fig. 2.5A, B left panels, Fig. 2.8A). These results demonstrate that pre-MBT embryos are indeed capable of detecting DNA damage and can activate the first major step in the checkpoint signaling pathway in response to double strand breaks, suggesting that the ATM-Chk2 pathway is intact. In contrast, failure to phosphorylate H2AX in response to replication stress after HU treatment suggests that the ATR-Chk1 arm of the DNA damage checkpoint is unresponsive prior to the MBT.

To directly test activation of the ATM and ATR signaling pathways, we examined Chk2 and Chk1 activation after DNA damage. We used phospho-specific antibodies against human Chk2-Thr68 (pChk2) or human Chk1-Ser345 (pChk1), well established markers for Chk2 or Chk1 activation that are also used in zebrafish (Gao et al., 2011; Zhao and Piwnica-Worms, 2001). Embryos were treated with either IR or HU, then fixed and stained with the phospho-specific antibodies. Pre-MBT embryos phosphorylate Chk2 in response to IR but not HU (Fig 2.6A, left panels), consistent with the γH2AX response (Fig. 2.5). Chk1 protein is present prior to the MBT (Fig. 2.8B), but Chk1 phosphorylation increases only slightly in response to IR in pre-MBT embryos, likely a result of weak Chk1 activation by ATM (Helt et al., 2005; Gatei et al., 2003), and not at all in response to HU (Fig 2.6B, left panels). The failure to activate Chk1 is consistent with a previous finding in *Xenopus* embryos with aphidicolin, another DNA damage-inducing replication inhibitor (Kappas et al., 2000). Post-MBT embryos showed robust pChk2 and pChk1 staining in response to either IR or HU (Fig. 2.6A, B right panels), indicating that both pathways are intact after the MBT as expected. Taken together, our results demonstrate a key difference in DNA damage signaling in pre-MBT versus post-MBT embryos. The ATR-Chk1 pathway is active only after the MBT, as shown by

analyses of H2AX and Chk1 phosphorylation. In contrast, ATR-Chk2 signaling is intact even prior to the MBT, although activation of this pathway alone does not lead to cell cycle delay. These results, together with our finding that premature Chk1 activation leads to cell cycle delay before the MBT (Fig 2.2), indicate that Chk1 activity is the limiting factor in checkpoint function prior to the MBT.

DISCUSSION

Our findings address how embryos orchestrate the maternal to zygotic transition, specifically ZGA and cell cycle remodeling at the MBT. Despite their simultaneous emergence, we show that these major events are governed by independent mechanisms. Our data are consistent with the model that zygotic transcription is coupled to the N:C ratio. Premature Chk1 activation in cleavage-stage embryos lengthens the cell cycles and therefore slows the increase in N:C ratio. In these embryos, transcriptional activity, as assayed by RNA polymerase II phosphorylation, increases in proportion to the N:C ratio.

Drosophila Chk1 mutants do not slow their cell cycles and do not undergo ZGA (Sibon et al., 1997). However, *Drosophila* Chk1/Chk2 double mutants maintain rapid cell cycles like Chk1 single mutants yet still activate transcription of several zygotic genes, suggesting that neither Chk1 activation nor cell cycle elongation is required for ZGA in *Drosophila* (Takada et al., 2007). On the other hand, evidence from other systems like zebrafish indicates that cell cycle elongation is involved in ZGA, based on the observation that the production of gene products is limited by the time it takes to transcribe and process RNA (Swinburne and Silver, 2008; Heyn et al., 2014). The rapid cell cycles of pre-MBT embryos might not support this process, as mitosis is not

compatible with transcription (Shermoen and Farrell, 1991). Consistent with this idea, overexpression of replication factors in *Xenopus* embryos leads to continuation of rapid cell cycles and delays the expression of a large number of zygotic genes (Collart et al., 2013), while precocious RNA synthesis occurs when cell cycles are artificially lengthened in Xenopus pre-MBT embryos treated with cycloheximide, an inhibitor of protein synthesis (Kimelman et al., 1987). In addition, the majority of zygotic genes expressed before the MBT in zebrafish are short, underscoring the idea that cell cycle elongation may be required to create a permissive environment for the expression of longer gene products after the MBT (Heyn et al., 2014). Although it may contribute to ZGA, our results show that cell cycle elongation is not sufficient for ZGA, as premature cell cycle lengthening does not lead to transcriptional activation at a lower N:C ratio.

Instead, our findings are consistent with previous studies that suggest that the N:C ratio governs ZGA. Work in *Xenopus* embryos demonstrated that transcription in pre-MBT embryos can be triggered precociously either in polyspermic embryos or by the addition of exogenous DNA to reach an N:C ratio characteristic of the MBT (Newport and Kirschner, 1982b). Precocious transcription also occurs in polyploid cells, with a high N:C ratio, of zebrafish embryos with a mutation that prevents chromosome segregation but leaves cleavage intact (Dekens, 2003). How transcriptional activation depends on the N:C ratio remains unclear. A cytoplasmic ZGA repressor may inhibit zygotic transcription until it is titrated out by a critical amount of DNA, and candidate transcriptional repressors have been identified in *Xenopus* and *Drosophila* (Stancheva and Meehan, 2000; Pritchard and Schubiger, 1996). Alternatively, the N:C ratio could indirectly control ZGA if it affects other MBT events not addressed in the present study,

such as maternal transcript degradation and chromatin modifications, which have also been suggested to regulate ZGA (Benoit et al., 2009; Pérez-Montero et al., 2013). The influence of the N:C ratio on these events remains a question for future work.

An implication of our data is that transcriptional activity does not dramatically increase at a specific N:C ratio, which suggests that ZGA is not a single, abrupt event. Rather, pRpb1 signal gradually increases, even throughout the cleavage divisions, suggesting transcriptional activity also increases gradually. Indeed, analyses of RNA synthesis and large-scale gene expression have found zygotic gene expression in *Drosophila*, *Xenopus* and zebrafish well before the MBT (Collart et al., 2014; Mathavan et al., 2005; Skirkanich et al., 2011; Blythe et al., 2010; Heyn et al., 2014; Lu et al., 2009; Yang, 2002; Shiokawa et al., 1994). Additionally, gene expression profiling data from haploid and diploid *Drosophila* embryos suggest more complex regulation of zygotic transcription, with distinct subsets of zygotic transcripts that depend either on time or on the N:C ratio (Lu et al., 2009).

One potential caveat to our work is the validity of assaying pRpb1 for transcriptional activity. pRpb1 is indicative of active, elongating RNA polymerase II, but it does not directly look at the increase or appearance of specific zygotic transcripts, which are the advantages to microarray analysis or classic pulse-chase experiments with radiolabelled UTP. However, pRpb1 staining allowed us to RNA polymerase II activity in individual embryos on a single-cell basis, which is not possible with the aforementioned techniques. Further, this assay has been used as a readout for transcriptional activity in zebrafish previously (Dekens, 2003; Nogare et al., 2009).

Our studies also provide insight into cell cycle remodeling at the MBT. We show that checkpoint acquisition can be uncoupled from zygotic transcription, and that checkpoint components are maternally supplied. Rather, differences in Chk1 activation underlie checkpoint function in pre- vs. post-MBT embryos. We demonstrate that pre-MBT embryos can detect DNA damage and activate Chk2 but not Chk1. Although there are mixed results on the ability of Chk2 to support a checkpoint without Chk1 (Xu et al., 2002; Brown and Baltimore, 2003; Cimprich and Cortez, 2008), our findings indicate that the lack of Chk1 activity accounts for the absence of DNA damage checkpoints during cleavage cycles. In addition, we show that premature Chk1 activation lengthens cell cycles, likely through inhibition of Cdc25 (Shimuta et al., 2002), consistent with previous findings in *Xenopus* (Shimuta et al., 2002; Kappas et al., 2000). Together, these results indicate that signaling downstream of Chk1 is intact before the MBT, but the checkpoint is not functional because Chk1 is not activated after DNA damage.

The principal goal of cleavage-stage cell cycles is to rapidly amass enough cells for later developmental stages. Uninterrupted cell cycle progression takes priority in pre-MBT stage embryos, even in the face of DNA damage. Absence of Chk1 activity is a strategy for embryos to avoid cell cycle delays prior to the MBT. Furthermore, embryos hijack the ability of Chk1 to delay cell cycle progression by transiently activating Chk1 at the MBT, which contributes to cell cycle elongation (Gotoh et al., 2011; Shimuta et al., 2002). We suggest a unifying model for cell cycle remodeling in which Chk1 gain-of-function dictates timing of both cell cycle elongation and DNA damage checkpoint acquisition in early embryogenesis. Chk1 gain-of-function at the MBT has been attributed to replication stalling and the phosphorylation of Claspin, an adaptor kinase

that promotes Chk1 activation (Gotoh et al., 2001; Newport and Dasso, 1989), which are both sensitive to the N:C ratio (Pogoriler and Du, 2004; Conn et al., 2004), but further work is required for a full understanding of this process.

Figure 2.1

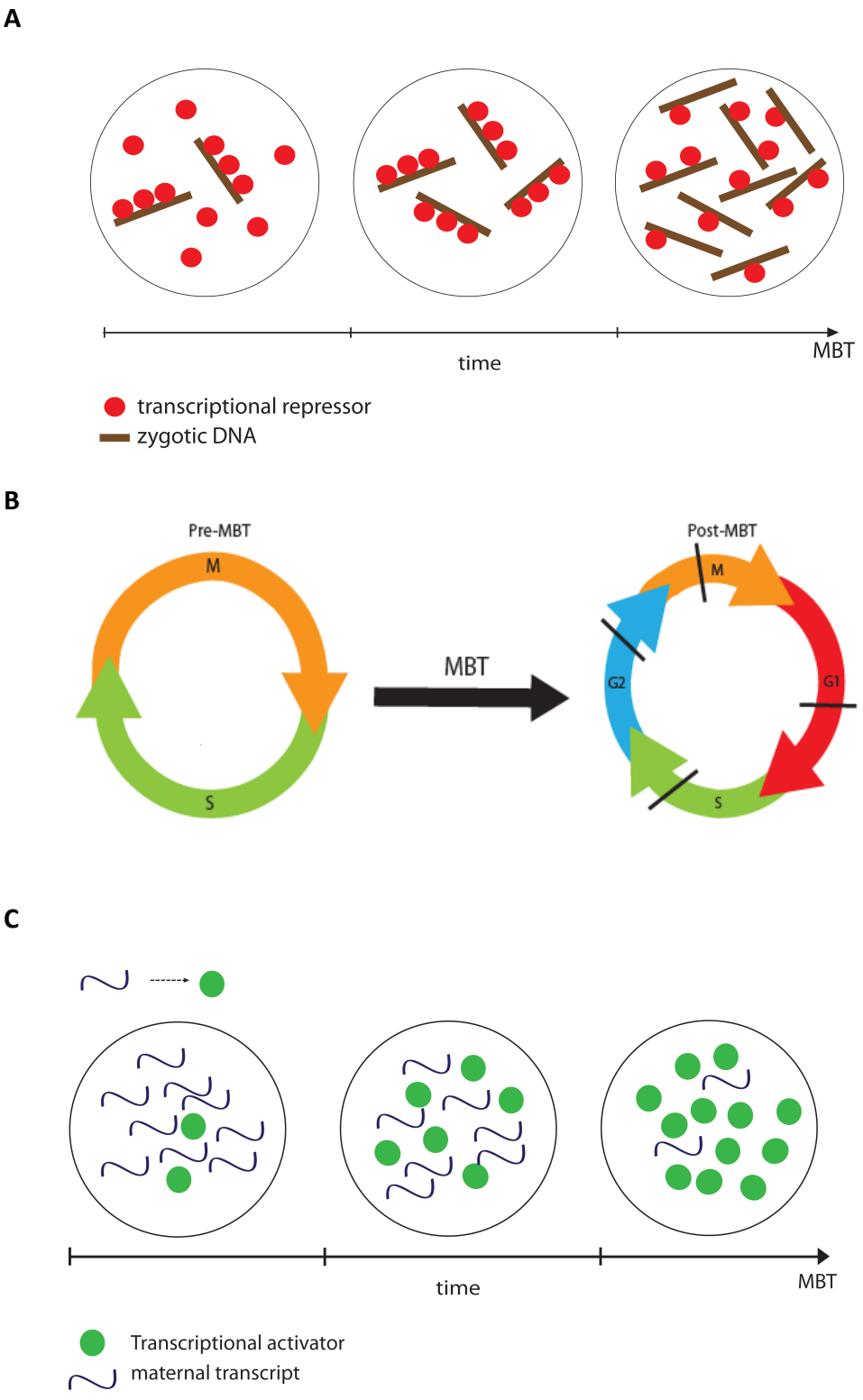
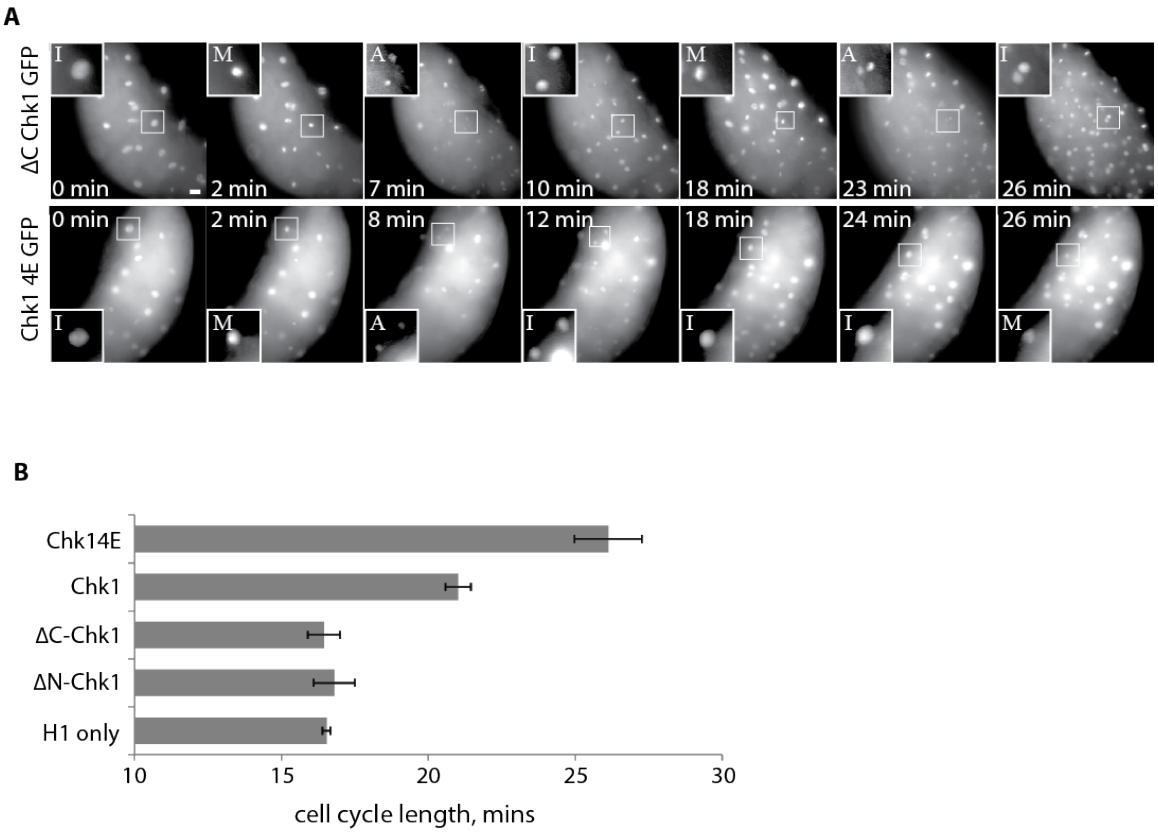

