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Yekaterina Brandt-(Webb)
Candidate

Cell Biology & Physiology
Department

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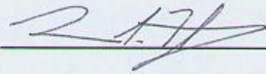
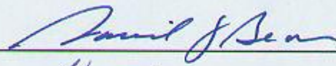
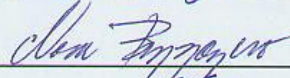
, Chairperson





by

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

The University of New Mexico
Albuquerque, New Mexico

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by

ABSTRACT OF DISSERTATION

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Regulation of Cyclin E stability in Xenopus laevis embryos

Yekaterina Brandt

M.D./ M.S. Biochemistry, Russian state Medical University 1997

Abstract

Cyclin-Cdk complexes positively regulate cell cycle progression. Cyclins are regulatory subunits that bind to and activate cyclin-dependent kinases or Cdks. Cyclin E associates with Cdk2 to mediate G1/S phase transition of the cell cycle. Cyclin E is overexpressed in breast, lung, skin, gastrointestinal, cervical, and ovarian cancers. Its overexpression correlates with poor patient prognosis and is involved in the etiology of breast cancer. We have been studying how this protein is downregulated during development in order to determine if these mechanisms are disrupted during tumorigenesis, leading to its overexpression. Using *Xenopus laevis* embryos as a model, we have shown previously that during the first 12 embryonic cell cycles Cyclin E levels remain constant yet Cdk2 activity oscillates twice per cell cycle. Cyclin E is abruptly destabilized by an undefined mechanism after the 12th cell cycle, which corresponds to the midblastula transition (MBT). Based on work our work and work by others, we have hypothesized that differential phosphorylation and a change in localization result in Cyclin E degradation by the 26S proteasome at the MBT. To test this, we generated a series of point mutations in conserved threonine/serine residues implicated in degradation of human Cyclin E. Using Western blot analysis, we show that similarly to human Cyclin E, mutation of

these residues to unphosphorylatable alanine stabilizes Cyclin E past the MBT when they are expressed *in vivo*. Cyclin E localization was studied by immunofluorescence analysis of endogenous and exogenous protein in pre-MBT, MBT, and post-MBT embryos. In addition, we developed a novel method of conjugating recombinant His_6-tagged Cyclin E to fluorescent (CdSe)ZnS nanoparticles (quantum dots) capped with dihydrolipoic acid. Confocal microscopy was used to visualize His_6Cyclin E-quantum dot complexes inside embryo cells in real time. We found that re-localization at the MBT from the cytoplasm to the nucleus precedes Cyclin E degradation. Mutations in phosphorylation sites did not abrogate nuclear accumulation of Cyclin E at the MBT. Inhibition of nuclear import and proteasome function resulted in accumulation of Cyclin E in the cytoplasm. We also show that Cyclin E is ubiquitinated at the MBT corresponding to its downregulation. These results suggest conservation of mechanism of degradation between human and Xenopus Cyclin E. In addition, our results show that the nuclear presence of Cyclin E is necessary for its downregulation at the MBT. Taken together with the fact that Cyclin E is often mislocalized to the cytoplasm in human cancers that overexpress it, our work suggests that a similar nuclear presence may be necessary for human Cyclin E degradation, and that this process could be disrupted during the process of tumorigenesis.

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Chapter 1. Introduction

The cell cycle

The cell cycle is the orderly sequence of events by which a cell duplicates its contents and divides into two. The basic cell cycle consists of two main phases, DNA synthesis or S phase, when duplication of the chromosomal DNA occurs; and mitosis or M phase, when the duplicated chromosomes are segregated into two daughter cells. There are also two gap phases, G1 and G2, which separate M and S phases and during which the cell grows and monitors the extra- and intracellular environment to ensure that conditions are appropriate before proceeding to the next phase (Fig. 1). When cells stop proliferating, either due to the lack of mitogenic stimuli or the presence of anti-mitogenic ones, they exit the cell cycle and enter a non-dividing quiescent state, called G0.

In most cells there are several important quality control points, called checkpoints, when cells assess the quality and completion of the previous phase before progressing further though the cell cycle. Cells can arrest at the checkpoints if something goes wrong. One of the checkpoints, called the restriction point in mammalian cells, occurs in mid-G1 phase. At the restriction point the cell determines if extracellular conditions are favorable (essential nutrients and growth factors are present) and makes the decision of whether the cell should commit to the next S-phase, delay division, or enter G0. Once the restriction point is passed, the cell is committed to division. Two of the other checkpoints evaluate the presence of DNA damage, one in late G1 phase to guard the entry into S phase, and the other in G2 phase, preventing entry into mitosis.

There is also an intra-S checkpoint, called the DNA structure checkpoint that ensures that DNA replication occurs with fidelity. Finally, the M phase checkpoint detects the failure of mitotic spindles to attach to the chromosomes and improper alignment of the spindle itself. Cells arrest the cell cycle in response to checkpoint activation and either fix the problem or undergo apoptosis if the problem cannot be fixed.

Progression through the cell cycle is mediated by proteins known as cyclins complexed to cyclin dependent kinases (Cdks). Cyclins are the activating subunit and their concentration rises when the Cdk activity is needed and falls when it is not. The cell cycle-regulating cyclins fall into three main families, based of the phase of cell cycle that they facilitate: the G1 cyclins (D1, D2, D3 and E1 and E2), the S phase cyclins A1 and A2 and the G2/M cyclins B1, B2, and B3 (Sherr, 1993) Cdks are a family of serine/threonine protein kinases that are activated upon binding of their cyclin partner occurs through a two step process (Kaldis et al., 1996). Their phosphorylation of target proteins mediates a series of events leading to the regulated step by step progression of the cell cycle. The role of the cyclin protein is not limited just to the activation of the Cdk partner but also includes directing it to the specific target proteins. This is illustrated by the fact that, when in complex with cyclin A, Cdk2 targets a wider variety of substrates than when partnered with Cyclin E (Kelly et al., 1998; Petersen et al., 1999; Sarcevic et al., 1997; Zarkowska et al., 1997); there are few overlapping targets between the two complexes, such as the G1 restriction point protein retinoblastoma (pRb) and the Cdk inhibitor p27kip1.

Regulation of Cyclin-Cdk2 Complexes.

Activation of the Cdk subunit occurs through a two step process (Espinoza et al., 1996; Fisher and Morgan, 1994; Kaldis et al., 1996) as shown in Figure 2. The first step is binding of the cyclin protein, which mediates a conformational change of Cdk2 that result in partial activation. The second step, phosphorylation of Cdk by the Cdk-activating kinase (CAK) and dephosphorylation of a different residue by Cdc25 phosphatase, results in full activation (Pavletich, 1999; Russo et al., 1996a; Russo et al., 1996b). The Wee1 protein kinase acts in opposition to Cdc25, phosphorylating the same residue and thus inactivating cyclin-Cdk complexes.

Cyclin-Cdk activity can be regulated by several mechanisms in addition to the rise and fall of cyclin levels during the cell cycle and phosphorylation. Additional fine-tuning of Cdk activity and cellular responses at specific stages of the cell cycle also takes place. Proteins known as Cdk inhibitors (CKIs) can counteract either the active kinase or its activation process (Fig. 2). Mammalian CKIs are divided into two families. Members of the Cip/Kip family include p21Cip1, p27Kip1, and p57Kip2 (Canepa et al., 2007). They negatively regulate cyclins E and A partnered with Cdk2, and cyclin B-Cdk1, but can also positively regulate cyclin D-Cdk4/6 by mediating its assembly in G1 (Massague, 2004). Cip/Kip proteins act by binding and inhibiting the active cyclin-Cdk complex (Fig. 2). The second CKI family, the INK4 family, comprising p16 (INK4a), p15 (INK4b), p18 (INK4c), and p19 (INK4d) targets only Cdk4 and Cdk6, inhibits the action of cyclin D. Members of the INK4 family compete with D cyclins for binding to the isolated Cdk subunit, thereby preventing complex assembly and subsequent activation.

INK4 members have also been shown to bind the cyclin-Cdk holoenzyme without dissociating or titrating the cyclin (Serrano, 1997).

Cyclin E-Cdk2 regulation of the cell cycle

In mid-G1 phase, Cyclin D is upregulated by mitogens or growth factors downstream of the Ras GTPase signaling pathway (Lee and Juliano, 2004). Active Cyclin D-Cdk4/6 phosphorylates and inactivates Rb, the central enforcer of the restriction point. Phosphorylated Rb releases the transcription factor E2F, leading to E2F activation. E2F facilitates transcription of S phase genes, including Cyclin E. Active Cyclin E-Cdk2 further phosphorylates and inactivates Rb, which removes mitogen dependency for further cell cycle progression. E2F also increases the transcription of its own gene, providing a positive feedback loop for Cyclin E expression and the G1/S-phase transition.

Substrates of Cyclin E in S phase include a regulatory protein Cdc6, which activates formation of pre-replication complexes that bind to chromatin and "license" DNA replication in S phase, and p220(NPAT), which leads to activation of histone H2B and histone gene transcription. Histones are important components of nucleosomes that are synthesized on demand to ensure proper packaging of DNA and completion of DNA replication. Cyclin E-Cdk2 is also required for centrosome duplication during S-phase (Chen et al., 2002; Cowan and Hyman, 2006; Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Schnackenberg et al., 2008) via its substrates nucleophosmin (NPM/B23) and CP110. NPM/B23 associates with unduplicated centrosomes and phosphorylation by Cyclin E-Cdk2 promotes its release thereby stimulating centrosomal

duplication (Okuda et al., 2000; Tokuyama et al., 2001). Moreover, phosphorylation of NPM by Cyclin E/A-Cdk2 directs it to nuclear speckles, dynamic sub-nuclear structures located in the interchromatin regions of the nucleoplasm, where pre-mRNA splicing factors are stored and assembled. This increases NPM's RNA binding activity and represses pre-mRNA splicing (Fukasawa, 2007). Among other Cyclin E substrates are several splicesome-associated proteins (SAP) and snRNP core proteins B' and B (Seghezzi et al., 1998), at least as shown by *in vitro* studies.

Recent studies indicate that effects of SIRT2, a member of Sirtuin family of NAD^+- dependent deacetylases are antagonized by Cyclin E-Cdk2 (Panditage R., 2008). SIRT2 promotes cell motility and modulates/inhibits neurite outgrowth of hippocampal neurons. Phosphorylation of SIRT2 by Cyclin E-Cdk2 inactivates it and increases cellular adhesion via downstream effects on tubulin. HIRA, named after its homology to Hir1p and Hir2p transcriptional repressors of *S. cerevisiae* (Lamour et al., 1995) is yet another substrate of Cyclin E -Cdk2. The exact mechanism of HIRA regulation of the cell cycle remains unclear, but its ectopic expression causes S-phase cell cycle arrest (Hall et al., 2001). Lastly, ankyrin repeat protein 17 (Ankrd17) is phosphorylated by and an important effector of Cyclin E-Cdk2. Ankrd17 positively regulates the G1/S phase transition. It is found in the nucleus and interacts with MCM proteins, Cdc6, and PCNA, proteins involved in DNA replication. Ankrd17 may be directly involved in DNA replication process since its depletion results in decreased binding of Cdc6 and PCNA to chromatin (Deng et al., 2009).

As shown by the studies summarized above, the list of Cyclin E targets has grown within the last few years and includes proteins that function not only directly in DNA replication, but also in centrosome duplication, histone activation and transcription, mRNA splicing, cell motility, and transcription. The identification of Cyclin E-Cdk2 substrates is an ongoing process, emphasizing its central role in coordinating many cellular processes with cell cycle progression.

Regulation of Cyclin E

Changes in human Cyclin E abundance are controlled via transcriptional, post-transcriptional and post-translational regulation. As mentioned previously, Cyclin E is an E2F target gene (Le Cam et al., 1999; Ohtani et al., 1995) and E2F-independent Cyclin E transcription has also been described (Lukas et al., 1997). Transcription of the Cyclin E gene is restricted to late G1 and early S-phase, helping to ensure the periodicity of its expression. E2F binding sites are found in the Cyclin E1 promoter, whose activity is affected by E2F in a cell-cycle dependent manner (Leone et al., 1999; Ohtani et al., 1995), as discussed previously. E2F recruits histone acetylases to the promoters of genes required for G1/S including Cyclin E. The transient induction of Cyclin E is also controlled via a Cyclin E repressor module (CERM), which has an E2F-binding site and an AT-rich sequence that cooperate during G0/G1 to delay Cyclin E expression until late G1 (Le Cam et al., 1999; Polanowska et al., 2001). In addition, Cyclin E-Cdk2 phosphorylates and regulates the activity of E2F itself (Morris et al., 2000) Another transcription factor, LRH-1, can directly regulate Cyclin E transcription in pancreatic and

hepatic cells (Botrugno et al., 2004). NFkB and transforming growth factor-β were shown to transcriptionally repress Cyclin E (Geng et al., 1996; Janbandhu et al., 2010), whereas Rho family GTPase Cdc42 can selectively induce Cyclin E (Chou et al., 2003). Moreover, viruses such as adenovirus E1A, human papillomavirus type 16, and human cytomegalovirus transform cells by transcriptionally activating Cyclin E (Botz et al., 1996; Bresnahan et al., 1998; Vogt et al., 1999). This subverts the cell cycle restriction point, before the G1/S phase transition.

Post-transcriptionally, Cyclin E is regulated at the levels of pre-mRNA splicing, mRNA stability, and translation. Nine alternatively spliced variants of Cyclin E1 mRNA have been identified. One encodes a 45 kDa isoform that was originally considered the full-length protein (Koff et al., 1991; Lew et al., 1991), but the full-length protein is now known to be 50 kDa (Ohtsubo et al., 1995). A 43 kDa splice variant lacking the cyclin box necessary for Cdk activation is expressed at 1/10 of the level of full length Cyclin E1 in several cell lines (Sewing et al., 1994). Four other splice variants can bind to and activate cdk2 *in vitro* (3 have unknown molecular weights), while one splice variant and one unspliced variant are not translated into protein.

Changes in levels of mRNA can mediate changes in the levels of protein present in cells. In turn, the steady state abundance of many mRNA, including that of Cyclin E, can be influenced by changes in mRNA stability (increased or decreased half-lives) (Moore et al., 2005). Regulation of mRNA stability is achieved by RNA binding proteins (RBP) or microRNAs (trans factors) (Bhattacharyya et al., 2006; Wilusz and Wilusz, 2004) that bind specific mRNA sequences (cis elements). This binding can elicit either

stabilizing or destabilizing effects on mRNA. One well characterized cis element located in the 3'untranslated region of the mRNA is represented by sequences enriched in A and U nucleotides, called AU-rich elements (AREs). Our lab has shown that Cyclin E mRNA stability is regulated by two RNA binding proteins that are overexpressed in several different cancers (Guo and Hartley, 2006; Guo and Hartley, 2010). Human antigen R (HuR) and cold-inducible RNA binding protein (CIRP) bind to the 3' untranslated region of Cyclin E mRNA to increase its stability and enhance its expression. HuR plays an essential role in tumorigenesis by stabilizing mRNAs involved in many cancer cell traits (Kim et al., 2009). We showed that HuR regulates Cyclin E mRNA both in human breast cancer cells and in Xenopus (Guo and Hartley, 2006; Slevin et al., 2007). CIRP is overexpressed in several breast cancer cell lines (Guo et al., 2009) as well as in about 30% of primary breast tumors (Artero-Castro et al., 2009). We found that CIRP increases Cyclin E1 mRNA stability partially by facilitating the binding of HuR to Cyclin E1 mRNA (Guo and Hartley, 2010). Conversely, the small regulatory RNA, microRNA miR-16, was reported to downregulate Cyclin E expression (Wang et al., 2009). Recent studies by our group show that miR-16 directly opposes and can inhibit HuR regulation of Cyclin E1 (Guo and Hartley, in prepraration). We also recently showed that the extracellular environment can stimulate Cyclin E expression directly, bypassing cyclin D1 (Wu et al., 2010). Lastly, the protein kinase Akt was recently shown to promote the translational efficiency of Cyclin E1 mRNA in hepatocytes (Mullany et al., 2007). This novel translational mechanism is distinct from the cyclin D1-E2F transcriptional pathway, suggesting that cells can respond to environmental stimulation (such as

mitogens or nutrients) by direct stimulation of Cyclin E1 translation by Akt to affect cell cycle progression.

Post-translational regulation of Cyclin E is largely via ubiquitin dependent-proteolysis, a mechanism providing regulated protein destruction and thus a rapid change of protein concentration in response to the altered state of a cell. This process targets proteins for degradation by covalent attachment of chains of the conserved 76 amino acid polypeptide called ubiquitin. Recognition of multi-ubiquitinated proteins and degradation of them into small peptides is performed by the 26S proteasome, a complex protease that also removes and recycles ubiquitin (Hershko, 1983). Ubiquitin ligases covalently attach ubiquitin to specific lysines of the target protein, targeting them for degradation. Cyclin E, as well as other targets, such as c-jun, and c-Myc, needs to be phosphorylated on specific sites to be recognized by the ubiquitin ligase $SCF^{Fbw7/Cdc4}$. Members of the SCF (Skp1-Cullin1-Fbox) family of ubiquitin ligases are composed of a linker protein Skp1, a scaffold protein Cul1/Cdc53, a ring-finger protein Rbx/Roc1, and a variable Fbox protein that determines substrate specificity. The human Fbox protein that recognizes phosphorylated Cyclin E as well as other phosphorylated targets is hCdc4; also known as Fbw7, hSEL-10, and hAgo (Gupta-Rossi et al., 2001)

There are three isoforms of Cdc4 in mammals, produced by alternative splicing: α, β and γ. They are localized to different parts of the cell, α and γ are found in the nucleus, whereas the β isoform is cytoplasmic (Ye et al., 2004). When overexpressed at a comparable level, all of the Cdc4 isoforms are capable of reducing the abundance of Cyclin E without affecting levels of Cdk2. Phosphorylation of human Cyclin E on

threonine 380 (T380) by GSK3β and on serine 384 (S384) by Cdk2 is required for Cyclin E recognition by SCF^{Cdc4} and its subsequent degradation (Ye et al., 2004) . Phosphorylation of T62 may serve as a priming event for T380 phosphorylation (Ye et al., 2004) or Cyclin E turnover may be directly dependent on T62 phosphorylation (Welcker et al., 2003), although its phosphorylation has not been shown *in vivo*.

Cyclin E is also regulated post-translationally by intracellular localization. In mammalian cells, the localization of Cyclin E and Cyclin E-Cdk2 is a dynamic process mediated by its nuclear to cytoplasmic shuttling (Jackman et al., 2002). This shuttling makes sense as two of the best defined functions of Cyclin E, initiation of DNA replication and centrosome duplication, require its presence in the nucleus and cytoplasm, respectively.

In Xenopus cycling egg extracts, in which cytoplasmic extract derived from Xenopus eggs drives several rounds of DNA replication and mitosis in sperm nuclei, Cyclin E is nuclear when the chromatin has begun to decondense and before initiation of DNA replication. During DNA replication, Cyclin E associates with chromatin. After termination of replication, Cyclin E remains in the nucleus, but no longer associates with chromatin. During mitosis, Cyclin E is excluded from the nucleus and is again detected in nuclei with the following S phase (Chevalier et al., 1996). A basic NLS, RSRKRK comprising residues 24-29, drives this nuclear accumulation (Moore et al., 2002). This sequence is completely conserved in human and mouse Cyclin E and varies by only one amino acid in chicken, zebrafish and goldfish accumulation (Moore et al., 2002). This NLS is also similar to the NLS of v-jun protein, KSRKRKL (Chida and Vogt, 1992).

In egg extracts, a conventional importin-α/β-dependent pathway is responsible for import of *Xenopus* Cyclin E -Cdk2 complexes into the nucleus (Moore et al., 1999). The importin-α component of the heterodimer complex associates directly with Cyclin E via its NLS (Moore et al 1999, Moore et al, 2002). Importin α/β (also known as karyopherin) functions as a nuclear receptor. They direct hundreds of proteins to the nuclear-pore complex (NPC) and transport them into the nucleus through nuclear pores. Importin-α recognizes its substrates in the cytoplasm, by binding to their classical nuclear localization signal (cNLS), and links them to importin-β, which ferries this ternary complex through the nuclear pore (Cingolani et al., 1999). The Xenopus NLS is essential for nuclear accumulation of Cyclin E and Cdk2 as its mutation leads to cytoplasmic localization of Cyclin E-Cdk2 complexes, which are not able to support DNA replication in egg extracts (Moore et al., 2002). Fusing a heterologous NLS to Cdk2 restores both DNA replication and nuclear import of Cyclin E-Cdk2 complexes lacking a functional Cyclin E NLS.

Despite the evidence in Xenopus Cyclin Extracts that the Cyclin E NLS is essential for nuclear localization, the NLS might not be required for nuclear entry of Cyclin E-Cdk2 in mammalian somatic cells. When the NLS is mutated, transiently transfected human Cyclin E accumulates in the nucleus and promotes S phase entry in murine cells (Kelly et al., 1998). Another study noted that Cyclin E mutants lacking the NLS are equally distributed between the nucleus and cytoplasm, whereas wild type Cyclin E remains nuclear in mouse embryonic fibroblasts (Geisen and Moroy, 2002). This study also showed that in malignantly transformed cells co-expressing Ha-ras

oncoprotein and Cyclin E, Cyclin E is mostly nuclear, as are NLS deletion mutants. Collectively, these studies indicate that localization of Cyclin E with or without its putative NLS can depend on the status of the cell and may even correlate with the process of malignant transformation. More support for the possible involvement of Cyclin E localization in malignant transformation will be discussed in the following section.

In addition to its nuclear to cytoplasmic shuttling, in Xenopus egg extracts Cyclin E also localizes to the centrosomes during S-phase, consistent with the requirement of Cyclin E-Cdk2 activity for the repeated reproduction of centrosomes (Chen et al., 2002; Hinchcliffe et al., 1999). A 20 amino acid sequence (from 231 to 250) in Xenopus Cyclin E is a centrosomal localization signal (CLS), which is responsible for loading/binding of Cyclin E onto the centrosomes (Matsumoto et al., 1999; Matsumoto and Maller, 2004). Cyclin E that is defective in its ability to bind Cdk2, but contains the intact CLS still localizes to the centrosome and promotes entry into S phase in a Cdk-independent fashion. When expressed in cells, intact GFP-tagged CLS peptides associate with the centrosome, inhibit localization of endogenous Cyclin E and cyclin A to the centrosome, and inhibit DNA synthesis. Mutant Cyclin E that is defective in its ability to bind Cdk2, but containing intact CLS still localized to centrosome and promoted entry into S phase in the Cdk independent fashion.

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Cyclin E in cancer

Regulation of G1 progression mediated by Cyclin E-Cdk2 is frequently disrupted in cancer. During this time the cell integrates multiple mitogenic, or growth factor

dependent, and anti-mitogenic stimuli from diverse growth regulatory pathways and coordinates the events leading to cell cycle progression. Key cell cycle regulators, such as Cyclin E-Cdk2, play a crucial role in controlling G1 progression. Therefore they and the proteins regulating their activities or levels are frequently mutated or affected in cancer.

Levels of Cyclin E are elevated in many types of human malignancy, where it is often present throughout the cell cycle (Hwang and Clurman, 2005). Experimental evidence indicates that Cyclin E overexpression is a cause rather than an effect of tumorigenesis, since increase in its abundance in primary tumors is out of proportion to the increase in markers of cellular proliferation (Dutta et al., 1995; Keyomarsi et al., 1994). In breast cancer it has been shown to be elevated in up to 40% of cases (Sutherland and Musgrove, 2004) and is implicated in its etiology via promoting genomic instability (Spruck et al., 1999). Constitutive expression of human Cyclin E in the mammary glands of mice during pregnancy and lactation using a specific ovine β-lactoglobulin promoter, resulted in development of adenocarcinomas (Bortner and Rosenberg, 1997). It has been proposed as a prognostic marker in breast cancer; high levels of Cyclin E correlate with high grade, advanced stage and decreased patient survival (Berglund and Landberg, 2006; Lopez-Beltran et al., 2006; Potemski et al., 2006). Immunohistochemical analysis revealed that morphological progression from normal breast tissue through ductal carcinoma in situ (DCIS) to invasive ductal carcinoma parallels the corresponding increase in Cyclin E expression; invasive ductal carcinomas also had a higher number of Cyclin E-positive cells(Scott and Walker, 1997). Comparison of expression levels of genes between normal tissue and tumor samples

established that high Cyclin E gene expression is a part of "proliferation signature" commonly associated with neoplasias (Whitfield et al., 2006). Taken together these studies strongly implicate Cyclin E as an oncogene involved in breast cancer and likely other cancers.