A
time
MBT
transcriptional repressor
zygotic DNA
B
Pre-MBT
M
S
MBT
Post-MBT
M
G2
G1
S
C
time
MBT
Transcriptional activator
maternal transcript

Figure 2.1 Models for the regulation of zygotic transcription at the MBT Three models exist for the regulation of zygotic transcription. (A) ZGA could rely on the N:C ratio if transcription is inhibited during the cleavage stages by a transcriptional repressor that is bound to DNA that can be titrated out by increasing amount of DNA. (B) Alternatively, ZGA could require the accumulation of a transcriptional activator protein over time. (C) Large-scale activation of the zygotic genome may only occur after cell cycles have been elongated during cell cycle remodeling. Rapid cell cycles may not be permissive to transcription of many genes, particularly long ones, as transcript abortion occurs upon mitotic entry.

Figure 2.2


A
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18 min
24 min
26 min
I
M
A
I
I
I
M
B
Chk14E
Chk1
ΔC-Chk1
ΔN-Chk1
H1 only
10
15
20
25
30
cell cycle length, mins

Figure 2.2 Expression of a constitutively active Chk1 kinase elongates pre-MBT cell cycles (A) Embryos were injected with Histone H1-594 protein and Chk1-4E-GFP mRNA. GFP was then imaged live to show Chk1-4E-GFP protein expression. (B) 1-cell stage embryos were injected with AlexaFluor 594-conjugated Histone H1 (Histone H1-594) protein and either Chk1-GFP mRNA or Chk1-4E-GFP mRNA, then imaged live. Fewer nuclei appear in the later timepoint (2.5 HPF) in embryos injected with Chk1-GFP or Chk1-4E-GFP, demonstrating fewer cleavages as a result of cell cycle elongation. (C) Cell cycle lengths for the first 10 cleavage divisions embryos injected with Histone H1-594 alone, H1-594 and Chk1-GFP mRNA or H1-594 and Chk1-4E-GFP mRNA. Schematic shows one representative embryo for each condition. Cell cycles after the first cleavage are ~15 min for control embryos but progressively lengthen as more Chk1-GFP or Chk1-4E-GFP protein is expressed, with Chk1-4E-GFP having the most dramatic lengthening after cleavage 5. Cell cycles lengths were measured as time between metaphases, based on Histone H1 morphology. (D) Cell cycles lengths were measured as time between metaphases, based on Histone H1 morphology and averaged over cleavages 6-10 for multiple embryos ($n \geq 9$). (E) Embryos were injected with Chk1-4E-GFP mRNA, then fixed and stained for DNA at indicated times ($n \geq 24$).

Figure 2.3

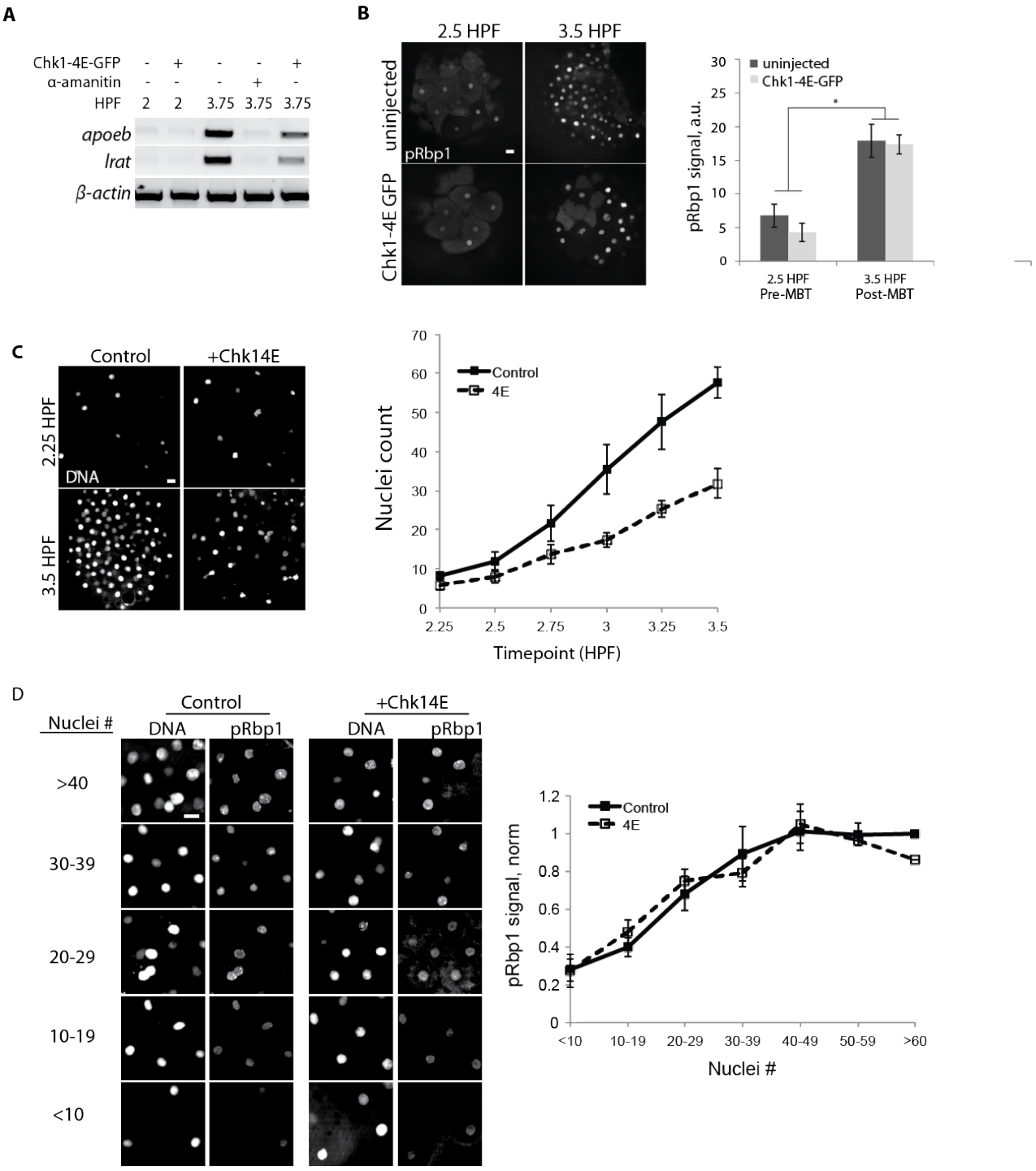
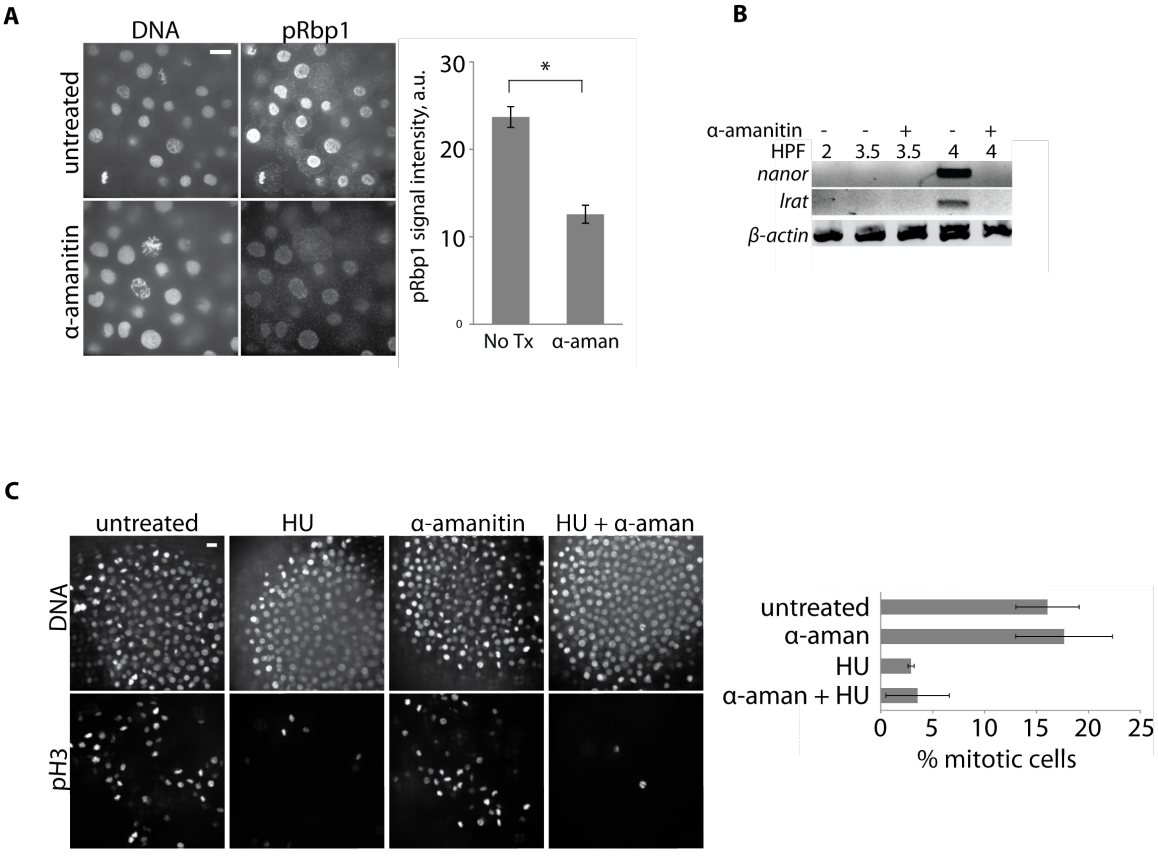

A
Chk1-4E-GFP - + - - +
α-amanitin - - - + -
HPF 2 2 3.75 3.75 3.75
apoeb
lrat
β-actin
B
2.5 HPF
3.5 HPF
uninjected
Chk1-4E GFP
pRbp1
uninjected
Chk1-4E-GFP
pRbp1 signal, a.u.
2.5 HPF Pre-MBT
3.5 HPF Post-MBT
C
Control
+Chk14E
2.25 HPF
3.5 HPF
DNA
Control
4E
Nuclei count
Timepoint (HPF)
D
Nuclei #
Control
+Chk14E
DNA
pRbp1
>40
30-39
20-29
10-19
<10
Control
4E
pRbp1 signal, norm
Nuclei #

Figure 2.3 Transcriptional activity is coupled to the N:C ratio.

(A) Expression of the zygotic genes *nanor, apoeb,* and *lrat* was monitored by RT-PCR at the times indicated, with or without Chk1-4E-GFP expression. α-amanitin injected embryos serve as a transcriptionally silent post-MBT control. *β-actin* is the loading control. (B) Embryos with our without Chk1-4E-GFP expression were fixed and stained for phosphorylated RNA Polymerase II (pRpb1) and DNA at the indicated times. pRpb1 staining was quantified and averaged over multiple embryos ($n \geq 10$). Error bars indicate s.e.m., $*p<0.05$. (C, D) Control and Chk1-4E-GFP embryos were fixed and stained for DNA and pRpb1 at 15-minute intervals between 2.25 and 3.5 HPF. Representative images show nuclei density at the first and last timepoints (C), and the plot shows average nuclei density ($n \geq 37$) at each timepoint, pooled from four independent experiments. Chk1-4E-GFP embryos have fewer nuclei as a result of elongated cell cycles. pRpb1 staining was calculated for embryos grouped by nuclei density (D). Representative images are shown for each group, and the plot shows average pRpb1 staining intensity for each group, calculated as a fraction of the ≥60 control group ($n \geq 9$ for Chk14E with >50 nuclei, $n \geq 13$ for all other groups). Error bars indicate s.e.m. All scale bars 20 μm.

Figure 2.4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DNA
pRbp1
untreated
α-amanitin
pRbp1 signal intensity, a.u.
30
20
10
0
*
No Tx
α-aman
B
α-amanitin - - + - +
HPF 2 3.5 3.5 4 4
nanor
lrat
β-actin
C
untreated
HU
α-amanitin
HU + α-aman
DNA
pH3
untreated
α-aman
HU
α-aman + HU
0 5 10 15 20 25
% mitotic cells

Figure 2.4 DNA damage checkpoint acquisition at the MBT does not depend on zygotic transcription (A) 1-cell stage embryos were injected with α-amanitin, incubated until ~3.75 HPF, then fixed and stained for pRpb1 and DNA. pRpb1 staining intensity was quantified and averaged over multiple embryos (n≥9). (B) Expression of the zygotic genes *nanor* and *lrat* was monitored by RT-PCR at the times indicated. *B-actin* is the loading control. (C) 1-cell stage embryos were injected with α-amanitin, treated with 250 mM HU at 3.25 HPF for 45 min, fixed at 4 HPF, and stained for pH3 and DNA. The % of nuclei positive for pH3 was calculated and averaged over multiple embryos (n≥21).

Figure 2.5

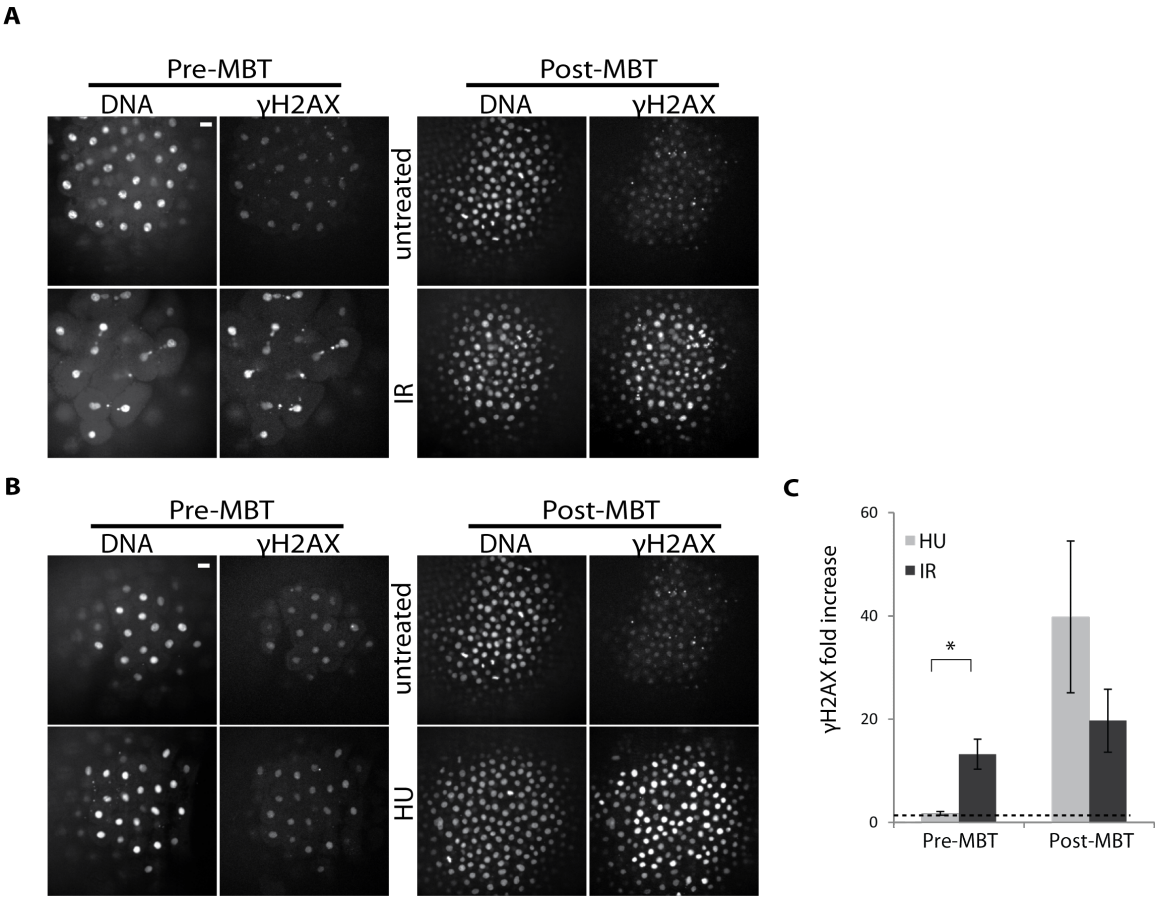
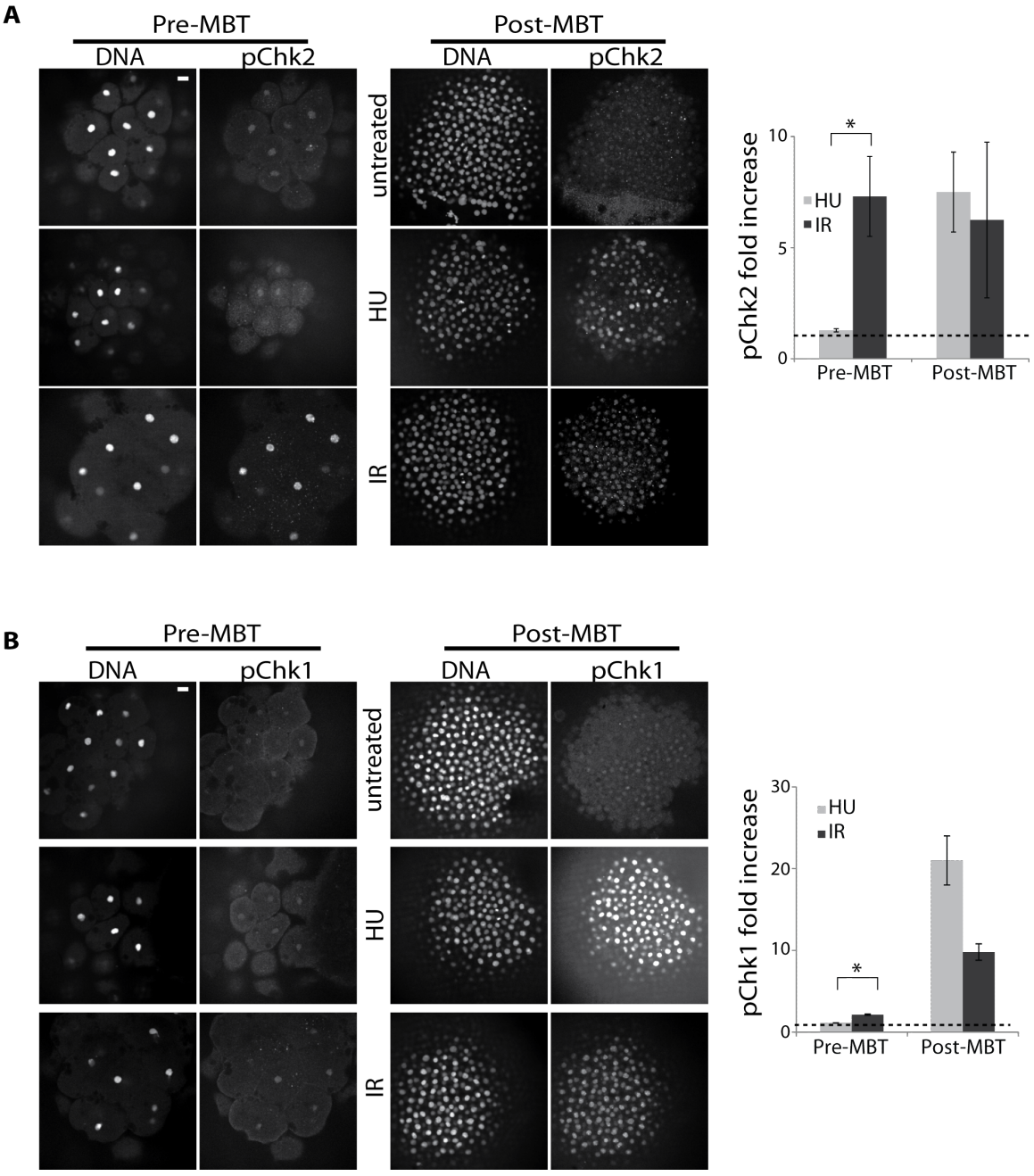

A
Pre-MBT
Post-MBT
DNA
γH2AX
DNA
γH2AX
untreated
IR
B
Pre-MBT
Post-MBT
DNA
γH2AX
DNA
γH2AX
untreated
HU
C
γH2AX fold increase
60
40
20
0
HU
IR
*
Pre-MBT
Post-MBT

Figure 2.5 Pre-MBT embryos can detect DNA damage caused by ionizing radiation and initiate a DNA damage response Pre-MBT (1.5 HPF) or post-MBT (4 HPF) embryos were treated with 10 Gy IR (A) or with 250 mM HU for 45 min (B), then fixed and stained for γH2AX and DNA. Scale bars 20 μm. Arrowheads show examples of nuclear fragmentation and lagging chromosomes from DNA damage. (C) γH2AX staining was quantified and averaged over multiple embryos ($n \geq 13$) Fold change indicates treated over untreated signal intensity for each timepoint and DNA damaging treatment. Error bars indicate s.e.m., $*p<0.05$. The dashed line represents no increase over untreated control.

Figure 2.6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Pre-MBT
Post-MBT
DNA
pChk2
DNA
pChk2
untreated
HU
IR
pChk2 fold increase
10
5
0
*
HU
IR
Pre-MBT
Post-MBT
B
Pre-MBT
Post-MBT
DNA
pChk1
DNA
pChk1
untreated
HU
IR
pChk1 fold increase
30
20
10
0
*
HU
IR
Pre-MBT
Post-MBT

Figure 2.6 Pre-MBT embryos can activate ATM-Chk2 but not ATR-Chk1 Pre-MBT (1.5 HPF) or post-MBT (4 HPF) embryos were treated with 10 Gy IR or with 250 mM HU for 45 min, then fixed and stained for pChk2 (A) or pChk1 (B) and DNA. pChk1 and pChk2 staining were quantified and averaged over multiple embryos ($n \geq 8$). Fold change indicates treated over untreated signal intensity for each timepoint and DNA damaging treatment. Error bars indicate s.e.m., $*p<0.05$, scale bars 20 μm. The dashed line represents no increase over untreated control.