Mechanisms of Cyclin E deregulation in cancer

Defects in Cyclin E turnover lead not only to increased abundance of Cyclin E, but also to deregulation of its expression relative to the cell cycle (Ekholm-Reed et al., 2004; van Drogen et al., 2006). These defects are often mediated by loss of function mutation in the Fbox proteins that are responsible for Cyclin E ubiquitination. Mutations in the Fbox protein Cdc4 have been described not only in breast and ovarian cancer cell lines (Moberg et al., 2001; Strohmaier et al., 2001; Willmarth et al., 2004), but also in primary pancreatic, endometrial and colon cancers (Calhoun et al., 2003; Rajagopalan et al., 2004; Spruck et al., 2002). This evidence is in agreement with the idea that the impaired Cyclin E degradation may be the cause of malignant transformation (Ekholm-Reed et al., 2004; Erlandsson et al., 2003). In addition to mutations in Cdc4, deletion of chromosomal region 4q32 containing the Cdc4 gene has been reported in over 30% of human tumors (Spruck et al., 2002).

In addition to high Cyclin E level, cancer cells often have low expression of the CKI p27Kip1. As mentioned previously, p27Kip1 negatively regulates cyclins E-Cdk2, by binding and inhibiting the active cyclin-Cdk complex during late G1-phase. The combined profile of low p27Kip1 and high Cyclin E predicts low survival rate in breast

cancer (Donnellan and Chetty, 1999; Shou and Dunphy, 1996) even when patients are node-negative (Cariou et al., 1998)

Localization of Cyclin E is also perturbed in cancer. In normal mammalian cells Cyclin E-Cdk2 is found in the nucleus together with Cdc4 α and γ, nuclear isoforms of the ubiquitin ligase that targets it for destruction (van Drogen et al., 2006; Ye et al., 2004). Mammalian Cyclin E is synthesized and degraded in the cytoplasm, but accumulates in the nucleus to facilitate DNA replication. Immunolabeling of tumors shows Cyclin E localization as nuclear in the majority of tumor tissues tested, similar to normal tissues (Donnellan and Chetty, 1999; Kim et al., 2001; Peters et al., 2004; Reed et al., 2003; Scott and Walker, 1997). Cytoplasmic staining has been noted, but for the most part has been disregarded as having no significance. Since nuclear staining was present with the cytoplasmic, some papers reported it as aberrant and still counted it as positive for Cyclin E in breast cancers (Dutta et al., 1995). A study by Shaye et al. provided preliminary evidence in favor of increased cytoplasmic Cyclin E contributing to breast tumorigenesis (Shaye et al., 2009). They showed that in *in situ* and invasive carcinomas, but not in non-neoplastic breast epithelial cells, increased cytoplasmic localization of Cyclin E strongly correlates with its overexpression in the nucleus. Several different breast cancer cell lines also exhibited similar altered Cyclin E localization. The authors conclude that in the initial stages of tumorigenesis, loss of regulation can result from either Cyclin E overexpression or its increased cytoplasmic localization. As the tumor develops and becomes more advanced, both forms of dysregulation take place. They

propose that increased Cyclin E localization to the cytoplasm serves as a novel form of dysregulation on par with overexpression.

Lastly, post-translational processing of Cyclin E into low molecular weight isoforms is associated with tumorigenesis. Full-length 50 kDa Cyclin E can be cleaved from the N-terminus by elastase (Porter et al., 2001) or calpain (Wang et al., 2003) generating products, called low molecular weight (LMW) isoforms, with molecular weights ranging from 33 to 45 kDa. LMW Cyclin E was originally observed in breast cancer cell lines (Wingate et al., 2005), then in ovarian cancers (Bedrosian et al., 2004), colorectal cancres (Corin et al., 2006), melanomas (Bales et al., 2005), and renal cell carcinomas (Nauman et al., 2007). These LMW isoforms are only overexpressed in aggressive and highly metastatic cancers. They exhibit altered biological properties that differ from those of the full-length Cyclin E including the ability to induce higher Cyclin E-Cdk2 activity (Harwell 2000; Keyomarsi et al, 1995, 2002), resistance to CKIs p27 and p21 (Akli et al., 2004), and insensitivity to anti-estrogens (Wingate et al., 2005). Overexpression of LMW Cyclin E shortens mitosis, leading to chromosome mis-segregation and results in centrosome amplification, which in turn is a prerequisite for genomic instability (Bagheri-Yarmand et al., 2010). LMW Cyclin E can promote angiogenesis and metastasis of human melanoma cells *in vivo* (Bales et al., 2005). LMW Cyclin E in tumors has been proposed to serve as a prognostic factor (Corin et al., 2010; Milne et al., 2008). In non-metastatic colon cancer, high expression of LMW isoforms alone, and especially together with full-length Cyclin E, have higher risks of recurrence and thus worse survival (Corin et al., 2010).

LMW isoforms of Cyclin E mislocalize to the cytoplasm, although some still are present in the nucleus despite their lack of an NLS (Delk et al., 2009). Even a form missing a significant portion of the N terminus, including the NLS localizes to the nucleus and is able to facilitate G1 to S progression (Porter et al., 2001). It is not surprising that they are also less susceptible to degradation mediated by Cdc4 α and γ, which are normally found in the nucleus. The altered localization of LMW Cyclin E results in aberrant protein-protein interactions that induce high Cdk2 activity and are proposed to have amplified oncogenic activity. That human Cyclin E localizes to the nucleus without an NLS differs from results in Xenopus extracts where the NLS is required for nuclear entry. This apparent contradiction may be explained by the fact that systems more complex than egg extracts likely have multiple mechanisms that govern important cellular processes such as nuclear translocation and their involvement and interplay with one another has not been delineated so far.

The significance of LMW isoforms of Cyclin E remains controversial. One study suggests that LMW isoforms are not tumor specific, but are formed when Cyclin E is present at high levels and cleaved by the proteolytic machinery. In this study, when Cyclin E protein levels in breast tumors and normal tissues were normalized on Westerns, LMW isoforms were detected in the same quantities. These results led to the conclusion that LMW Cyclin E does not represent a tumor specific property (Spruck et al., 2006).

Despite recent advances in understanding of Cyclin E's role in normal events of the cell cycle, its regulation during the normal cell cycle, and its dysregulation during neoplastic progression, the molecular basis of Cyclin E-mediated tumorigenesis remains

elusive. As our knowledge on this subject evolves, so should our ability to discover effective anti-proliferative agents that directly target dysregulated Cyclin E and to select the appropriate combination therapies derived from specific biomarkers identified for each tumor type. The long term goal of our studies is to increase understanding of how Cyclin E1 protein is downregulated in the cell cycle, in order to inform and develop future cancer therapies.

Cyclin E in embryonic development

In mammals, there are two E type cyclins, Cyclin E1, known as Cyclin E and the more recently described Cyclin E2. They share 47% overall amino acid homology and 75% homology within the conserved cyclin box (Gudas et al., 1999; Lauper et al., 1998; Zariwala et al., 1998). Both of the isoforms are present in virtually all proliferating cells. So far they seem to share similar activities and regulation. Knockout mice deficient in Cyclin E1 or E2 are viable and appear normal, whereas double knockout mouse embryos do not survive past 11.5 days of gestation (Geng et al., 2003; Parisi et al., 2003). Embryonic lethality did not result from defects in embryogenesis itself but was due to severe abnormality of the trophoblast giant cells of the placenta (Parisi et al., 2003). In the absence of both E type cyclins, these cells fail to undergo endoreplication (repeated rounds of DNA synthesis without intervening of mitosis) leading to profound defects in placental physiology. E2-/- but not the E1-/- males presented defects in spermatogenesis, decreased fertility, and reduced testicular size resulting from testicular atrophy (Geng et al., 2003); evidence implicating cycin E2 in regulation of meiotic cell cycles.

Diverse cell types are affected by ablation of both E cyclins as impaired endoreplication was also found in megacaryocytes and murine embryonic fibroblasts (MEFs) were not able to re-enter the cell cycle from G0 because of failure to incorporate MCM helicase into DNA replication origins (Geng et al., 2003). This step becomes important when re-entering cell cycle from quiescence since MCM proteins are displaced from chromatin during G0. Knockout MEFs were also less susceptible to transformation by oncogenes, implicating E cyclins in cellular transformation. The results of these studies were unexpected, since type E cyclins did not appear to be essential for either embryonic development or continuous cell cycle progression. Cdk2 knockout mice showed that this subunit was similarly dispensible for embryogenesis, as the mice are viable and live up to two years. The only detectable abnormality in these mice was severe atrophy of their gonads (both testis and ovaries) leaving them completely sterile (Ortega et al., 2003). The male and female germ cells from Cdk2 lacking mice arrest in prophase I of meiosis. The lack of requirement for E cyclins and Cdk2 can be attributed to the in-built redundancy of various cyclin-Cdk complexes (Malumbres and Barbacid, 2009). The differences in cellular phenotypes resulting from Cdk2 and Cyclin E ablation indicate that Cyclin E can function independently of Cdk2.

In contrast to its G1/S phase restricted expression in the adult somatic cell cycle, Cyclin E is expressed constitutively through the cell cycle in all early embryos studied to date. Cyclin E mRNA and protein is supplied maternally and stored in the egg prior to fertilization. Maternal Cyclin E protein level is constant in the early Drosophila, sea urchin, mouse and *C. elegans* embryo and downregulated during development (Brodigan

et al., 2003; Damjanov et al., 1994; Knoblich et al., 1994; Sumerel et al., 2001). In *Xenopus laevis*, sea urchin, and Drosophila embryos, maternal Cyclin E level is high and constant following fertilization through the first 12 rapid cleavage divisions, and is abruptly destabilized in the midblastula embryo (Hartley et al., 1996; Rempel et al., 1995). This downregulation occurs at the midblastula transition (MBT), which is when global transcription initiates, cell motility begins, and the rapid embryonic cell cycle is remodeled to the adult cell cycle. In contrast, A and B cyclins fluctuate with each cell cycle as in adult somatic cells (Hartley et al., 1996). Given its markedly distinctive pattern of expression during the embryonic and adult cell cycle with the time of its disappearance corresponding to cell cycle remodeling in the embryo, maternal Cyclin E degradation has been proposed to be necessary for establishment of the adult cell cycle (Hartley et al., 1997; Howe and Newport, 1996; Rempel et al., 1995; Richardson et al., 1993).

Similar to results with Cyclin E knockout mice, Cyclin E has also been implicated in regulation of endoreplication in Drosophila embryos. Most postmitotic cells during Drosophila development undergo rounds of endoreplication during the second half of embryogenesis (Smith and Orr-Weaver, 1991). Cyclin E is required for entry into S phase for both mitotic and endoreplicative cell cycles in Drosophila (Knoblich et al., 1994; Richardson et al., 1993) While Cyclin E is present continuously during the early embryonic cleavage divisions, it is periodically expressed during the endoreduplication cycle. This periodic expression results in periodic S phases; ectopic expression of Cyclin E is sufficient to force S phase in endopreplicating tissues but not in postmitotic cells

after the last mitotic division. Hence, in Drosophila embryos the normal pattern of Cyclin E expression determines the execution of spatial and temporal programs of endoreplication (Sauer et al., 1995).

In sea urchin embryos, constitutively expressed Cyclin E is not required for initiation of DNA replication, but functions in sperm maturation (Schnackenberg et al., 2007) and in centrosome duplication (Schnackenberg et al., 2008), as does Cyclin E in mammalian cells, Xenopus extracts, and *C. elegans* embryos (Cowan and Hyman, 2006; Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999). Moreover, embryos lack or have very short G phases when Cyclin E is constitutively present, and acquire them when Cyclin E is periodically expressed. In mammalian fibroblasts, constitutively overexpressing Cyclin E shortens G1 phase, leading to premature S phase entry (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994). These data point to a requirement for Cyclin E periodicity to acquire and maintain a normal G1 phase. During the first embryonic cleavages when the zygote produces an increasing number of genomically identical but smaller cells without growth, the best way to complete this simple and redundant task is to keep divisions rapid and synchronous. After the MBT, the embryo undergoes gastrulation and patterning/differentiation, which require specialized expression of various genes in different cells provided by the beginning of global transcription. In Xenopus and other embryos, cell cycle regulators, such as cyclins and cdks are now expressed in tissue specific patterns that are unique for each cell cycle component (Vernon and Philpott, 2003). The cell cycle becomes asynchronous and includes G

phases to accommodate these requirements for the individuality and complexity within different cells and tissues.