Figure 2.7

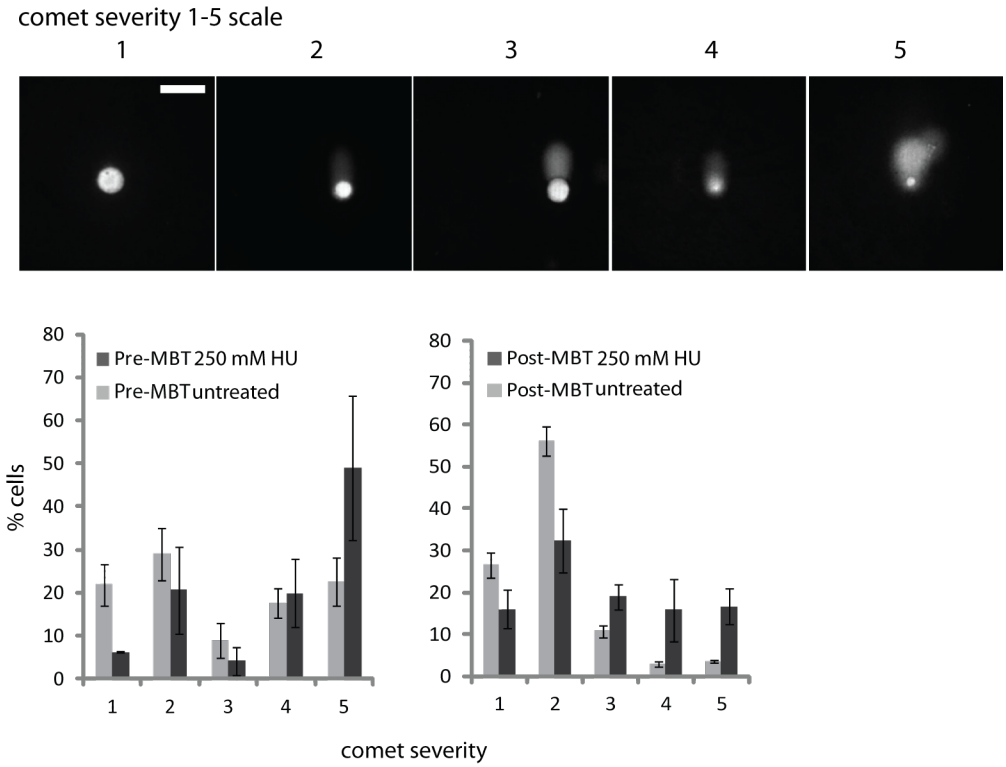

comet severity 1-5 scale
1
2
3
4
5
Pre-MBT 250 mM HU
Pre-MBT untreated
Post-MBT 250 mM HU
Post-MBT untreated
% cells
comet severity

Figure 2.7 Comet assay to assess DNA damage after treatment with HU Comet assay for DNA damage after treatment with HU. Images demonstrate DNA damage severity on a scale of 1-5 based on comet appearance; graphs show frequencies of comets for each condition ($n \geq 150$). High levels of replication and replication fork "bubbles" in pre-MBT cells impede DNA mobility resulting in the appearance of more severe comets (Olive and Banáth, 1993) in pre-MBT, untreated conditions. Error bars indicate s.e.m.,*$p<0.05$, scale bars 20 μm.

Figure 2.8

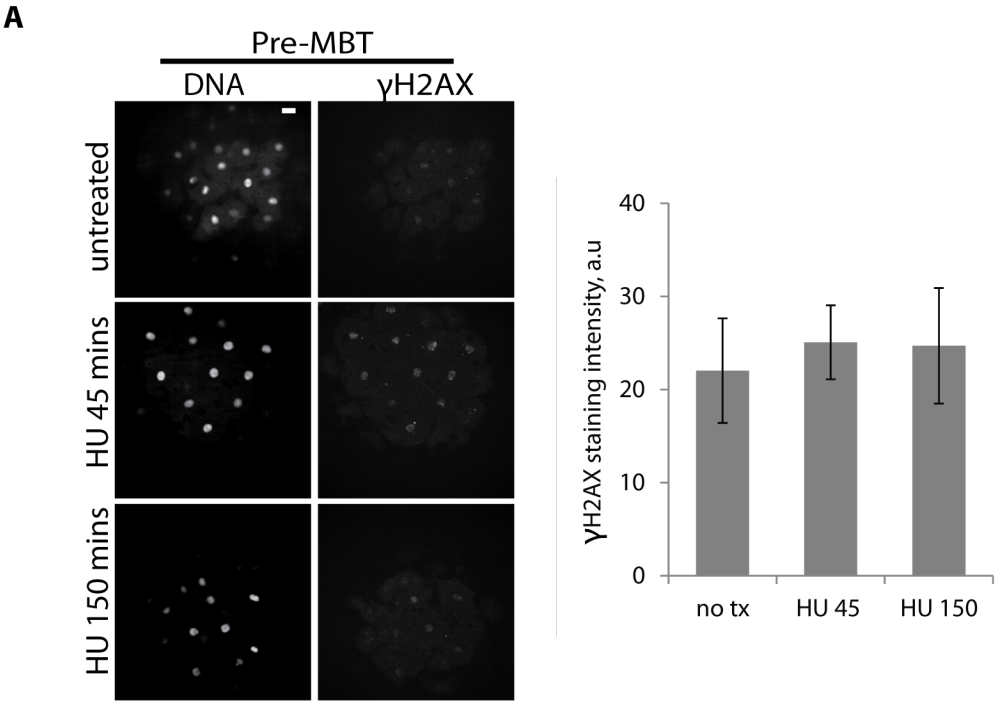

A
Pre-MBT
DNA
γH2AX
untreated
HU 45 mins
HU 150 mins
γH2AX staining intensity, a.u
40
30
20
10
0
no tx
HU 45
HU 150
B
WT
+ Chk1 MO
zChk1
1 dpf, 75 ug per lane
4 HPF
2.75 HPF
zChk1
70 ug per lane

Figure 2.8 Supplemental figure relating to figures 2.4 and 2.5 (A) Embryos were treated with 250 mM HU starting at 1 HPF for either 45 minutes or 1.5 hours, then fixed and stained for γH2AX and DNA. γH2AX staining was quantified and averaged over multiple embryos (n≥6). Error bars indicate s.e.m., scale bar 20 μm. (B) Western blots for Chk1 using zebrafish-specific anti-Chk1 antibody. Top blot: zChk1 polyclonal antibody was validated first in 1 day post fertilization (DPF) embryos. Lanes show 75 ug of total protein from control embryos and 75 ug of total protein from embryos injected with a Chk1 morpholino to confirm the correct band. Bottom blot for zChk1 is in 4 and 2.75 HPF embryos, with 70 ug of total protein loaded into each lane.

CHAPTER 3

Spindle assembly checkpoint acquisition at the mid-blastula transition

This work has been submitted for publication

ABSTRACT

The spindle assembly checkpoint (SAC) maintains the fidelity of chromosome segregation during mitosis. Nonpathogenic cells lacking the SAC are typically only found in cleavage stage metazoan embryos, which do not acquire functional checkpoints until the mid-blastula transition (MBT). It is unclear how proper SAC function is acquired at the MBT, though several models exist. First, SAC acquisition could rely on transcriptional activity, which increases dramatically at the MBT. Embryogenesis prior to the MBT relies primarily on maternally loaded transcripts, and if SAC signaling components are not maternally supplied, the SAC would depend on zygotic transcription at the MBT. Second, checkpoint acquisition could depend on Chk1, which is activated at the MBT to elongate cell cycles and is required for the SAC in somatic cells. Third, SAC function could depend on a threshold nuclear to cytoplasmic (N:C) ratio, which increases during pre-MBT cleavage cycles and dictates several MBT events like zygotic transcription and cell cycle remodeling. Finally, the SAC could by regulated by a timer mechanism that coincides with other MBT events but is independent of them. Using zebrafish embryos we show that SAC acquisition at the MBT is independent of zygotic transcription, cell cycle lengthening, and Chk1 activity, indicating that the checkpoint program is maternally supplied. Furthermore, we show that SAC acquisition can be uncoupled from the nuclear to cytoplasmic (N:C) ratio. Together, our findings indicate that a maternally programmed, developmental timer regulates SAC acquisition.

INTRODUCTION

The spindle assembly checkpoint (SAC) ensures that sister chromatids are correctly attached to spindle microtubules before anaphase onset. In the presence of unattached kinetochores, the SAC is active and inhibits the anaphase promoting complex/cyclosome (Lara-Gonzalez et al., 2012). This checkpoint maintains genomic integrity by preventing chromosome segregation errors.

Intriguingly, newly fertilized embryos of most metazoans, like fish, flies and frogs, lack SAC function. Immediately following fertilization, *Xenopus* embryos undergo metasynchronous cleavage divisions that cause surface contraction waves on the embryo, which are easily visualized. When spindle assembly is inhibited by microtubule depolymerizing agents like colchicine and vinblastine, embryos continue to have periodic surface contraction waves, indicating that cell cycle progression is not affected (Hara et al., 1980; Kimelman et al., 1987). Furthermore, Maturation Promoting Factor (MPF) activity continues to oscillate in embryos after microtubule depolymerization, providing further evidence for lack of a spindle checkpoint (Gerhart et al., 1984).

In *Xenopus* and zebrafish, cell cycles elongate dramatically and are extensively remodeled at the mid-blastula transition (MBT): rather than the rapid replication-mitosis cycles typical of cleavage divisions, cells acquire gap phases (Kane and Kimmel, 1993) and cell cycle checkpoints. When treated with DNA damaging agents or spindle poisons, post-MBT embryos arrest their cell cycles similarly to somatic cells (Zhang et al. manuscript submitted; Ikegami et al. 1997a; Ikegami et al. 1997b). Furthermore, the MBT marks a period of robust transcriptional activity, when developmental control switches from maternal to zygotic (Newport & Kirschner 1982a; Newport & Kirschner

1982b; Clute & Masui 1992). The simultaneous appearance of multiple changes at the MBT makes it difficult to determine which may control SAC acquisition, and we considered several possible models.

First, SAC function at the MBT could be under either maternal or zygotic control. In oviparous organisms where embryogenesis occurs outside the mother, embryos rely on maternal transcripts loaded during oogenesis to drive many early developmental events after fertilization. Therefore, the SAC may not function in pre-MBT embryos simply because checkpoint components are not maternally supplied and depend on zygotic transcription, which is induced robustly at the MBT.

Second, Chk1 kinase activity could promote SAC function at the MBT. Chk1 kinase is a regulator of cell cycle progression that is well known for its role in the DNA damage checkpoint. Upon activation after DNA damage, Chk1 phosphorylates multiple substrates to promote cell cycle delay (Bartek and Lukas, 2003). However, Chk1 also plays an important role at the MBT. In *Xenopus,* Chk1 is transiently activated at the MBT and targets Cdc25 phosphatase for degradation, inducing cell cycle elongation (Shimuta et al., 2002). Moreover*, Drosophila* embryos with a mutation in *grapes*, the Chk1 homolog, do not lengthen their cell cycles at the MBT, undergoing two additional syncytial pre-MBT-like divisions (Sibon et al., 1997). Chk1 is also required for the SAC in somatic cells (Carrassa et al., 2009; Petsalaki et al., 2011; Zachos et al., 2007), which suggests that Chk1 activation at the MBT could lead to SAC acquisition.

Third, many MBT events are governed by the nuclear-to-cytoplasmic ratio (N:C ratio). Because cleavage-stage embryos divide without cell growth, cell volumes halve at each division until a threshold N:C ratio is achieved at the MBT (Newport and Kirschner,

1982b; Edgar and Schubiger, 1986). Several MBT events, such as transcriptional activation and DNA damage checkpoint acquisition, occur prematurely if the N:C ratio is precociously increased in embryos (Newport and Kirschner, 1982b; Pritchard and Schubiger, 1996).

Alternatively, SAC function could be regulated by a cell cycle-independent timer mechanism, uncoupled from the N:C ratio, that begins at fertilization or egg activation. Several MBT events seem to be controlled temporally (Tadros and Lipshitz, 2009). For example, degradation of cyclins A and E1 in *Xenopus* embryos contributes to cell cycle lengthening at the MBT and is independent of the N:C ratio and zygotic transcription (Howe and Newport, 1996, 1995).

Using zebrafish embryos, which are easily manipulated and amenable to fixed and live cell imaging, we investigated the influence of large-scale changes that occur at the MBT on SAC acquisition. We demonstrate that the SAC does not rely on transcriptional activity. We also show that Chk1 activity and cell cycle elongation before the MBT are not sufficient for precocious checkpoint function, and that SAC acquisition does not depend on a threshold N:C ratio. We conclude that while occurring concomitantly with cell cycle remodeling and an increase in zygotic transcription, SAC function is independently regulated by a developmental timer.

RESULTS

Pre-MBT Zebrafish embryos do not delay mitosis after microtubule disruption

The SAC delays mitosis in response to microtubule disruption, which we used to test for SAC function in early embryos. To determine the time in mitosis, we used a live-cell

imaging assay to monitor nuclear localization of Proliferating cell nuclear antigen (PCNA) during the cleavage cycles in zebrafish embryos. PCNA is a replication factor that localizes to the nucleus soon after anaphase, when the nuclear envelope is reformed. It remains in the nucleus throughout S-phase, and then disperses into the cytoplasm when the nuclear envelope breaks down during prometaphase (Kisielewska et al., 2005). We injected GFP-tagged PCNA protein into 1-cell stage embryos and measured the times of nuclear envelope breakdown and reformation for successive cell cycles (Fig. 3.1A). Embryos were co-injected with fluorescently labeled Histone H1 protein to serve as a chromatin marker during M-phase when the PCNA-GFP is diffuse throughout the cytoplasm.

To test for a SAC response, pre-MBT embryos were treated with nocodazole. Upon drug treatment, cells formed compact nuclei and were unable to separate their chromosomes or complete cytokinesis (Fig. 3.1A). Despite these catastrophic failures, PCNA-GFP continued to localize to and disperse from nuclei, indicating nuclear envelope breakdown and reformation. Importantly, the total cell cycle time (data not shown) and length of mitosis were unchanged (Fig. 3.1A), demonstrating lack of a SAC in cleavage-stage embryos, consistent with previous findings (Gerhart et al., 1984; Hara et al., 1980; Kimelman et al., 1987).

SAC acquisition at the MBT is a maternal program

To distinguish whether SAC acquisition is governed by a maternal or zygotic program, we inhibited transcriptional activity by injecting one-cell stage embryos with α-amanitin, an inhibitor of RNA polymerase II (Dalle Nogare et al. 2007; Meinecke, B. and

Meinecke-Tillmann 1993), which inhibits transcription until 4 HPF (hours post fertilization) (Zhang et al., manuscript submitted). To test for a mitotic checkpoint response in the absence of zygotic transcription, embryos were injected with α-amanitin at the 1-cell stage, then treated with nocodazole at 3.25 HPF, when the MBT has already occurred and the SAC is functional in control embryos.

Cell density and motility and cell cycle asynchrony limit our ability to measure mitotic timing live in post-MBT embryos. Instead, to assay for SAC function after nocodazole treatment, embryos were fixed and stained for phosphorylated Serine 10 on Histone H3 (pH3), a well-established marker for mitosis (Paulson and Taylor, 1982). The accumulation of pH3-positive cells serves as a readout for cells arrested in mitosis. The mitotic index increased in control post-MBT embryos treated with nocodazole, indicating a functional SAC (Fig. 3.2). Inhibition of zygotic transcription with α-amanitin did not change the mitotic index of post-MBT embryos and did not affect the cell cycle response to nocodazole after the MBT: cells still accumulated in mitosis after nocodazole treatment (Fig. 3.2). These data demonstrate that transcription is unnecessary for SAC acquisition at the MBT. Rather, SAC components are maternally supplied but are not functional until the MBT.

Precocious Chk1 activity and cell cycle elongation do not lead to premature SAC function

Given the activation of Chk1 at the MBT and its role in the SAC, we hypothesized that Chk1 activity could control SAC acquisition at the MBT. To test the effect of precocious Chk1 activity on SAC function prior to the MBT, embryos were injected with an mRNA encoding an active, phosphomimetic Chk1 mutant (Chk1-4E) (Katsuragi et al. 2004;

Zhang et al. manuscript submitted), together with fluorescently labeled PCNA and Histone H1 proteins. Total cell cycle times and time spent in mitosis were then measured live with our PCNA-GFP localization assay. Exogenous Chk1-4E progressively lengthens cell cycles in pre-MBT embryos starting from the 5-6th cleavage cycle at ~2 HPF (Fig. 3.3A). When pre-MBT embryos expressing Chk1-4E are treated with nocodazole at 2.25 HPF, PCNA-GFP nuclear localization and dispersion is unperturbed, and there is no increase in the duration of mitosis (Fig. 3.3B). These data demonstrate that precocious Chk1 activity and cell cycle elongation are not sufficient to mount a proper SAC response prior to the MBT.

SAC acquisition depends on a developmental timer

The N:C ratio increases more slowly in Chk1-4E injected embryos compared to control embryos due to their elongated cell cycles, which allowed us to test whether SAC activation is coupled to the N:C ratio or to a developmental timer. Control embryos complete 10 cleavages at 3 HPF, which marks the onset of the MBT. However, embryos injected with Chk1-4E mRNA complete only 8 cleavages by 3 HPF (Fig. 3.4A). If SAC function requires that embryos obtain the threshold N:C ratio achieved after 10 cleavages, we would not expect SAC acquisition in Chk1-4E embryos until they reach this threshold N:C ratio. In contrast, if SAC acquisition is controlled by a developmental timer that is independent of the N:C ratio, we expect SAC function in Chk1-4E embryos at a lower N:C ratio, at ~ 3 HPF as in control embryos.

To distinguish between the timer and N:C ratio models, we compared control embryos at 2.75 HPF, before the MBT, to Chk1-4E embryos just past 3 HPF. To compare

N:C ratios, we measured the nuclei density in Chk1-4E and control embryos. The nuclei density in Chk1-4E embryos at 3.1 HPF is much lower than the nuclei density in control embryos at 2.75 HPF (Fig. 3.4B, and compare nuclei densities in Fig. 3.1 vs Fig. 3.4). Whereas control embryos at 2.75 HPF lack SAC function (Fig. 3.1), Chk1-4E embryos at 3.1 HPF spend significantly longer in mitosis (16.3 vs. 10.7 min) after nocodazole treatment (Fig. 3.4B), indicating that the SAC is functional. Thus, Chk1-4E embryos have a functional SAC at 3.1 HPF, at an N:C ratio that is lower than that of control pre-MBT embryos at 2.75 HPF, which do not have a SAC. These data indicate that SAC function depends on a developmental timer rather than a threshold N:C ratio.

DISCUSSION

Our findings provide insight into how the early embryo acquires SAC function. Specifically, we investigated whether SAC acquisition is coordinated with other major developmental changes during the MBT, including cell cycle elongation and Chk1 activity, a threshold N:C ratio, and zygotic transcription. Despite the coincident appearance of these events in the embryo, we show that SAC function can be uncoupled from other MBT events.

We first investigated whether SAC acquisition is coupled with transcriptional activity, as past studies have suggested that zygotic transcription and certain aspects of cell cycle remodeling are coordinated. For example, addition of the G2 phase of the cell cycle during MBT cell cycle remodeling in zebrafish relies on zygotic transcription (Nogare et al., 2009). Conversely, cell cycle elongation may be required for zygotic transcription, as transcripts are often aborted in rapid cell cycles in *Drosophila* due to

time constraints (Shermoen and Farrell, 1991). Despite the clear co-regulation of cell cycle remodeling and transcriptional activity at the MBT, we show that zygotic transcription is not required for SAC acquisition, implying that SAC components are maternally loaded. Similarly, our previous work shows that DNA damage checkpoint acquisition occurs independently of transcriptional activity (Zhang et al., manuscript submitted).

Our data are consistent with similar findings in *Xenopus*, which showed that blocking transcription after the 8-cell stage in dissociated blastomeres does not prevent SAC acquisition (Clute and Masui, 1995). However, gene expression profiling has revealed that many zygotic genes are expressed during the cleavage stages, some as early as the 4-cell stage (Tan et al., 2012). Thus, the previous experiments did not fully account for possible early zygotic transcription of SAC components, which may provide a sufficient pool of mRNA for SAC protein synthesis and accumulation. In contrast, we inhibited transcription immediately after fertilization, at the 1-cell stage, ruling out the possibility of a zygotic contribution of SAC components.

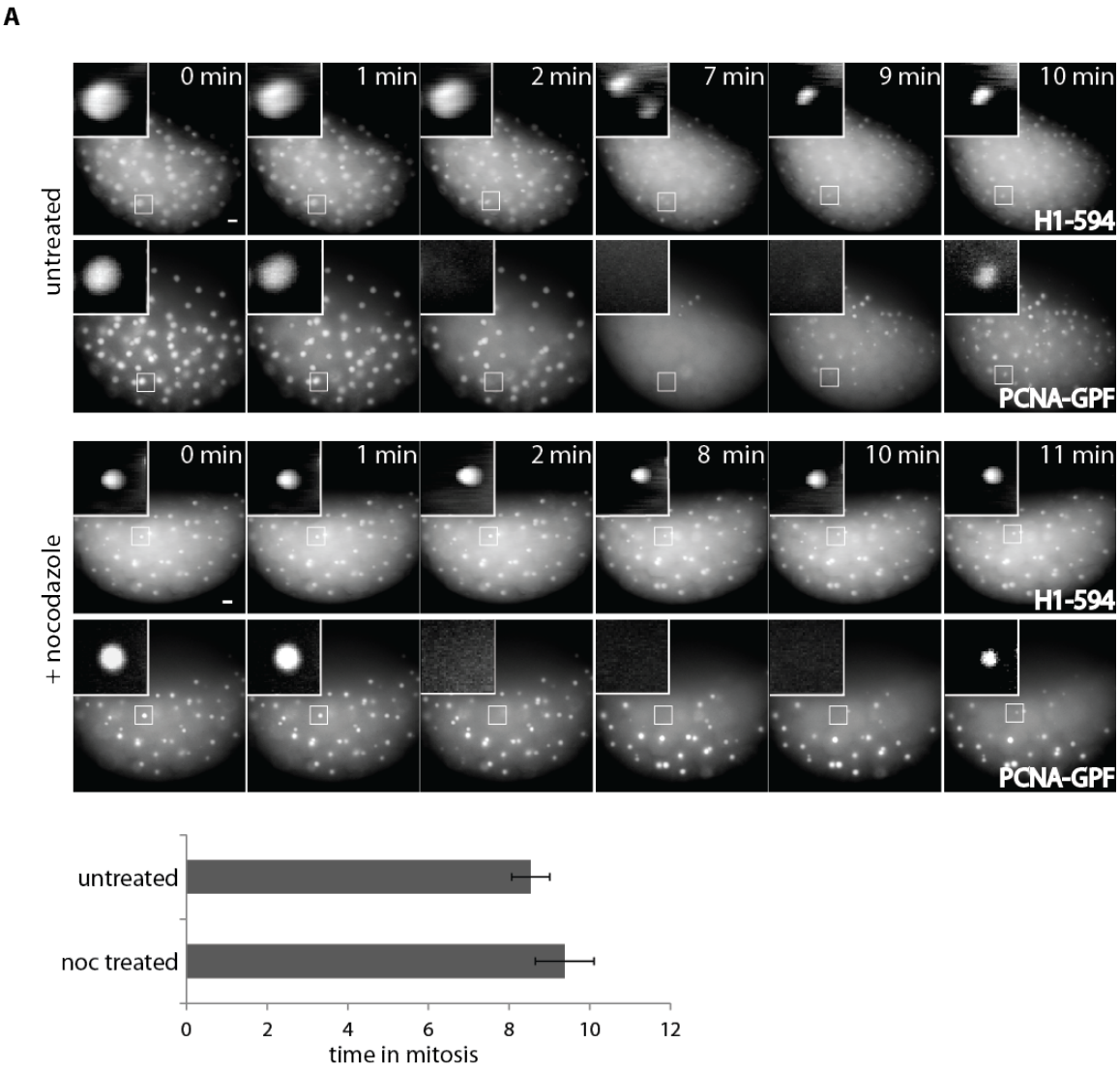
We also investigated the role of the N:C ratio in SAC acquisition. The coordination of many MBT events seems to stem from the N:C ratio, which increases with every cleavage cycle. For example, the replication factors which account for the fast S-phase in pre-MBT embryos are titrated as the N:C ratio increases, leading to slowed replication at the MBT and increased interphase duration (Collart et al., 2013). Additionally, the N:C ratio can affect DNA damage checkpoint acquisition: addition of exogenous DNA to pre-MBT Xenopus embryos, to mimic the N:C ratio typical of the

MBT, can lead to precocious checkpoint function after DNA damage (Conn et al., 2004; Peng et al., 2007).

By precociously increasing cell cycle lengths in pre-MBT embryos with Chk1, and therefore slowing the N:C ratio increase, we show that SAC acquisition does not depend on a threshold N:C ratio. This result is consistent with previous experiments which showed that individual blastomeres isolated from dissociated *Xenopus* embryos acquire a functional SAC with varying N:C ratios (Clute and Masui, 1995, 1997). Our results indicate that SAC acquisition is regulated by a timer mechanism that does not rely on Chk1 activity or zygotic transcription.

Our findings raise the question of how a maternally-controlled developmental timer regulates SAC acquisition at the MBT. For example, time could be required for either accumulation of SAC proteins from maternally supplied transcripts or degradation of a SAC inhibitor. Future work is required to determine the molecular differences between checkpoint signaling before and after the MBT and to elucidate the molecular basis for the developmental timer.