We have been studying Cyclin E regulation in the *Xenopus laevis* embryo. In *Xenopus laevis* embryos, three isoforms of Cyclin E have been cloned: E1 (Chevalier et al., 1996; Rempel et al., 1995), E2 (Gotoh et al., 2007) and E3 (Chevalier et al., 1996). E1 and E3 are thought to be variants of the same gene, differing only in their untranslated regions (Chevalier et al., 1996). Cyclin E1 was the first cloned and is the best characterized in Xenopus. Cyclin E1 is abundant in eggs and its level remains high until the onset of the MBT when it drops precipitously (Gotoh et al., 2007; Hartley et al., 1996; Rempel et al., 1995). In contrast, Cyclin E2 is barely detectable until early gastrula stages, when it increases and remains constant until tadpole stages.

In Xenopus, the first cell cycle following fertilization is 75 minutes in length while cell cycles 2-12 are 25 minutes, alternating rapidly between mitosis and DNA synthesis with no detectable G phases (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). These divisions are mitogen-independent and lack transcription. After the 12th division, (~6 hours post fertilization, hpf) during the MBT, the rapid embryonic cell cycle begins remodeling to the adult cell cycle, containing G phases and checkpoint controls. At the MBT, zygotic transcription begins and Cyclin E protein is abruptly destabilized by an undefined mechanism (Hartley et al., 1997; Howe and Newport, 1996; Rempel et al., 1995). Following the MBT, Cyclin E is expressed in a G1/S specific manner, cyclin D and the CKI p27 are produced (Shou and Dunphy, 1996; Su et al., 1995), and cells gain the ability to undergo cell cycle arrest and apoptosis in response to

injury (Anderson et al., 1997; Sible et al., 1997). This process can be viewed simplistically as the reverse of a normal human cell's progression towards cancer. Degradation of Cyclin E at the MBT does not require new transcription, an increase nuclear to cytoplasmic ratio, or cell cycle expansion (Hartley et al., 1997; Howe and Newport, 1996). We set out to determine the mechanism of Cyclin E downregulation in the MBT Xenopus embryo.

Hypothesis

As discussed earlier, phosphorylation of human Cyclin E on T62, T380 and S384 marks it for recognition by SCF ^{Cdc4} for ubiquitination and degradation (Welcker et al., 2003; Ye et al., 2004). These residues are conserved in Xenopus laevis, and correspond to T75, T394 and S398 (Fig. 3). While mammalian Cyclin E shuttles between the nucleus and cytoplasm (Jackman et al., 2002), in Xenopus embryos Cyclin E is found in the nucleus at the MBT, the time period corresponding to the beginning of its degradation (Chevalier et al., 1996). Its localization in earlier embryos has not been examined. **Based on these findings, we hypothesized that Cyclin E destabilization is dependent on a change in its phosphorylation, a change in its localization and is mediated by the 26S proteasome.** To test this hypothesis, we set out to complete the following aims:

Aim 1: Determine if differential phosphorylation plays a role in regulation of Cyclin E stability in *Xenopus laevis* embryos

Aim 2: Determine if a change in localization at the MBT regulates Cyclin E stability in *Xenopus laevis* embryos

Aim 3: Determine if Cyclin E degradation is triggered by its ubiquitination and mediated by the 26 S proteasome

Figures and Figure Legends

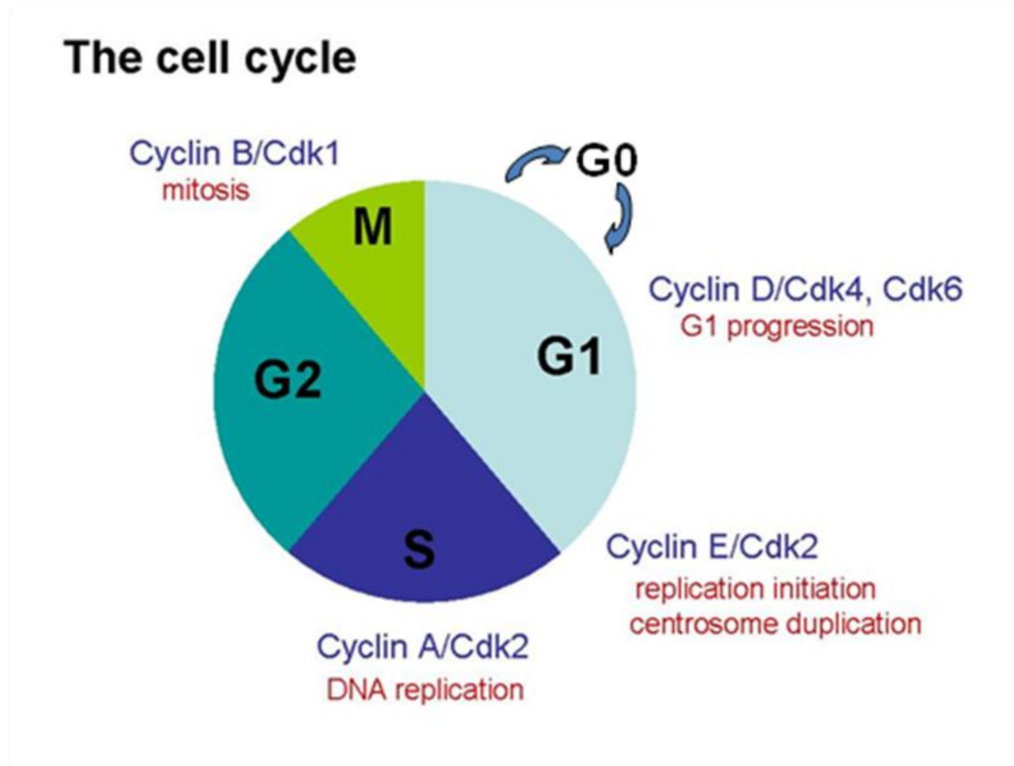

Figure 1. In mid-G1, cyclin D/Cdk4 (or Cdk6) activation is required to induce cyclin E synthesis. Cyclin E/Cdk2 regulates entry into S-phase, promoting initiation of DNA replication and centrosome duplication, among other functions. CyclinA/Cdk2 then functions in completion of DNA replication during S-phase. Once DNA is duplicated, cells enter G2 and synthesize proteins required for mitosis. Cyclin B/Cdk1 activation promotes entry into mitosis, in which the two DNA copies are separated. Cytokinesis occurs at the end of M phase. Cells can exit and re-enter the cell cycle in G1 to and from G0, a resting phase.

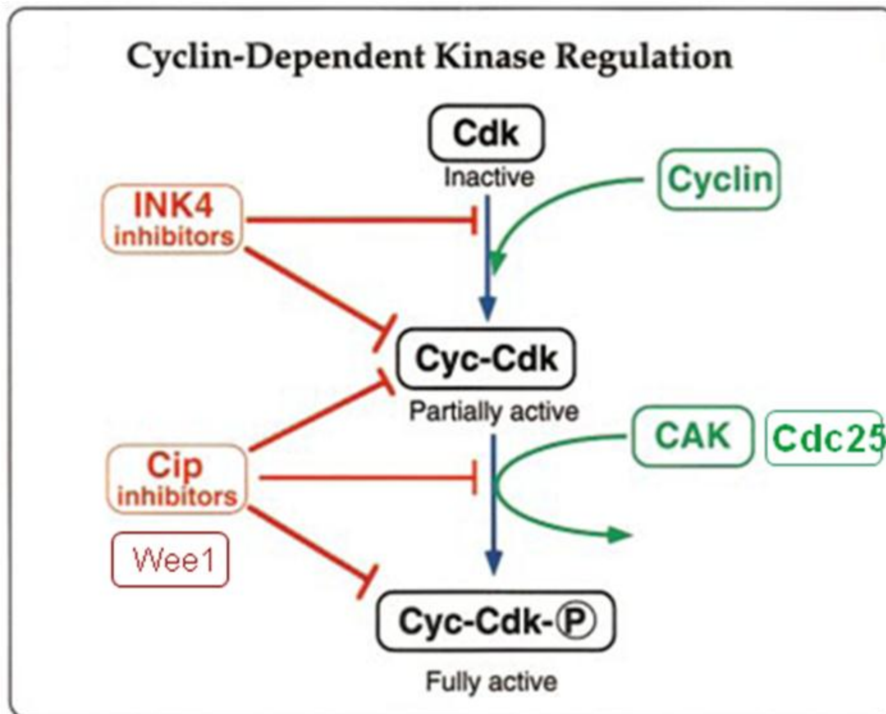

Figure 2. Flowchart of the major Cdk-regulatory mechanisms. CAK is a Cdk-activating kinase, Cdc25 is a Cdk activating phophatase, Wee1 is a Cdk inactivating kinase,and encircled letter P indicates a phosphate group. INK4 and Cip are families of Cdk inhibitors.

Modified from: Pavletich J.Mol.Biol (1999) 287, 821-8.

MPVISNPAVEKSTKDEGTASCSVRSRKRKADVAIFLQDPD 40

75

ETLDSLEMTKKKQYQDRGPWSNEMTCK**SP**HKLIP**TP**EKEE 80

HEPNPTNYSHFASLRFSPVSV**SP**LPRLGWANQDDVWRNML 120

NKDRIYLRDKNFFQKHPQLQPNMRAILLDWLMEVCEVYKL 160

HRETFYLAQDFFDRFMATQKNVIKSRLQLIGITSLFIAAK 200

LEEIYPPKLHQFSFITDGACTEDEITRMELIIMKDLGWCL 240

SPMTIVSWFNVFLQVAYIRELQQFLRPQFPQEIYIQIVQL 280

LDLCVLDICCLEYPYGVLAASAMYHFSCPELVEKVSGFKV 320

TELQGCIKWLVPFAMAIKEGGKSKLNFFKGVDIEDAHNIQ 360

394 398

THSGCLELMEKVYINQALLEEQNRT**SP**IPTGVL**TPP**QSNK 400

KQKSDRAD 408

Figure 3. Amino acid sequence of Xenopus cyclin E1. Potential phosphorylation sites that are conserved between Xenopus and humans, threonines (T) and serines (S) most often followed by prolines, are underlined and bold. Numbers to the right indicate amino acid residue. Sites mutated in this study (Chapter 2) are indicated by top numbering.

Chapter 2. Developmental downregulation of Xenopus Cyclin E is phosphorylation and nuclear import dependent and is mediated by ubiquitination

Abstract

Cyclins are regulatory subunits that bind to and activate catalytic Cdks. Cyclin E associates with Cdk2 to mediate the G1/S transition of the cell cycle. Cyclin E is overexpressed in breast, lung, skin, gastrointestinal, cervical, and ovarian cancers. Its overexpression correlates with poor patient prognosis and is involved in the etiology of breast cancer. We have been studying how Cyclin E is normally downregulated during development in order to determine if disruption of similar mechanisms could either contribute to its overexpression in cancer, or be exploited to decrease its expression. In *Xenopus laevis* embryos, Cyclin E protein level is high and constant until its abrupt destabilization by an undefined mechanism after the 12th cell cycle, which corresponds to the midblastula transition (MBT) and remodeling of the embryonic to the adult cell cycle. Since degradation of mammalian Cyclin E is regulated by the ubiquitin proteasome system and is phosphorylation dependent, we examined the role of phosphorylation in Xenopus Cyclin E turnover. We show that similarly to human Cyclin E, phosphorylation of serine 398 and threonine 394 plays a role in Cyclin E turnover at the MBT. Immunofluorescence analysis shows that Cyclin E relocalizes from the cytoplasm to the nucleus preceding its degradation. When nuclear import is inhibited, Cyclin E stability is markedly increased after the MBT. To investigate whether degradation of Xenopus Cyclin E is mediated by the proteasomal pathway, we used proteasome inhibitors and

observed a progressive accumulation of Cyclin E in the cytoplasm after the MBT. Ubiquitination of Cyclin E precedes its proteasomal degradation at the MBT. These results show that Cyclin E destruction at the MBT requires both phosphorylation and nuclear import, as well as proteasomal activity.