Figure 3.1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0 min
1 min
2 min
7 min
9 min
10 min
H1-594
PCNA-GPF
+ nocodazole
0 min
1 min
2 min
8 min
10 min
11 min
H1-594
PCNA-GPF
untreated
noc treated
0
2
4
6
8
10
12
time in mitosis

Figure 3.1 Pre-MBT embryos lack a functional spindle assembly checkpoint

Embryos were injected with Alexa 594-Histone H1 and PCNA-GFP proteins, treated with or without nocodazole before the MBT at 2.75 HPF, then imaged live. Images show cell cycle progression based on Histone H1 and PCNA. Insets are displayed with higher contrast settings to show the changing morphology of a single nucleus at different cell cycle stages: I, interphase; M, prometaphase/metaphase; A, anaphase. In the montage, time between metaphase and the next interphase is 8 min for untreated control embryos and 9 min for nocodazole-treated embryos. Graph shows average length of mitosis for each condition ($n \geq 14$ embryos for each, from three independent experiments). Error bars indicate S.D.; $P > 0.05$; scale bars 20 μm.

Figure 3.2

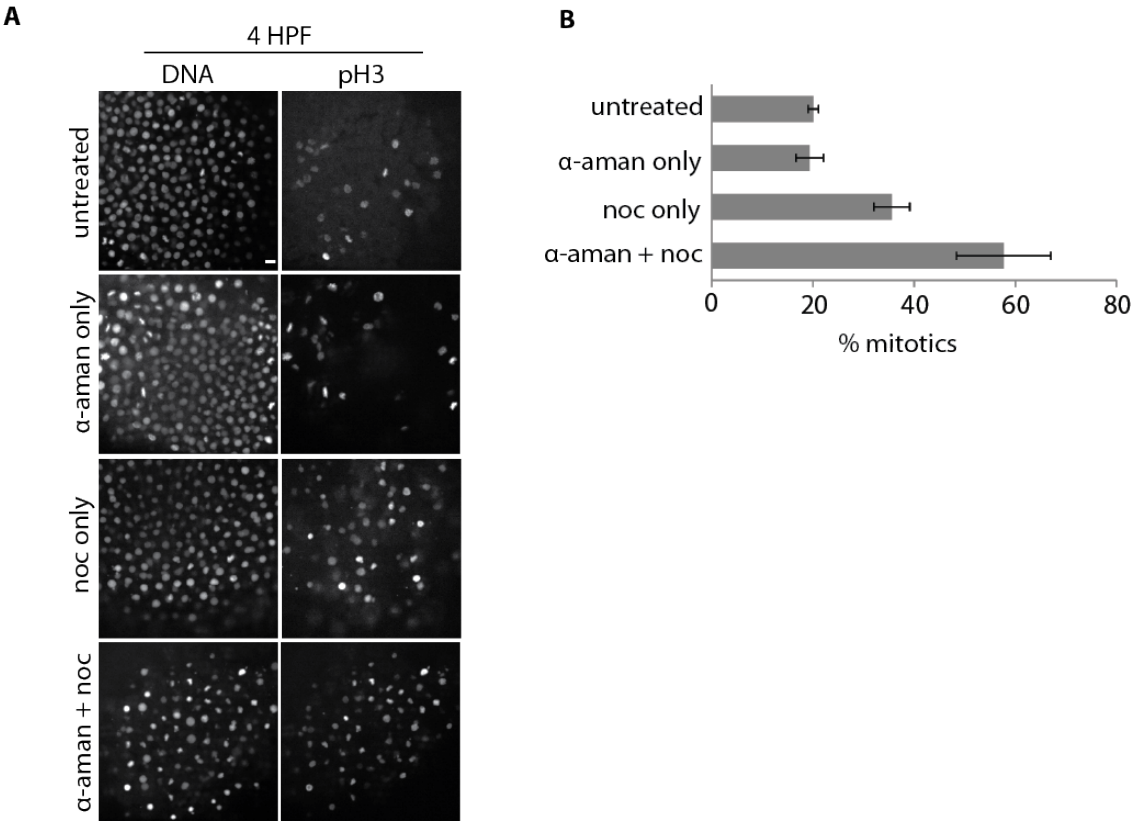
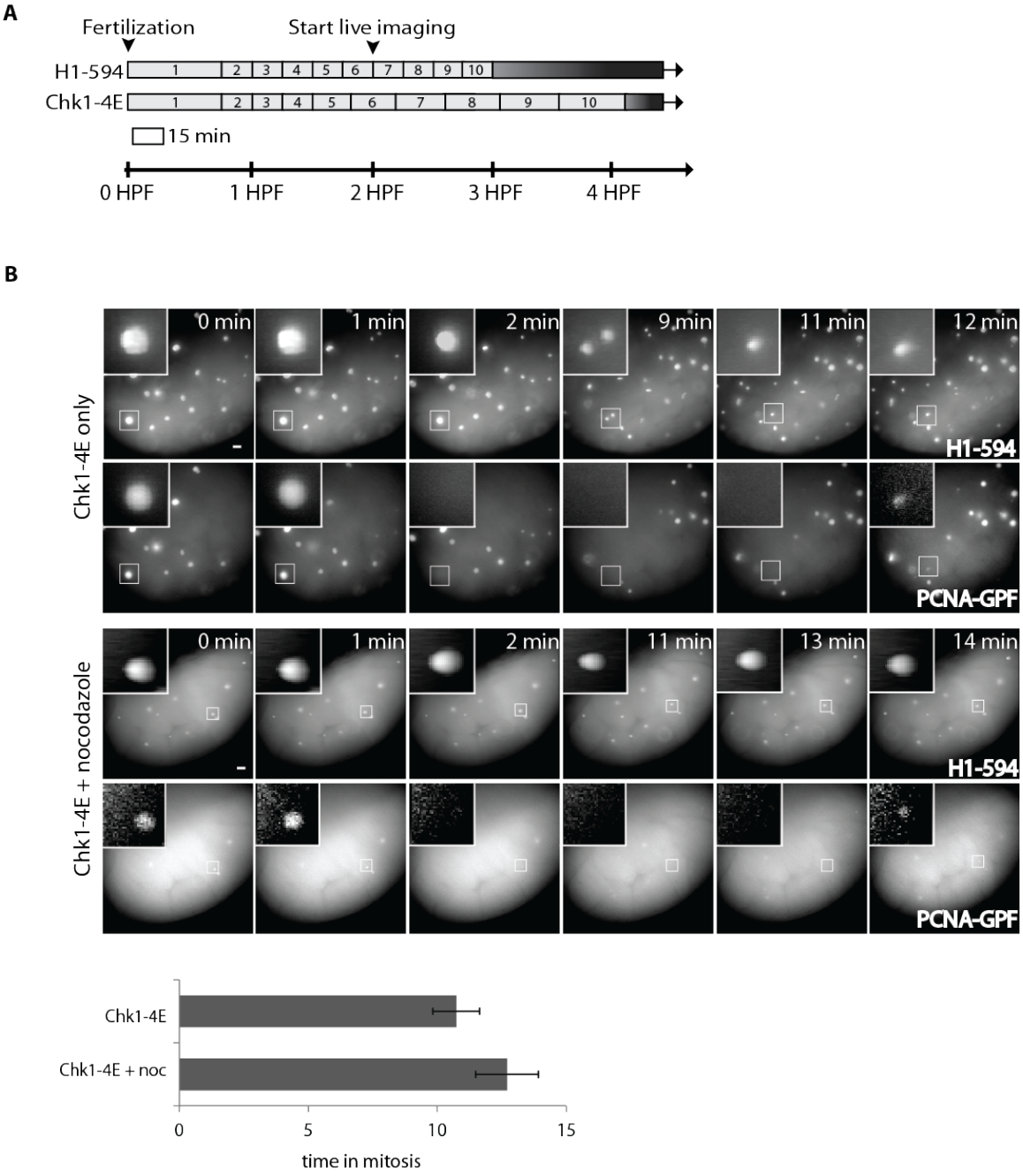


Figure 3.2 SAC acquisition does not rely on zygotic transcription 1-cell stage embryos were injected with α-amanitin as indicated, treated with or without nocodazole at 3.25 HPF for 45 min, fixed at 4 HPF, and stained for pH3 and DNA. The percent of nuclei positive for pH3 was calculated and averaged over multiple embryos (n≥18 for each condition, pooled from three independent experiments). Error bars indicate s.e.m.; scale bar 20 μm.

Figure 3.3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Start live imaging
H1-594
Chk1-4E
1
2
3
4
5
6
7
8
9
10
15 min
0 HPF
1 HPF
2 HPF
3 HPF
4 HPF
B
Chk1-4E only
0 min
1 min
2 min
9 min
11 min
12 min
H1-594
PCNA-GPF
Chk1-4E + nocodazole
0 min
1 min
2 min
11 min
13 min
14 min
H1-594
PCNA-GPF
Chk1-4E
Chk1-4E + noc
0
5
10
15
time in mitosis

Figure 3.3 Precocious Chk1 activity and cell cycle elongation are not sufficient for SAC acquisition (A) Schematic of cell cycle lengths for the first 10 cleavage divisions of embryos injected with Alexa 594-Histone H1, with or without Chk1-4E-GFP mRNA. Embryos were incubated until they reached the 5-6th cleavage, then imaged live. Cell cycles lengths were measured as time between metaphases, based on Histone H1 morphology. Embryos injected with H1-594 alone had consistent cleavage divisions that each lasted ~15 min, while embryos injected with Chk1-4E mRNA and H1-594 had cleavage cycles that progressively lengthened. (B) Embryos were injected with Alexa 594-Histone H1 and PCNA-GFP proteins and Chk1-4E mRNA, treated with our without nocodazole at 2.25 HPF, then imaged live during the pre-MBT, cleavage-stage cell divisions. Images show cell cycle progression based on Histone H1 and PCNA. Insets are displayed with higher contrast settings to show nuclear morphology as in Figure 1. In the montage, time between metaphase and the next interphase is 10 min without nocodazole and 12 min for nocodazole-treated embryos. Graph shows average length of mitosis for each condition ($n \geq 12$ for each condition, from three independent experiments). Error bars indicate S.D.; $P > 0.05$; scale bars 20 μm.

Figure 3.4

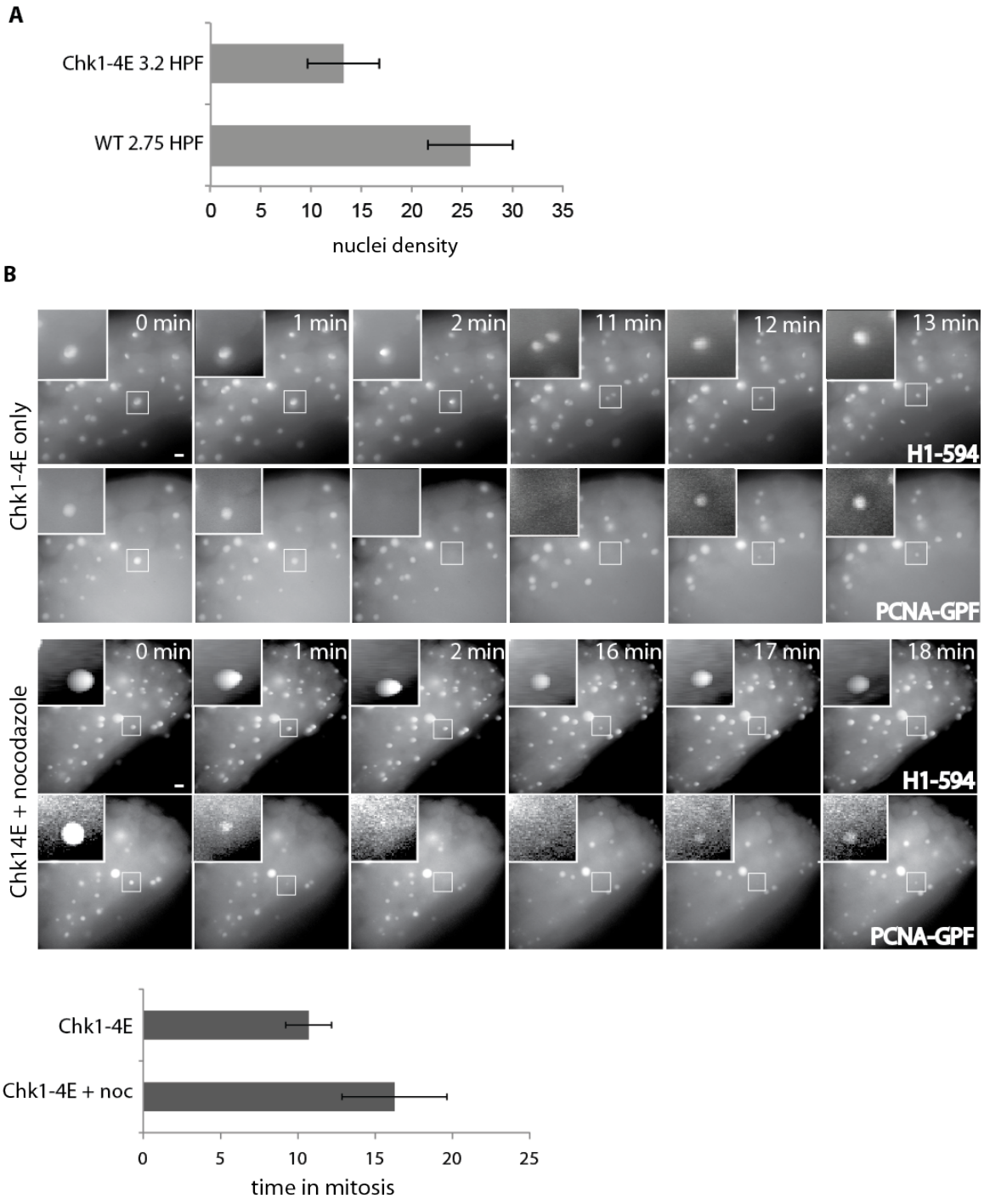
A
Chk1-4E 3.2 HPF
WT 2.75 HPF
0 5 10 15 20 25 30 35
nuclei density
B
Chk1-4E only
0 min
1 min
2 min
11 min
12 min
13 min
H1-594
PCNA-GPF
Chk14E + nocodazole
0 min
1 min
2 min
16 min
17 min
18 min
H1-594
PCNA-GPF
Chk1-4E
Chk1-4E + noc
0 5 10 15 20 25
time in mitosis

Figure 3.4 SAC acquisition is independent of the N:C ratio (A) Nuclei density was measured for embryos injected with Alexa 594-Histone H1 and PCNA-GFP proteins and Chk1-4E mRNA at 3.2 HPF, or embryos injected only with 594-H1 and PCNA-GFP proteins at 2.75 HPF. Error bars are S.D.; $n \geq 11$; $P \leq 0.001$.

(B) Embryos were injected with Alexa 594-Histone H1 and PCNA-GFP proteins and Chk1-4E mRNA, treated with or without nocodazole at 3.1 HPF, then imaged live. Images show cell cycle progression based on Histone H1 and PCNA-GFP. Insets are displayed with higher contrast settings to show nuclear morphology as in Figure 1. In the montage, time between metaphase and the next interphase is 10 min without nocodazole and 16 min for nocodazole-treated embryos. Graph shows average length of mitosis for each condition ($n \geq 13$ from four independent experiments). Error bars indicate S.D.; $P \leq 0.001$; scale bars 20 μm.

CHAPTER 4

Discussion and future directions

General conclusions and summary

In organisms where embryogenesis occurs outside the mother, survival requires swift embryogenesis that leads to motile, self-sufficient progeny. To achieve this, embryos employ specialized cell cycles that forgo high transcriptional activity, gap phases, and cell cycle checkpoints in favor of rapid cell proliferation. The purpose of this dissertation is to describe the regulation of the specialized cell cycles and zygotic transcription during early embryogenesis, with the emphasis on how regulation changes at the MBT. In Chapter 2, I describe the regulation of zygotic transcription in early embryogenesis and how embryos acquire the DNA damage checkpoint at the MBT. In Chapter 3, I show how SAC acquisition is regulated. The work presented here was performed in zebrafish, which are just beginning to be realized as a powerful model system in cell biology for its suitability in fixed and live single-cell imaging in an intact organism.

Regulation of zygotic transcription in early embryogenesis

In Chapter 2, I examined zygotic genome activation during early embryogenesis. Using RNA polymerase II phosphorylation as a readout for ZGA, I show that transcriptional activity increases gradually throughout the cleavage stages. My findings corroborate recent data from *Drosophila* and zebrafish, which show that zygotic transcripts appear during the cleavage stages (Aanes et al., 2011; Heyn et al., 2014; Lu et al., 2009). Similarly, zygotic transcripts have also been found prior to the MBT in *Xenopus* (Blythe et al., 2010; Skirkanich et al., 2011; Yang, 2002). Transcript detection assays are powerful because of their sensitivity, but are limited in their ability to view global

transcription on a single-cell basis with fine time precision. Thus, my data are a complement to and corroborate with the existing data.

To determine whether transcriptional activity increases with age (defined as time post fertilization) or the N:C ratio, we experimentally elongated pre-MBT cell cycles via premature activation of Chk1 so that the N:C ratio increases more slowly. The pattern of zygotic transcriptional activity shifted in parallel with the N:C ratio (Fig 2.3D). Therefore, ZGA is regulated by the N:C ratio rather than age. This result suggests that transcriptional activity relies on a factor that is titratable by DNA. As described in Chapters 1 and 2, one likely scenario is the presence of a DNA-binding, transcriptional repressor protein whose concentration stays constant but is redistributed across increasing amounts of DNA (Fig 2.1A). This transcriptional repressor could inhibit the transcriptional machinery directly, like the *Drosophila* protein Ttk (see Chapter 1). Alternatively, chromatin state could not be permissive to transcription. In *Xenopus*, depletion of the DNA methyltransferase xDnmt1 leads to precocious transcriptional activation (Stancheva and Meehan, 2000), making it a possible candidate of a titratable factor that can be influenced by the N:C ratio. It will be interesting for future work to explore whether these and other possible transcriptional repressors' activities can be regulated by the N:C ratio.

Though my work shows transcriptional activity increasing throughout the cleavage stages, previous work shows that the embryo experiences a large burst of transcriptional activity at the MBT, when cell cycles elongate (De Renzis et al., 2007). I show that premature cell cycle elongation does not cause more transcriptional activity than normal during the cleavage stages. However, cell cycle elongation may provide the

fully permissive environment for transcription of long genes, which are likely aborted during short cleavage divisions upon mitotic entry.

Initially, transcriptionally silent embryos were thought to suddenly undergo dramatic at ZGA at the MBT based on *Xenopus*, where newly synthesized RNA detected by incorporation of radiolabelled UTP only appears after the MBT (Newport and Kirschner, 1982a). However, as advances have been made in the detection of zygotic transcripts, it has become apparent that transcription of zygotic genes occurs well before the MBT in *Xenopus*, *Drosophila* and zebrafish (Skirkanich et al., 2011; Lu et al., 2009; Heyn et al., 2014). Our pRpb1 staining data (Fig 2.3D), together with these previous findings, suggest that zygotic transcriptional activity increases gradually throughout the cleavage stages, rather than suddenly at a particular N:C ratio.

In light of my data as well as others', I offer the following description of zygotic transcription: Immediately following fertilization, embryos are transcriptionally incompetent as a result of a chromatin-bound transcriptional repressor that occupies much of the DNA. With every subsequent round of replication, this transcriptional repressor is titrated more and more with increasing amounts of DNA. This is demonstrated by the appearance of short transcripts and increasing RNA Polymerase II activity (Heyn et al., 2014; Chapter 2). This continues until the end of the cleavage divisions, when the embryo is fully transcriptionally competent as a result of dilution of the transcriptional repressor. At this point, the MBT occurs and cell cycles are elongated. The fully transcriptionally competent embryo can now transcribe longer transcripts (and therefore activate a higher percentage of the zygotic genome), which would explain the spike in zygotic transcripts observed at the MBT.

Checkpoint function in early embryogenesis

Despite the presence of zygotic transcripts in early embryos, genetic materials driving cell cycle progression during the cleavage stages are maternally supplied. In Chapter 2, I examined whether the checkpoint program is also maternally supplied. Prior to this dissertation, Conn and colleagues showed that precocious checkpoint function could be induced by addition of DNA to increase the N:C ratio (Conn et al., 2004). This suggests that checkpoint proteins are maternally supplied, as no other components were added or altered to confer checkpoint function. However, addition of exogenous DNA also triggers precocious ZGA in *Xenopus* embryos and egg extracts (Newport and Kirschner, 1982a,b), making it feasible that precocious transcription occurred and elicited checkpoint response.

I show that inhibiting zygotic transcription throughout the cleavage divisions does not inhibit acquisition of either the spindle assembly or DNA damage checkpoints at the MBT. This demonstrates that checkpoint components are maternally supplied but cannot function during the cleavage stage. Additionally, transcription-inhibited embryos still elongate their cell cycles and lose cell cycle synchrony after the cleavage divisions. This is demonstrated by the diverse nuclear morphologies and mitotic index in α-amanitin treated embryos, which are indistinguishable from untreated control embryos (Fig 2.4c). Similarly, *Xenopus* embryos still elongate their cell cycles at the MBT after transcription inhibition (Clute and Masui, 1992). Our results and others' suggest that vertebrates like frogs and fish do not require ZGA for certain aspects of cell cycle remodeling at the MBT. *Drosophila* embryos, however, appear to rely more on zygotic transcription for cell cycle elongation at the MBT. Zygotic transcription is required for destruction of

Twine, a Cdc25 homolog, which is normally degraded at the MBT (Farrell and O'Farrell, 2013). Additionally, a *Drosophila* mutant that leads to precocious ZGA undergoes fewer cleavage divisions before cell cycle remodeling (Sung et al., 2013).

Given that checkpoint components are present in pre-MBT embryos, I next investigated why they are not functional. The molecular basis for cleavage divisions is well characterized, with high Cdk1 activity lying at the heart of rapid cell cycle progression. The lack of cell cycle arrest in pre-MBT after DNA damage indicates that Cdk1 is not properly inhibited. However, Cdk1 inhibition is one of the last steps in the checkpoint pathway, and it is unclear which other steps are dysfunctional in checkpoint pathways prior to the MBT. I tested function of two key steps in the checkpoint pathway: DNA damage detection/pathway initiation and effector kinase activation. These steps are upstream of cyclin/Cdk activity and are essential for Cdk1 inhibition. I found that pre-MBT embryos are competent at detecting DNA damage lesions and initiating the checkpoint response. Moreover, Chk2 is efficient phosphorylated after damage, demonstrating that the ATM-Chk2 arm of the DNA damage response is intact. However, Chk1 cannot be activated after damage, suggesting that Chk1 activation is the key element of checkpoint dysfunction prior to the MBT. As discussed in Chapter 3, previous work in *Drosophila* and *Xenopus* demonstrated that Chk1 activation at the MBT is important for cell cycle elongation, highlighting its role in a major MBT event. (Shimuta et al., 2002; Sibon et al., 1997) My data expands upon this by demonstrating that DNA damage checkpoint acquisition also relies heavily on Chk1 gain-of-function. Taken together, these data implicate Chk1 as the point of convergence for all aspects of cell cycle remodeling at the MBT.

Our findings are not mutually exclusive with earlier work which suggests that a critical N:C ratio is the determining factor for checkpoint acquisition. In fact, Chk1 is an ideal candidate for such a biochemical 'sensor' of N:C ratios. First, developmental activation of Chk1 at the MBT is triggered by replication delays that are thought to arise from the titration of replication factors by increasing amounts of DNA (Collart et al., 2013; Dasso and Newport, 1990; Newport and Dasso, 1989; Newport and Kirschner, 1982a; 1982b; Shimuta et al., 2002). These delays, which only arise from a specific concentration of DNA, trigger the full activation of the damage/replication checkpoint, including Chk1 activation.

Additionally, Chk1 gain-of-function could be caused by the availability of phosphorylated Claspin, an adaptor protein that is required for Chk1 activation (Kumagai and Dunphy, 2000; 2003; Chini and Chen, 2003). When phosphorylated, Claspin recruits Chk1 to ATR to promote Chk1 activation. In *Xenopus*, phospho-Claspin appears at the MBT (Gotoh et al., 2011). Claspin is usually phosphorylated by ATR after the replication checkpoint is activated. However, Gotoh and colleagues also showed that claspin phosphorylation occurred at the MBT even when ATR activity is inhibited with caffeine, suggesting that it can occur from ATR-dependent and independent mechanisms. Importantly, Claspin phosphorylation is sensitive to the N:C ratio, leading to the theory that a kinase inhibitor or phosphatase could also be titrated out with increasing DNA concentration. However, no specific candidates have been identified, and further work is required to understand the mechanism behind Chk1 activation at the MBT.

Alternatively, the lack of Chk1 activity suggests that the master kinase ATR may be nonfunctional prior to the MBT. When single-stranded breaks appear in somatic cells,

the protein RPA coats the ssDNA and recruits ATR/ATRIP to the site of the lesion, leading to ATR activation. However, it is likely this does not occur during cleavage cycles, as there was no increase in γH2AX signal after treatment with a replication inhibitor that normally triggers ATR activation (Fig. 2.5b).

Another outstanding question from my findings is why the ATM-Chk2 activation we observed in the early embryos after inducing damage is not sufficient to cause cell cycle delay in cleavage embryos. Like Chk1, Chk2 also mediates cell cycle arrest after its activation and has been shown to mediate Cdc25 destruction (Falck et al., 2001). In addition, Chk2 can also mediate cell cycle arrest by activating the transcription factor p53, an important tumor suppressor gene that promotes the expression of p21, which binds directly to cyclin/cdk complexes, inhibiting their kinase activity (Ahn et al., 2004). If the majority of Chk2's efficacy relies on p53 mediation, I speculate that the Chk2-p53-p21 axis may be non-functional in pre-MBT embryos since there is little capacity for transcriptional activity.