Introduction

Oscillations of Cyclin E-Cdk2 activity regulate the G1/S phase transition of the metazoan cell cycle. In mammalian cells Cyclin E1, also known as Cyclin E, is synthesized in late G1 phase, where its activation of Cdk2 stimulates proliferation by promoting G1 phase progression, initiation of DNA replication, and centrosome duplication (Hwang and Clurman, 2005). Cyclin E is degraded during S phase by ubiquitin-dependent proteolysis. The ubiquitin ligase SCF^{Cdc4} (Skp1-Cullin1-Fbox) targets Cyclin E for ubiquitination and subsequent degradation by the 26S proteasome (Koepp et al., 2001; Strohmaier et al., 2001). Members of the SCF family of ubiquitin ligases contain a linker protein Skp1, a scaffold protein Cul1/Cdc53, a ring-finger protein Rbx/Roc1, and a variable Fbox protein that determines substrate specificity. The human Fbox protein hCdc4 (also known as Fbw7, hSEL-10, and hAgo) recognizes phosphorylated Cyclin (Hao et al., 2007; Koepp et al., 2001; Sangfelt et al., 2008; van Drogen et al., 2006). The three alternatively spliced isoforms of hCdc4 localize differently within the cell; Cdc4 α and γ isoforms are nuclear, whereas the β isoform is cytoplasmic (Ye et al., 2004).

Phosphorylation of human Cyclin E on threonine 380 (T380) by GSK3β and on serine 384 (S384) by Cdk2 is required for Cyclin E recognition by SCF^{Cdc4} and its subsequent degradation (Ye et al., 2004). Phosphorylation of T62 may serve as a priming event for T380 phosphorylation (Ye et al., 2004) or Cyclin E turnover may be directly dependent on T62 phosphorylation (Welcker et al., 2003), although its phosphorylation has not been shown *in vivo*. Defects in Cyclin E turnover lead to increased protein and its

presence throughout the cell cycle (Ekholm-Reed et al., 2004). Cdc4 loss of function has been described in breast and ovarian cancer cell lines (Moberg et al., 2001; Strohmaier et al., 2001) and in primary endometrial, pancreatic, and colon cancers (Calhoun et al., 2003; Rajagopalan et al., 2004; Spruck et al., 2002); consistent with impaired Cyclin E degradation contributing to malignant transformation (Ekholm-Reed et al., 2004).

Similar to cancer cells with cdc4 defects, in *Xenopus laevis* embryos Cyclin E level is high and constant following fertilization through the first 12 rapid cell divisions (Hartley et al., 1996; Rempel et al., 1995). In contrast, cyclins A1, B1 and B2 fluctuate with each cell cycle in Xenopus embryos, similar to normal adult somatic cells, peaking during mitosis and then subsiding despite ongoing translation (Hartley et al., 1996). The first cell cycle following fertilization is 75 minutes in length while cell cycles 2-12 are 25 minutes, alternating rapidly between mitosis and DNA synthesis with no detectable G phases (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). These divisions are mitogen-independent and lack transcription. After the 12th division, (~6 hours post fertilization, hpf) during the period of development called the midblastula transition (MBT), the rapid embryonic cell cycle begins remodeling to the adult cell cycle, containing G phases and checkpoint controls. At the MBT, zygotic transcription begins and Cyclin E protein is abruptly destabilized by an undefined mechanism (Hartley et al., 1997; Howe and Newport, 1996; Rempel et al., 1995). Following the MBT, Cyclin E is expressed in a G1/S specific manner, cyclin D and the Cdk inhibitor p27 are produced (Shou and Dunphy, 1996; Su et al., 1995), and cells gain the ability to undergo cell cycle arrest and apoptosis in response to DNA damage (Anderson et al., 1997; Sible et al.,

1997). This process can be viewed simplistically as the reverse of a normal human cell's progression towards cancer. Degradation of Cyclin E at the MBT does not require new transcription, an increase nuclear to cytoplasmic ratio, or cell cycle expansion (Hartley et al., 1997; Howe and Newport, 1996).

Similar to Xenopus, maternal Cyclin E protein level is constant in the early Drosophila, sea urchin, mouse and *C. elegans* embryos and downregulated during development (Brodigan et al., 2003; Damjanov et al., 1994; Knoblich et al., 1994; Sumerel et al., 2001). Given its markedly distinctive pattern of expression during the embryonic and adult cell cycle with the time of its disappearance corresponding to cell cycle remodeling, maternal Cyclin E degradation has been proposed to be necessary for establishment of the adult cell cycle (Hartley et al., 1997; Howe and Newport, 1996; Rempel et al., 1995; Richardson et al., 1993).

We set out to define the mechanism of Xenopus Cyclin E degradation at the MBT in order to be able to determine its developmental significance, as well as to potentially identify novel ways of reducing Cyclin E protein levels in cancer in future studies.

Materials and Methods

Embryos: *X. laevis* embryos were obtained by inducing egg laying with hormones followed by *in vitro* fertilization using standard methods (Hartley et al., 1996). Embryos were microinjected with capped, *in-vitro* transcribed mRNA into the animal pole of one cell at the two-cell stage. Embryos were staged according to Nieuwkoop and Faber (1994) and collected at the stated time post-fertilization.

<u>Proteasome and nuclear import inhibition:</u> Proteasome inhibitors Clasto-Lactocystin β-Lactone (CLBL; Calbiochem, La Jolla, CA) and MG132 (Cayman Chemical Company, Ann Arbor, MI), or wheat germ agglutinin Alexa 633 Fluor conjugate (WGA; Invitrogen, Eugene, OR), were mixed with mRNA at the final concentration indicated and injected into 1-cell of 2-cell embryos.

<u>Constructs:</u> Cloning and site-directed mutagenesis primers are listed in Table 1. Cyclin E mutants T394A, T394D, S398A, S398D, and T75A were generated by site directed mutagenesis of the Cyclin E1 gene (Genbank accession no. Z13966) in the pET21A vector. T394A S398A and T394D S398D mutants were generated by site directed mutagenesis in the context of T394A and T394D mutants, respectively, in pET21A using the Stratagene (Santa Clara, CA) Quick Change Site-Directed Mutagenesis protocol. The open reading frames of wild type Cyclin E and its mutants were cloned by PCR out of pET21a without the stop codon. An EcoRI restriction site plus four additional nucleotides were added to the forward primer to ensure in-frame cloning and prevent generation of potential novel phosphorylation sites. An XbaI restriction site was added to the reverse primer. The PCR product was digested with XbaI and EcoRI and ligated into pCS2mt-GFP (Addgene, Cambridge, MA) cut with the same enzymes using a Quick ligation kit (New England Biolabs, Ipswich, MA). 10 ng of DNA was used for transformation of DH5α. Clones were selected and verified by restriction digest and sequencing.

<u>*In vitro* transcription:</u> Plasmids were linearized with Not I and transcribed with SP6 RNA polymerase according to the manufacturer's instructions (Promega, Madison, WI). 1-2 μg of the *in vitro* transcribed RNAs were analyzed on formaldehyde gels to check

quality. Capped transcripts were injected into 1-cell of a 2-cell embryo (0.5 ng), between 1.5-2 hours post-fertilization.

Western analysis: 3-5 embryos were collected on dry ice at the indicated hour post-fertilization (hpf). To prepare cytoplasmic extracts, embryos were homogenized in extraction buffer (EB, 10 μl/per embryo) containing protease inhibitors (Hartley et al., 1997) and centrifuged at 12,000 rpm for 10 min. Supernatant (cytoplasmic extract) was removed to a new tube, avoiding the yolk protein, and diluted with 6X loading buffer and heated at 95°C for 5 min. For detection of ubiquitinated Cyclin E, MG132 was added to the EB to the final concentration of 20 μM. One embryo equivalent (or 4.5 embryo equivalents for detecting ubiquitinated Cyclin E) was loaded per lane and separated on 10% SDS-polyacrylamide gels. Proteins were then transferred to a PVDF membrane using a semi-dry blotting apparatus. Membranes were blocked with 5% nonfat dry milk in Tris buffered saline, 0.05 % Tween 20 (TBST) and then incubated with Myc antibody (1:10, 000 in block; Cell Signaling Technology, Danvers, MA) for 1.5 hrs at room temperature (RT) or overnight at 4°C, followed by goat-anti-mouse horseradish peroxidase (HRP)-conjugated secondary (1:24,000 in TBST; Santa Cruz Biotechnology, Santa Cruz, CA). Signals were visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA). Blots were stripped using and reprobed with β-Actin antibody (1:1,000 in TBST, 5% BSA; Santa Cruz Biotechnology) followed by donkey-anti-goat HRP (1: 5,000 in TBST). Blots were imaged and quantified using a Kodak MI Image Station and associated software.

Immunofluorescence analysis: 3-5 embryos were collected at the indicated time points and fixed in 3.7% formaldehyde/phosphate buffered saline (PBS) for 2 hours on a nutator. Embryos were then transferred into Dent's fixative (4 parts of MeOH and 1 part DMSO), and incubated at -20°C for at least 48 hours. Embryos were rehydrated in a graded series of methanol and washed twice for 10 min in PBS. Rehydrated embryos were hemi-sectioned and all incubations and washes were performed at 4°C on a nutator. Blocked embryos were incubated in either anti-Myc (1: 2,000; Cell Signaling Technology) or anti-Cyclin E (1:50; Dr. Michel Philippe, University of Rennes, Rennes, France) in PBT overnight. The embryos were washed four times with PBT, and incubated in Alexa Fluor 488 conjugated goat-anti-mouse or goat-anti-rabbit (Molecular Probes, Eugene, OR) in PBT (1:200) overnight. Embryos were washed 3 times with PBT, nuclei counterstained with 4',6'-diamidino-2-phenylindole (DAPI) in PBS (1:3,000 dilution of 1 mg/ml) for 30-60 min, washed extensively with PBS, and mounted in depression slides using Vectashield mounting media (Vector Laboratories, Burlingame, CA) and coverslipped. An empirically chosen constant exposure time was used for imaging on a Zeiss LSM 510 confocal microscope equipped with a META detector, or on a Zeiss Axiovert 200 microscope equipped with a Hamamatsu camera.

Immunoprecipitation: Cytoplasmic extract was prepared from embryos injected with WT Cyclin E mRNA as described above in western analysis, except that EB contained 1.5X the concentration of protease inhibitors and was supplemented with MG132 to 20 μM final concentration. All procedures were carried out at 4°C, unless noted otherwise. Seven and a half embryo equivalents of cytoplasmic extract were diluted in a total

volume of 100 μl of EB and precleared with 25 μl of 50% protein A agarose in EB for 25 min on a rocker. After brief centrifugation, the supernatant was transferred into a fresh tube and 2 μl of anti-Myc or normal mouse IgG was added and incubated for 4 hrs. 25 μl of 50% protein A agarose was added and incubated with rocking for 2 hrs. Samples were briefly centrifuged and supernatant removed. The protein A agarose beads were then washed twice with both low and high salt TENT buffers (20 mM Tris-HCl, pH 7.4; 5 mM EDTA; 1% Triton X-100; containing either 100 mM or 1M NaCl, respectively), resuspended in 50 μl of 1X gel loading buffer and heated at 95°C for 5 min. Samples were loaded onto 10% polyacrylamide gels followed by western blotting with either anti-Myc (1:5,000) followed by goat-anti-mouse HRP (1:10, 000), or Lys48 specific anti-Ubiquitin antibody (Cell Signaling Technologies) followed by goat-anti-rabbit HRP (1: 3,000). To determine the relative efficiency of the immunoprecipitation (IP), the starting extract (input) and the supernatant remaining after IP (supernatant) were also analyzed by western analysis for anti-Myc.

Two-dimensional gel electrophoresis-Western analysis: IPs were carried out as described in the previous section, except that after immunoprecipitation the protein A agarose beads treated with Protein Phosphatase I (PP1; New England Biolabs, Ipswich, MA), or PP1 buffer alone for 30 min at room temperature. PP1 and buffer treated beads were washed twice with low and high salt TENT buffers, three times with dH_2O and resuspended in rehydration buffer (2% CHAPS, 20mM DTT. 9.5M urea, containing traces of bromophenol blue). Samples were absorbed overnight onto IPG strips (pH 3-10, non linear) (Biorad, Hercules, CA) at RT and subjected to isoelectric focusing. Strips

were then layered on top of 10% gels (Criterion precast Tris-HCl gel, Biorad) and proteins were separated by electrophoresis and transferred to PVDF membrane. Western blot analysis was performed using anti-Myc antibody (1:5,000) and goat-anti-mouse HRP conjugated secondary (1:4,000 dilution). Signals were visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation).

Results

Differential phosphorylation regulates Cyclin E stability in *Xenopus laevis* embryos.

The phosphorylation sites that are important for human Cyclin E recognition by SCF^{Cdc4}, T380, S384, and T62 (Welcker et al., 2003; Ye et al., 2004) are conserved in Xenopus and correspond to T394, S398 and T75. We observed previously that an unphosphorylatable T394 to alanine (T394A) mutation stabilizes Cyclin E and its associated Cdk2 activity past the MBT, when Cyclin E is usually degraded and Cdk2 activity drops (R.S. Hartley, unpublished). In addition, two Xenopus orthologs of hCdc4 have been cloned: α and β (Almeida et al., 2010). Both isoforms are present throughout development, can promote destruction of Cyclin E and therefore represent potential candidates for its degradation at the MBT. Based on the above findings, we hypothesized that phosphorylation of Cyclin E at T394, S398, and T75 destabilizes Cyclin E at the MBT in *Xenopus laevis* embryos.

To examine contribution of individual phosphorylation events to Cyclin E turnover, we mutated T394 and S398 to either alanine (prevents phosphorylation) or aspartic acid (mimics constitutive phosphorylation) and T75 to alanine by site-directed

mutagenesis. We also created double mutants affecting two of the potentially most important residues, T394 and S398. If phosphorylation at these residues is important for turnover, mutating them should affect Cyclin E stability. Wild type (WT) Cyclin E and its mutants were cloned into the pCS2mt-GFP vector that contains both Myc_6 and GFP tags, *in vitro* transcribed, and mRNAs injected into 2-cell embryos for *in vivo* translation.

Western blots of embryo time courses using an antibody against the Myc-tag are shown in Fig. 1. Cyclin E level was normalized to β-actin as a loading control, as its level remains constant throughout development. WT Cyclin E and its mutants accumulated to comparable levels (Fig 1A-I and J). Fig. 1A shows that WT Cyclin E accumulated until 6 hours post fertilization (hpf), corresponding to the MBT, when it reached it's maximum (taken as 100%) and started to decrease by 8 hpf. Its abundance was reduced to 69% of maximum by 8 hpf and was almost undetectable by 25.5 hpf when 1.3 % remained (Fig. 1K). This expression pattern is similar to that of endogenous Cyclin E (Hartley et al., 1997; Rempel et al., 1995), validating our approach of using exogenous protein to study Cyclin E turnover. In contrast, control Myc_6-GFP protein, translated from empty vector mRNA, increased in a linear fashion until 25.5 hpf (Fig. 1B and K). In. We detected no signal in uninjected embryos collected at any of the time points (Fig. 1I, the lane labeled Un, is an uninjected embryo at 6 hpf).

In comparison to WT Cyclin E, the T394A mutant accumulated more slowly, but maintained a higher level after the MBT through at least 25.5 hpf, when 70% of the maximum protein remained (Fig. 1C and L). The T394D phosphomimetic mutant partially restored Cyclin E turnover, as seen in Fig. 1D and L (47% of maximum

remained at 25.5 hpf). We next assessed the importance of S398 in Cyclin E turnover. The S398A mutant was even more resistant to degradation than the T394A mutant, resulting in the presence of 80% of maximal level of protein through 25.5 hpf. (Fig 1E and M). The S398D phosphomimetic mutation restored Cyclin E turnover more effectively than the T394D mutation, with only 19% of maximal Cyclin E protein present by 25.5 hpf (Fig 1F and M). The T75A mutant delayed but did not prevent Cyclin E turnover (Fig. 1I and K), consistent with observation that the corresponding residue T75 in human Cyclin E does not play a major role in its turnover (Ye et al., 2004). Mutating both T394 and S398 residues to alanine caused accumulation of Cyclin E until 25.5 hpf, when the majority of wild type Cyclin E is already degraded (Fig. 1G and N). Finally, T394D S398D double mutants exhibited decreased protein turnover (Fig. 1H and N) as compared to T394A S398A, with only 38% of maximum protein remaining at 25.5 hpf. These data indicate that similarly to human Cyclin E, corresponding residues in Xenopus Cyclin E serve as phosphodegrons, with their phosphorylation mediating Cyclin E destabilization, suggesting conservation of destruction pathways. Of the single mutants, S398A was the most stable followed by T394A and T75A. The fact that the T394AS398A mutant exhibited complete stabilization, points toward the requirement of at least two phosphorylation events marking Xenopus Cyclin E for destruction.

In order to determine if a change in phosphorylation accompanies the degradation of Cyclin E at the MBT *in vivo*, we analyzed the phosphorylation state of WT Cyclin E translated in pre-MBT (4 hpf) and MBT (6 hpf) embryos using two-dimensional gel electrophoresis followed by Western blotting. WT Cyclin E was immunoprecipitated

from cytoplasmic extracts prepared from pre-MBT or MBT embryos. Phosphatase treatment of WT Cyclin E from pre-MBT embryos did not result in significant mobility differences (Fig 2A, top and bottom). On the contrary, at the MBT (6 hpf), there is a pronounced shift of Cyclin E toward a more acidic pH, compared to the phosphatase treated sample (Fig 2B top and bottom). These results suggest that in pre-MBT (4 hpf) embryos phosphorylation of cytoplasmic Cyclin E is minimal, whereas at the MBT (6 hpf) its phosphorylation is dramatically increased, corresponding to the beginning of its degradation.

Nuclear accumulation at the MBT precedes Cyclin E degradation.

Mammalian Cyclin E-Cdk2 complexes reside in the nucleus, (Delk et al., 2009; Jackman et al., 2002), whereas Cyclin E by itself shuttles between the nucleus and cytoplasm in mammalian cells (Jackman et al., 2002). In mammalian cells, Cdc4 α and γ are found in the nucleus (Ye et al., 2004) where they can function sequentially to promote Cyclin E ubiquitination (van Drogen et al., 2006). First, Cdc4 α binds both Pin1, the prolyl cis/trans isomerase, and Cyclin E-Cdk2 complex and promotes Pin1 isomerization of Cyclin E. This modification renders Cyclin E susceptible to ubiquitination by Cdc4 γ. When Cyclin E is overexpressed, Cdc4 α alone is sufficient for its turnover (Grim et al., 2008; Sangfelt et al., 2008). Cdc4 β resides in the cytoplasm and is also capable of ubiquitinating Cyclin E, leading to its degradation (van Drogen et al., 2006; Ye et al., 2004). In sea urchin and Drosophila embryos, Cyclin E is nuclear (Richardson et al., 1993; Sumerel et al., 2001). In MBT Xenopus embryos, Cyclin E is found in the nucleus

just prior to its degradation (Chevalier et al., 1996). The localization of Xenopus Cyclin E has not been studied in the early embryonic cell cycle *in vivo*. We hypothesized that a change in localization contributes to Xenopus Cyclin E destabilization at the MBT. To test this hypothesis, Cyclin E localization within cells in whole mount pre-MBT (4 hpf), MBT (6 hpf) and post-MBT (8 hpf) embryos was assessed by confocal immunoflourescence analysis (IFA) using a Xenopus Cyclin E antibody (Chevalier et al., 1996) to detect endogenous Cyclin E (courtesy of Dr. Michel Philippe, University of Rennes, France).

Fig. 3A shows that in pre-MBT embryos, Cyclin E was primarily cytoplasmic. At MBT, when the abundance of Cyclin E starts to decrease, it began to appear in the nucleus, with some cytoplasmic staining still present (Fig. 3B), consistent with a previous report (Chevalier et al., 1996). Note that individual cells of the embryo become progressively smaller as embryos continue to divide without growth. Post-MBT, Cyclin E remained both nuclear and cytoplasmic (Fig. 3C), confirmed by colocalization of Cyclin E with nuclear DAPI staining (data not shown). Interestingly, neither DAPI nor TOPRO nuclear dyes appear to stain pre-MBT nuclei efficiently. However, since Cyclin E was cytoplasmic pre-MBT and did not localize to any intracellular compartments or structures, this did not interfere with interpretation of results. Localization of WT Myc-tagged Cyclin E was similar to that of endogenous Cyclin E; cytoplasmic pre-MBT, and both nuclear and cytoplasmic from the MBT on (Fig. 4B). Importantly, Myc_6GFP protein expressed from an empty vector remained cytoplasmic in pre-MBT, MBT and post MBT embryos (Fig 4D). Nonspecific fluorescence was not observed in control embryos

microinjected with H_2O (vehicle, for exogenous Cyclin E) or incubated with secondary Alexa-488 conjugated antibody alone (for both endogenous and exogenous Cyclin E detection, data not shown). Based on these results, we concluded that in agreement with our hypothesis, Cyclin E re-localizes from the cytoplasm to the nucleus at the MBT.

Phosphorylation of T394, S398 or T75 is not required for nuclear localization.

Phosphorylation can serve as a signal for nuclear localization of cyclins (Li et al., 1997). To determine if mutating the phosphorylation sites involved in Cyclin E degradation impairs its nuclear accumulation at the MBT, we used IFA to examine localization of our Cyclin E phospho-mutants. Fig. 4B and C show that T394A S398A and T394D S398D mutants were localized similarly to WT Cyclin E in pre-MBT, MBT and post-MBT embryos. Localization of T394A, T394D, S398A, S398D, and T75A mutants was also similar to WT Cyclin E (data not shown). The absence of obvious mislocalization of Cyclin E mutants does not exclude the possibility that the dynamics of accumulation of these mutants inside the nucleus might be affected, or that phosphorylation (or other post-translational modifications) at undefined sites may be required for nuclear localization.

Cyclin E degradation is dependent on its localization to the nucleus.

In cycling extracts, Xenopus Cyclin E-Cdk2 is taken into the nucleus via direct interaction of Cyclin E with the nuclear pore receptor importin α/β (Moore et al., 1999). A basic nuclear localization signal (NLS) is responsible for nuclear translocation of

Xenopus Cyclin E; RSRKRK spanning residues 24-29 in the N terminus (Moore et al., 2002). To determine if Cyclin E turnover is dependent on nuclear import we co-injected wheat germ agglutinin (WGA) into the embryos together with the WT Cyclin E mRNA. We chose WGA because it blocks nuclear pores (Yoneda et al., 1987) and is routinely used to inhibit nuclear import. First, we empirically determined the concentration of WGA that completely abolished nuclear accumulation of Myc_6-Cyclin E-GFP at the MBT. Figure 5A shows confocal images of live MBT embryos injected with the WT Cyclin E mRNA using a GFP filter. WT Cyclin E is cytoplasmic in WGA-treated embryos compared to the control without the WGA (Fig 5A). Figure 5B shows fixed WGA-treated MBT embryos stained with the Myc antibody and counterstained with DAPI to show nuclei. Blocking nuclear import resulted in an increase of cytoplasmic Cyclin E throughout the entire 25.5 hr time course, as seen in the Western blot in Fig 5C. Moreover, the maximum amount of Cyclin E in WGA injected embryos exceeded the maximum amount of Cyclin E in control embryos by1.6-fold (Fig. 5D). Therefore, we concluded that Cyclin E degradation after the MBT is dependent on its import into the nucleus.

Proteasome inhibition increases cytoplasmic Cyclin E levels.

Since Xenopus Cyclin E degradation requires similar phosphorylation events to that of mammalian Cyclin E degradation, we tested whether Cyclin E degradation is mediated by the 26S proteasome. Cyclin E stability was monitored by Western blot analysis of embryos injected with Clasto-Lactocystin β-Lactone (CLBL), a very specific

and potent inhibitor of the 20S proteasome (Gaczynska and Osmulski, 2005). 20S is a core complex of the 26S proteasome responsible for degradation of ubiquitinated target proteins (Konstantinova et al., 2008). CLBL was co-injected into one cell of a two cell embryo together with WT Cyclin E mRNA. CLBL injection caused progressive accumulation of Cyclin E past the MBT (Fig. 6A and C). In contrast, Cyclin E level in cytoplasmic extracts from control embryos injected with WT cyclin mRNA in DMSO (vehicle for CLBL), decreased following the MBT as expected (Fig 6B and C).