One caveat to this and most other studies of checkpoint function in embryogenesis relates to the semantics of what a 'properly functioning checkpoint' entails. Pre-MBT embryos clearly have no functional checkpoint, as cell cycles remain rapid and DNA damage is not successfully eliminated. However, cell cycle delay after checkpoint activation does not necessarily mean that genomic integrity is being maintained, as DNA damage or replication stress ultimately needs to be resolved. In eukaryotic cells, this often requires transcription of genes involved in DNA repair or apoptosis. Future work should address the *bona fide* fidelity of the DNA repair pathway in early embryogenesis.

Spindle assembly checkpoint acquisition at the MBT

My work also investigated the acquisition of the spindle assembly checkpoint, another signaling pathway found in most somatic cells that prevents cells from progressing to anaphase when there are unattached kinetochores. This checkpoint is active by default, and can only be turned off when all KT-MT attachments are made. Pre-MBT embryos do not have functioning SAC and attempt cell cycle progression even when catastrophic nuclear defects arise after treatment with spindle poisons like nocodazole or taxol.

My work demonstrated that, like the DNA damage checkpoint, components of the SAC are maternally provided, as inhibition of ZGA did not prevent SAC acquisition (Fig 8). Moreover, I find that the SAC is not acquired at a specific N:C ratio. Using the constitutively active Chk1 once again to increase cell cycle lengths during the cleavage stage and thus slow the rate of N:C ratio increase, I show that full SAC function appears at the same age in Chk1-4E injected embryos, even though they have not attained the MBT N:C ratio (Fig 9). This argues against the titration of any proteins that may be inhibiting SAC. Instead these results suggest that early embryos may require an absolute time in order to accumulate a protein that is required for activation of the SAC, which occurs by the MBT.

Although we find that a threshold N:C ratio is not necessary for SAC acquisition, the SAC can become functional prematurely in *Xenopus* egg extracts if sperm nuclei are added to increase the N:C ratio to a threshold level (Minshull et al., 1994). Together these findings suggest that both a developmental timer and increases in the N:C ratio can contribute to SAC regulation. Increasing N:C ratio may artificially titrate as-yet unidentified cytosolic SAC inhibitors that are present during the cleavage stages, or

increased numbers of kinetochores may enhance production of a SAC signal. During normal development, when the number of kinetochores is fixed, a set time may be required for the synthesis and accumulation of SAC proteins that amplify signaling downstream from initial SAC activation at kinetochores. However, the need for accumulation of these proteins could be bypassed if large numbers of kinetochores amplify SAC signaling. While biochemistry is difficult in zebrafish due to limited antibody availability, further work in *Xenopus* could determine SAC protein synthesis and regulation during the cleavage stages.

CHAPTER 5

Materials and methods

Fish husbandry

Embryos were collected from natural mating and incubated in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at 28˚C. All experiments were carried out in the Tuebingen long fin strain.

Cell cycle length measurements using PCNA-GFP

Recombinant protein was purified as described [18] from a GFP-fused human PCNA gene in pENeGFP-PCNA2 provided by Dr Michael Whitaker (Institute of Cell and Molecular Biosciences, University of Newcastle upon Tyne). Histone H1 from calf thymus (Sigma) was conjugated to Alexa-Fluor 594 (Invitrogen) or Alexa-Fluor 647 following the manufacturer's instructions. 1-cell stage embryos were injected with GFP-PCNA and AlexaFluor-tagged histone H1 and incubated in E3 buffer at 28°C until live imaging. For live imaging, embryos were dechorionated with 1 mg/mL pronase in E3 buffer for 10 min, washed 2x in E3 buffer, then mounted on a 4-well fluorodish (Grenier Bio-One) in 0.4% agarose dissolved in E3 buffer. For nocodazole-treated embryos, nocodazole was included in the E3 buffer to maintain the working concentration in the agarose. Images were acquired using a 20x 0.7 NA objective on an inverted fluorescence microscope (DM6000, Leica Microsystems) equipped with an automated XYZ stage and a charge-coupled device camera (Orca-AG, Hamamatsu Photonics), controlled by Metamorph Software (MDS Analytical Technologies). Embryos were imaged every minute by fluorescence. At each time point a z series of 10 images was collected at 10 µm intervals. Cell cycle lengths were measured by manually tracking shuttling of GFP-

PCNA into and out of nuclei defined by AlexaFluor-tagged histone H1, using Metamorph and ImageJ software.

Cell cycle length measurements using Histone H1-594

Histone H1 from calf thymus (Sigma) was conjugated to Alexa-Fluor 594 (Invitrogen) following the manufacturer's instructions. 1-cell stage embryos were injected with Alexa 594-histone H1 and incubated in E3 buffer at 28°C until live imaging. For live imaging, embryos were dechorionated with 1 mg/mL pronase in E3 buffer for 10 min, washed 2x in E3 buffer, then mounted on a 4-well fluorodish (Grenier Bio-One) in 0.4% agarose dissolved in E3 buffer.. Images were acquired using a 20x 0.7 NA objective on an inverted fluorescence microscope (DM6000, Leica Microsystems) equipped with an automated XYZ stage and a charge-coupled device camera (Orca-AG, Hamamatsu Photonics), controlled by Metamorph Software (MDS Analytical Technologies). Embryos were imaged every 1-2 minutes by fluorescence. At each time point a z series of 10 images was collected at 10 µm intervals. Max intensity projections are shown. Cell cycle lengths were measured by manually tracking nuclear morphology visualized by Alexa 594-histone H1, using ImageJ and Metamorph software. For cell cycle length measurements in embryos treated with IR, embryos were irradiated immediately before mounting and imaging.

DNA damage and embryo drug treatments

For pH3 staining, post-MBT embryos were treated with 250 mM HU dissolved in E3 buffer or 0.125 ug/uL nocodazole dissolved in DMSO at 3.25 HPF for 45 min, then fixed.

For γH2AX, pChk1 and pChk2 staining embryos were treated with 10 Gy ionizing radiation with a Gammacell 40 irradiator (Nordion International), using cesium-137 as the radiation source. Pre-MBT embryos either fixed immediately after irradiation or after 7 min as above, and interphase nuclei were analyzed. Post-MBT embryos were all fixed immediately after irradiation. To inhibit transcription, 2 nL of 1 mg/mL α-amanitin dissolved in ddH2O was injected into the cell of 1-cell stage embryos. Embryos were incubated in E3 buffer at 28˚C until drug treatment with HU or RNA extraction.

Reverse transcription-polymerase chain reaction

RNA was isolated using Trizol (Invitrogen) from embryos at 2, 3.5, 3.75 and 4 HPF following the manufacturer's instructions, using 50 embryos were used for each condition and timepoint. 3.75 HPF embryos were used for testing transcription activity in Chk1-4E embryos as transcripts did not appear until then. Single-stranded cDNA was synthesized from total RNA extracted, using the SuperScript III First Strand Synthesis System (Invitrogen) following the following the manufacturer's instructions, using random hexamer primers. *β-actin* was used as the loading control. Primers used for PCR reaction were as follows:

nanor:

Fwd: CAGCGAGCAGCGTTTACAGCGG

Rev: GAGGGAATCACCGCTCTGGTCTG

lrat:

Fwd: ACGGGTCCAATATTTTGCTG

Rev: AGRCATCCACAAGATGAAGG

apoeb:

Fwd: AGCAGAATGCAGATGACGTG

Rev: TCAGAGAGGTGCGTAGGTT

β-actin:

Fwd: ACGCTTCTGGTCGTACTA

Rev: GATCTTGATCTTCATGGT

Immunofluorescence

Embryos were fixed with 4% paraformaldehyde in PBST (PBS with 0.1% Tween-20) overnight at 4˚C, then manually dechorionated and dehydrated in 100% methanol overnight at -20 ˚C. Embryos were rehydrated the next day sequentially with 75%, 50% and then 25% methanol in PBST (5 min each), then permeabilized with 100% acetone at -20 ˚C for 7 min, then blocked with buffer containing 20% Heat-inactivated FBS, 20% Blocking reagent (Roche) and 1% DMSO in PBST for 1 hr at room temperature. Antibodies were diluted in blocking buffer, applied to embryos and incubated overnight at 4 ˚C. Embryos were then washed four times (30 min each) with PBST, incubated with secondary antibody in blocking buffer, washed another three times, stained for 5 min with SYTOX Green (Invitrogen), then washed once with PBST. Embryos were mounted on fluorodishes (World Precision) in 4% methylcellulose dissolved in E3 buffer.

Primary antibodies were: mouse monoclonal against phospho-Ser10 Histone H3 (1:1000, Millipore cat #05-806); rabbit polyclonal against RNA Polymerase II subunit B1 CTD phospho-Ser2/5 (pRpb1) (1:1000, Cell Signaling cat #4735); rabbit polyclonal anti-γH2AX (1:1000, gift from Dr. James Amatruda, University of Texas Southwestern);

rabbit monoclonal against human Chk1 phospho-Ser345 (1:500, Cell Signaling cat #2348); rabbit polyclonal against human Chk2 phospho-Thr68 (1:250, Cell Signaling cat #2661). Secondary antibodies were Alexa-Fluor 594-conjugated anti-mouse or anti-rabbit (1:200, Invitrogen).

All fixed embryos were imaged with a spinning disk confocal: a microscope (DM4000, Leica) with a 20x 0.7 NA objective or a 63x 1.3 NA glycerol objective, an XY piezo-z stage (Applied Scientific Instrumentation), a spinning disk (Yokogawa), an electron multiplier charge-coupled device camera (ImageEM, Hamamatsu Photonics), and an LMM5 laser merge module equipped with 488 and 593 nm lasers (Spectral Applied Research) controlled by Metamorph software. To quantify γH2AX, pRpb1, pChk1, or pChk2 staining, nuclei were defined based on DNA staining, and phospho-antibody staining intensity was averaged over all nuclei in each field after subtracting background based on cytoplasmic intensity using ImageJ software. % mitotic cells was quantified by calculating pH3 positive nuclei as a fraction of total nuclei (by DNA stain) in a field.

Nuclei density measurements

Control and Chk14E-injected embryos were fixed at 15-minute intervals between 2.25 and 3.5 HPF and stained with STYOX Green to label nuclei. Embryos were imaged as described above, and a z-series of 10 images was collected at 10 μm intervals for each embryo. We used ImageJ Object 3D Counter to count the number of nuclei within an isolated field measuring 157 x 157 μm across three z-slices for individual embryos.

mRNA constructs and injections

Wildtype zebrafish Chk1 cDNA was purchased from ATCC (Cat no. 5410666) and cloned into a GFP-pCS2+ mRNA expression vector. The constitutively active, phosphomimetic zChk1 (Katsuragi et al., 2004) was created by mutating four residues (S256E, S280E, T292E, S301E) in zebrafish Chk1 in a 492 basepair gBlock gene fragment (IDT). This fragment replaced residues 193-358 of wildtype zChk1 in GFP-pCS2+. A cDNA encoding the N-terminal kinase domain of Chk1 (amino acids 1-99) was amplified from wildtype zChk1 and cloned into GFP-pCS2+ to create ΔC-Chk1-GFP construct. Another cDNA encoding a C-terminal domain of Chk1 (amino acids 215-410) was amplified from wildtype zChk1 and cloned into GFP-pCS2+ to create the ΔN-Chk1-GFP construct. The Ambion mMessage mMachine SP6 in vitro transcription kit was used to make Chk1-GFP and Chk1-4E-GFP, ΔC-Chk1-GFP, and ΔN-Chk1-GFP mRNA. All constructs were injected into the cell of the 1-cell stage embryo. mRNAs were diluted in 0.2M KCl. Each embryo was injected with a 6.75 ng of mRNA. For live imaging, Histone H1 protein was also added to the injection mix. Expression of Chk1 constructs was monitored by GFP expression.

Morpholino injections

The morpholino oligonucleotide (MO) for Chk1 was synthesized by Gene Tools™ LLC, resuspended in sterile water at a concentration of 1mM and delivered into zebrafish embryos at the one-cell stage by microinjection. Chk1 MO sequence is: aggcacagccattatgcaatcttcg (Sidi et al., 2008). Working concentration of the MO was 0.75 mM

Chk1 Immunoblotting

Frozen embryo samples were thawed on ice, diluted in 6x Laemmli sample buffer and loaded on 4-20% Ready Gel Tris-HCl Gels (Bio-Rad). 70 ug of protein was loaded onto each lane. Samples were electrophoresed at 100 mA, transferred to Hybond ECL nitrocellulose membrane (GE Healthcare), and blocked for 1 hour in PBS plus 0.1% (v/v) Tween-20 and 3% Blocking Agent (ECL Advance; GE Healthcare) at 25°C. Zebrafish-specific anti-Chk1 antibody (60B253; Antagene; 1:500 dilution) was used to detect Chk1. Horseradish peroxidase-conjugated secondary antibody (Amershame Biosciences; 1:100,000 dilution) were detected with chemiluminescence (ECL Advance; Amersham Biosciences)

CHAPTER 6

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