To confirm that CLBL did not perturb nuclear translocation of Cyclin E at the MBT, we examined the localization of WT Cyclin E at the MBT (6 hpf) by IFA. Unexpectedly, we observed that nuclear accumulation of Cyclin E did not occur in the presence of CLBL; Cyclin E remained cytoplasmic (data not shown). Since we established previously that nuclear import is imperative for Cyclin E degradation starting at the MBT, it remained unclear if cytoplasmic accumulation of Cyclin E following CLBL treatment was a direct result of proteasome inhibition or a nonspecific impairment of nuclear import. Therefore we tested whether a chemically distinct inhibitor, the peptide aldehyde MG132, could impede Cyclin E degradation by the proteasome without adversely affecting its nuclear import. Similar to CLBL, MG132 inhibited destruction of Cyclin E in a dose-dependent manner (Fig 6D-F). Embryos microinjected with 20 μM MG132 exhibited maximal accumulation of Cyclin E at the MBT (6 hpf) followed by a rapid decrease (Fig 6D). Although Cyclin E accumulated to higher levels up to the MBT in embryos microinjected with 20 μM MG132 compared to control DMSO injected embryos (compare Fig 6D with 6B), it was still efficiently eliminated following the

MBT, suggesting that the capacity of the cellular degradation machinery was not compromised by Cyclin E overexpression alone. At an MG132 concentration of 30 μM, there was a significant increase in Cyclin E remaining in the cytoplasm after the MBT (Fig 6E). 40 μM of MG132 completely inhibited Cyclin E destruction after the MBT causing further accumulation of Cyclin E in the cytoplasm throughout the duration of the time course, until it reached its maximum at 12 hpf (Fig 6F and G). IFA of fixed embryos showed that 40 μM of MG132 allowed proper nuclear redistribution of WT Cyclin E at the MBT (6hpf) (Fig 6H), same as observed in control DMSO injected embryos (Fig 6I). Neither CLBL nor MG132 treated embryos survived past 12 hpf, likely due to the previously reported induction of apoptosis via multiple secondary mechanisms (Mimnaugh et al., 2001). Our findings suggest that the reduction in Cyclin E levels after the MBT is mediated through proteasomal degradation.

Cyclin E is ubiquitinated *in vivo* starting at the MBT.

Phosphorylation of human Cyclin E marks it for ubiquitination and degradation by the 26S proteasome (Clurman et al., 1996; van Drogen et al., 2006; Won and Reed, 1996). Since both phosphorylation and the proteasome appear to play a role in Cyclin E turnover in *Xenopus laevis* embryos, we next asked whether proteasomal degradation of Cyclin E is preceded by its ubiquitination. Detection of ubiquitination of endogenous proteins is problematic due to their rapid degradation via proteasomal catabolism and trimming of linear or branched ubiquitin chains by ubiquitin-cleaving isopeptidases (Weissman, 2001). To overcome this limitation, we overexpressed WT Cyclin E and

overexposed Western blots to detect higher molecular weight ubiquitinated isoforms of Cyclin E in cytoplasmic embryo extracts. These isoforms were detected starting at MBT (6 hpf), but not in pre-MBT embryos collected at 4 hpf (Fig 7A). These slower migrating forms become more prominent with progression of time at 8 and 10 hpf and no bands were observed in uninjected control embryos (data not shown).

To further confirm the presence of ubiquitinated Cyclin E, we immunoprecipitated WT Cyclin E from cytoplasmic extracts treated with MG132 to inhibit destruction of ubiquitinated proteins. Half of the immunoprecipitated protein was subjected to Western blotting with anti-Myc and half with anti-ubiquitin, Lys-48-specific, a modification associated with proteasomal degradation. In this experiment slower migrating forms of Cyclin E were detected by immunoblotting with anti-Myc starting at the MBT in 6 and 8 hpf samples, but not at 4 hpf (pre-MBT) (Fig 7C, αMyc). No signal was detected in precipitates using control IgG (Fig 7C, IgG) or in anti-Myc immunoprecipitates from embryos in which WT Cyclin E mRNA was not injected (Fig 7C, αMyc, Un lane). Cyclin E was efficiently immunoprecipitated from the extracts since only trace amounts of it remained in the supernatants after the anti-Myc IP as compared to the IgG IP and the total before IP (Fig 7D, compare with total, Fig 7B). We also observed a series of higher molecular weight isoforms in the ubiquitin blots from MBT (6 hpf) and post-MBT (8 hpf) embryos, but not in pre-MBT (4 hpf) and uninjected embryos (Fig 7E), confirming that Cyclin E is ubiquitinated beginning at the MBT.

Discussion

The objective of this study was to define the mechanism of maternal Cyclin E degradation in *Xenopus laevis* embryos. Xenopus Cyclin E is 90% identical to human Cyclin E at the nucleotide level, and the phosphorylation sites responsible for human Cyclin E turnover are conserved in the Xenopus protein. Therefore, we tested the hypothesis that the mechanism of Cyclin E degradation is conserved between the two species. We provide evidence that timely destruction of Xenopus Cyclin E at the MBT is dependent on phosphorylation, is preceded by its ubiquitination, and occurs via the proteasomal pathway. These results suggest that the mechanism of Cyclin E downregulation is conserved between species. Unlike spatial regulation of Cyclin E in humans, for which interplay between localization and degradation is far from being completely understood (Delk et al., 2009; Grim et al., 2008; Welcker et al., 2004; Ye et al., 2004), in Xenopus embryos Cyclin E is relocalized from the cytoplasm to the nucleus preceding its degradation and this relocalization is required for its turnover.

Point mutations of conserved phosphorylation sites S398, T394 and T75 to unphosphorylatable alanine each decrease Xenopus Cyclin E turnover to various extents, reflecting the individual importance of each residue in this process. S398A and T394A mutations each render Cyclin E more resistant to degradation than T75A, while the double T394A S398A mutation completely stabilizes Cyclin E, resulting in its accumulation in the cytoplasm past the MBT. Mutation of the above-mentioned residues to phosphomimetic aspartic acid restores Cyclin E turnover. There are several examples of multiple phosphorylation events being required for efficient protein degradation,

including human Cyclin E. Structural analysis of human Cyclin E bound to Skp1-hCdc4 complex identified simultaneously phosphorylated T380 and S384 motif as an optimal high affinity degron (Hao et al., 2007). Individual phosphorylation of these residues mediates only weak binding to Cdc4, which can be compensated for by Cdc4 dimerization, resulting in increased ubiquitination of Cyclin E. Complete stabilization of the Xenopus Cyclin E T394A S398A mutant (corresponding to T380 and S384 of human Cyclin E) compared to partial stabilization of the single mutants provides evidence that the requirement for multiple phosphorylation events for efficient turnover is conserved between species. Another example is the cdk inhibitor Sic1, a substrate of SCF^{Cdc4} in yeast. Sic1 contains nine suboptimal phosphorylation sites, of which a minimum of six need to be phosphorylated for tight binding to a single phosphodegron binding site on Cdc4. Each separate phosphorylation event is suboptimal for recognition by Cdc4, which delays Sic1 degradation (Nash et al., 2001; Orlicky et al., 2003) and sets the threshold of cdk activity (the kinase responsible), creating an ultrasensitive response. This flexibility of substrate recognition allows fine-tuning of irreversible regulatory switches. In the case of transcription factors IkBα and β-catenin, phosphorylation of two serines in their destruction motif serves as a prerequisite for the Fbox protein β-TRCP binding (Wu et al., 2003).

In addition to the requirement for phosphorylation for Cyclin E turnover, Cyclin E is cytoplasmic prior to the MBT and begins to accumulate in the nucleus in MBT embryos. The timing of this change in localization is consistent with a possible role in facilitating Cyclin E turnover, and agrees with the previously reported nuclear

localization of Cyclin E in MBT Xenopus embryos (Chevalier et al., 1996). Phosphorylation often serves as a signal for nuclear localization of proteins and is important for Cyclin E degradation, yet the phosphomutants examined were correctly localized to the nucleus at the MBT. This result suggests that these phospho-sites are not required for Cyclin E nuclear accumulation. We cannot exclude the possibility that changes in the rate of nuclear import versus nuclear export of Cyclin E is altered in these mutants, skewing their nuclear to cytoplasmic distribution, which our experiments may not have the sensitivity to detect. Interestingly, an increased nuclear to cytoplasmic ratio was previously observed for the S384A human Cyclin E mutant (corresponding to the most stable of our single phosphorylation mutants in Xenopus, S398A), which logically resulted in its decreased turnover by Cdc4 β, the cytoplasmic isoform of Cdc4 (Ye et al., 2004). The reason for its preferential accumulation in the nucleus was not investigated.

Phosphorylation on specific sites can facilitate nuclear export, affecting its rate. For instance, phosphorylation of the transcription factor Pho4 and of Cyclin D promotes their binding to their nuclear exportins, Msn5 (Kaffman et al., 1998) and CRM-1 (Alt et al., 2000), respectively. Similar to nuclear export, nuclear import can also be positively and negatively regulated by phosphorylation. Phosphorylation of chicken lamin B2 (Hennekes et al., 1993), yeast transcription factor SWI5 (Moll et al., 1991) and SV40 T antigen (Jans et al., 1991) promotes their retention in the cytoplasm. Phosphorylation of cytoplasmic retention signals can also promote translocation into the nucleus, as is seen for Xenopus cyclin B1 at the beginning of M phase. Given the significant increase in cytoplasmic Cyclin E phosphorylation at the MBT, when it begins to accumulate in the

nucleus, another possibility is that phosphorylation of different residue(s) from the ones tested in our study facilitate nuclear import. Once in the nucleus, other phosphorylation events could facilitate its export into the cytoplasm for ubiquitination and degradation.

In addition to the potential phosphorylation dependent mechanisms that can influence its subcellular localization, Xenopus Cyclin E contains a basic NLS (RSRKRK), spanning residues 24-29 in its N-terminus. This sequence is thought to be essential for nuclear accumulation of Cyclin E-Cdk2 complexes during S-phase in egg extracts, promoting DNA replication (Moore et al., 2002). Cdk2 does not possess its own NLS and "piggy-backs" into the nucleus with Cyclin E (Jackman et al., 2002). In contrast, nuclear localization of human Cyclin E cannot be attributed entirely to its NLS (Delk et al., 2009; Geisen and Moroy, 2002; Kelly et al., 1998; Porter et al., 2001). A recent study compared the intracellular localization of Cyclin E with that of Cyclin E truncation mutants missing the NLS, also known as low molecular weight (LMW) Cyclin E isoforms (Delk et al., 2009). This study showed that some LMW isoforms are cytoplasmic while others are still nuclear, despite of their lack of an NLS. We speculate that nuclear accumulation of Cyclin E even in a simple model such as Xenopus embryos might not be as straightforward as what current knowledge suggests, with both phosphorylation and NLS dependent mechanisms involved. The role of individual phosphorylation sites and the corresponding kinases involved in this process remain to be elucidated.

Degradation of Xenopus Cyclin E was dependent on its nuclear accumulation at the MBT. It was previously reported that Xenopus Cyclin E degradation is impaired by

activating the MAPK pathway in early embryos (Howe and Newport, 1996). More recent work has shown that activation of the MAPK pathway decreases classical nuclear import of NLS containing proteins (Faustino et al., 2008). Taken together with our results, its possible that MAPK activation may impair Cyclin E degradation by impeding its nuclear import. In support of this, the bulk of MAPK is inactivated after fertilization in Xenopus but is active at this time of development, participating in mesoderm induction (Gotoh et al., 1995; Hartley et al., 1994)).

Destruction of several cell cycle regulators has been shown to depend on their phosphorylation dependent translocation into or out of the nucleus. For example, the mammalian Cdk2 inhibitor $p27^{Kip1}$, is phosphorylated by Cdk2 inside the nucleus, which triggers its export into the cytoplasm and subsequent degradation (Muller et al., 2000). Mammalian cyclin D is also exported out of the nucleus at the beginning of S phase, after being phosphorylated by GSK-3β and is then degraded by the 26S proteasome in the cytoplasm (Alt et al., 2000). Interestingly, both Cdk2 and GSK-3β phosphorylate human Cyclin E, on S384 and T380, respectively, triggering its ubiquitination and degradation (Welcker et al., 2003). Moreover, in Xenopus Cyclin E, T75 fits the consensus site for Cdk2, T/SPXK/R (Kitagawa et al., 1996), while a C-terminal cluster starting with S386 and ending with S398 that also contains T390 and T394, presents a perfect match for the unique substrate specificity requirement of GSK-3β (Kim and Kimmel, 2000). Phosphorylation or "priming" of the substrate at a serine or threonine located at the +4 position from the GSK-3β site allows GSK-β to phosphorylate its target residue, which in turn can serve as a priming event for the subsequent phosphorylation of another target

residue four residues upstream. Therefore, Cdk2 and GSK-3βare also primary candidates for marking Xenopus Cyclin E for MBT destruction by phosphorylation.

Finally, we show that Cyclin E is ubiquitinated at the MBT and is degraded by the proteasomal pathway. Proteasome inhibitors CLBL and MG132 abolished Cyclin E turnover after the MBT, resulting in progressive increase of its cytoplasmic abundance. Unlike MG132, CLBL treatment also prevented nuclear accumulation of Cyclin E at the MBT. Since we established that nuclear import is necessary for Cyclin E degradation, CLBL might be acting via this nonspecific but relevant mechanism, rather than proteasome inhibition. Support for this idea is provided by our observation that ubiquitination of Cyclin E at and after the MBT is abolished in CLBL injected embryos (data not shown). This observation also suggests that Cyclin E is ubiquitinated in the nucleus, or that nuclear passage is necessary for its ubiquitination. Cyclin E may first be modified by phosphorylation in the nucleus, similar to cyclin D1 (Alt et al., 2000), and then ubiquitinated either in the nucleus or in the cytoplasm. Blocking Cyclin E nuclear export would allow us to test where ubiquitination and subsequent degradation of Xenopus Cyclin E take place. However, unlike cyclin D1 or cyclin B1 (Hagting et al., 1998; Jackman et al., 2002) export of Cyclin E from the nucleus is independent of the nuclear export receptor CRM-1 and is thus insensitive to the CRM-1 inhibitor leptomycin B, which blocks nuclear export of these cyclins (Jackman et al., 2002). Therefore resolution of these important questions awaits the ability to prevent nuclear export of Xenopus Cyclin E as well as studies on the localization of Xenopus Cdc4 isoforms.

Why is Cyclin E constitutively expressed in early embryos yet restricted to the G1/S transition in the adult cell cycle? Several studies have examined the role of Cyclin E during early development. In mice, elimination of both E1 and E2 cyclins results in early embryonic lethality via endoreplication defects in placental trophoblast giant cells (Geng et al., 2003). During endoreplication, cells undergo repeated rounds of DNA replication without subsequent mitoses resulting in formation of giant nuclei containing up to 1000 N of DNA (Zybina and Zybina, 1996). As was mentioned earlier, Cyclin E also regulates endoreplication in flies; a change in Cyclin E expression from constitutive to the G1/S phase only mediates the transition from a mitotic to an endoreplicative cell cycle during Drosophila embryogenesis (Knoblich et al., 1994; Richardson et al., 1993). Constitutively expressed Cyclin E1 in sea urchin embryos is not required for initiation of DNA replication, but functions in sperm maturation (Schnackenberg et al., 2007) and in centrosome duplication (Schnackenberg et al., 2008), as does Cyclin E in mammalian cells, Xenopus extracts, and *C. elegans* embryos (Cowan and Hyman, 2006; Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999). Moreover, both Drosophila and Xenopus embryos lack G phases when Cyclin E is constitutively present (Rempel et al., 1995; Richardson et al., 1993), and acquire them when Cyclin E is periodically expressed. In mammalian fibroblasts, constitutively overexpressing Cyclin E shortens G1 phase, leading to premature S phase entry (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994). These data point to a requirement for Cyclin E periodicity to acquire and maintain a normal G1 phase. During the first embryonic cleavages when the zygote produces an increasing number of genomically identical but smaller cells, the best way to complete

this simple and redundant task is to keep divisions rapid and synchronous. After the MBT, the embryo undergoes gastrulation and patterning/differentiation, which require specialized expression of various genes in different cells provided by the beginning of global transcription. In Xenopus and other embryos, cell cycle regulators, such as cyclins and cdks are now expressed in tissue specific patterns that are unique for each cell cycle component (Vernon and Philpott, 2003). The cell cycle becomes asynchronous and includes G phases to accommodate these requirements for the individuality and complexity within different cells and tissues. Ubiquitination provides a rapid way of eliminating maternal Cyclin E whose constant presence throughout the cell cycle is no longer required for pushing cells through the accelerated mitotic divisions and therefore serves a cell cycle remodeling function.

Cyclin E is often expressed constitutively in cancer cells as well, consistent with the increasing evidence that disease often results when developmental programs are activated at the wrong time and place. The pre-MBT embryonic cell cycle with its rapidity, constitutively high Cyclin E, lack of cdk inhibitors, and mitogen independence due to lack of cyclin D, resembles the cancer cell cycle. At the MBT regulatory mechanisms “kick in”, maternal Cyclin E is promptly destabilized, the cell cycle is remodeled to include gap phases, and cyclin D and the cdk inhibitor p27 are produced. Cells in the embryo now have the ability to arrest and undergo apoptosis in response to DNA damage. In contrast, as some cells advance towards tumorigenesis, their cell cycle loses checkpoint controls, becoming rapid and autonomic as they lose growth factor dependency due to Cyclin E overexpression and/or loss of cdk inhibition.

In normal mammalian cells, Cyclin E-Cdk2 complexes are nuclear, as are Cdc4 α and γ (van Drogen et al., 2006; Ye et al., 2004). In primary breast tumors, Cyclin E is often elevated in the cytoplasm (Shaye et al., 2009). This altered localization has been suggestesd as a novel form of deregulation on par with Cyclin E overexpression. Furthermore, LMW isoforms of Cyclin E, but not full length Cyclin E, are often localized to the cytoplasm, although some are still nuclear despite of their lack of an NLS (Delk et al., 2009). These LMW isoforms induce high Cdk2 activity and are proposed to have amplified oncogenic activity via aberrant protein-protein interactions and regulation due to their altered localization (Delk et al., 2009). They are also less susceptible to degradation mediated by Cdc4 nuclear subtypes α and γ. Further elucidating the signals directing Cyclin E into the nucleus for degradation may increase our understanding of how deregulation occurs in human cancer cells, leading to a skewed nuclear to cytoplasmic distribution of Cyclin E. The regulation of spatial distribution is an important issue in regard to orchestration of Cyclin E degradation in Xenopus development and the relevance of its disruption to malignant transformation in human cells.

Acknowledgements

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P20 RR11830, NCI P30 CA118100, NCRR S10 RR19287, NCRR S10 RR016918, the UNM HSC, and the UNM Cancer Center. The Cyclin E antibody was a kind gift of Dr. Michel Philippe (University of Rennes, France).

Tables

Table 1. PCR primer pairs for cloning and site-directed mutagenesis of Cyclin E	
Mutant	Primer Pair
Cloning ORFs out of pet21A	Forward: CACGAATTCTGCACCAGTGATAAGCAATCCTGC Reverse: CCGTCTAGAGTCTGCTCGATCAGTTTT
S398A	Forward: GGTGTTCTGACTCCTCCCCAGGCGAACAAGAAACAGAAATCTGATC Reverse: GATCAGATTTCTGTTTCTTGTTCGCCTGGGGAGGAGTCAGAACACC
S398D	Forward: GGTGTTCTGACTCCTCCCCAGGATAACAAGAAA CAGAAATCTGATC Reverse: GATCAGATTTCTGTTTCTTGTATCCCTGGGGAGGAGTCAGAA CACC
T394A	Forward: GGC GAG GCG CCA GAA CAC Reverse: GTGTTCTGGCGCCTCCCC
T394D	Forward: GTTCTGGATCCTCCCCAGAG Reverse: CTCTGGGGAGGATCCAGAACACC
S398A T394A	Forward: GTTCTGGCGCCTCCCCAGGCTAACAAGAAACAGAAATCT Reverse: AGATTTCTGTTTCTTGTTAGCCTGGGGAGGCGCCAGAAC
S398D T394D	Forward: GTGTTCTGGATCCTCCCCAGGATAACAAGAAACAGAAATCTG Reverse: CAGATTTCTGTTTCTTGTTATCCTGGGGAGGATCCAGAACAC
T75A	Forward: GTTCCTCTTTTTCTGGAGCAGGAATCAATTTGTGGGG Reverse: CCCCACAAATTGATTCCTGCTCCAGAAAAAGAGGAAC

Figures and Figure Legends

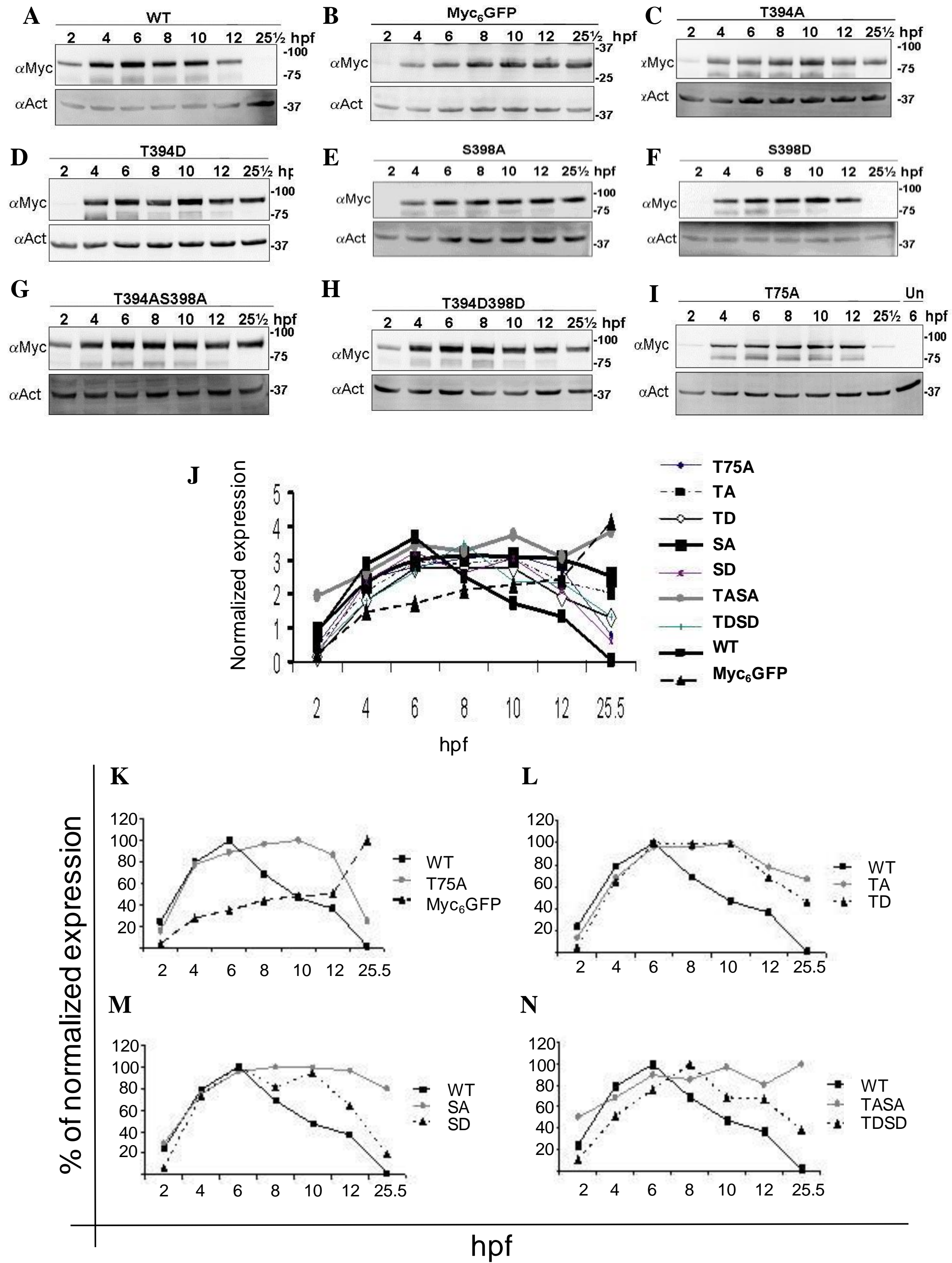


Figure 1. Differential phosphorylation regulates Cyclin E stability in *Xenopus laevis* embryos**.** Western blot analysis of cytoplasmic extracts from embryos injected with mRNA encoding A) exogenous wild type (WT) Cyclin E, B) Myc_6-GFP, C-I) Cyclin E phosphorylation mutants, as indicated. 2-cell embryos (1.5-2 hours post fertilization, hpf) were microinjected with 0.5 ng of *in vitro* transcribed mRNA and collected at time points indicated on the top of each blot (hpf). One embryo equivalent was analyzed by immunoblotting with a Myc antibody (αMyc). Blots were stripped and re-probed for actin (αAct) as a loading control. In panel I, Un indicates uninjected embryos used as a negative control. Markers are to the right in kDa. Representative blots are shown from five experiments for A, D, F, and I; four experiments for C, and 3 experiments for B, E, G, and H. J) Graph of normalized Cyclin E level for experiments represented in panels A-I, averaged over all experiments. K-N) Graphs showing Cyclin E levels as percent of normalized expression based on mean values from all experiments. For each plot the maximal normalized value was taken as 100% and relative intensities of all bands were represented as percent of normalized expression. Each graph has WT Cyclin E plotted for easy comparison.

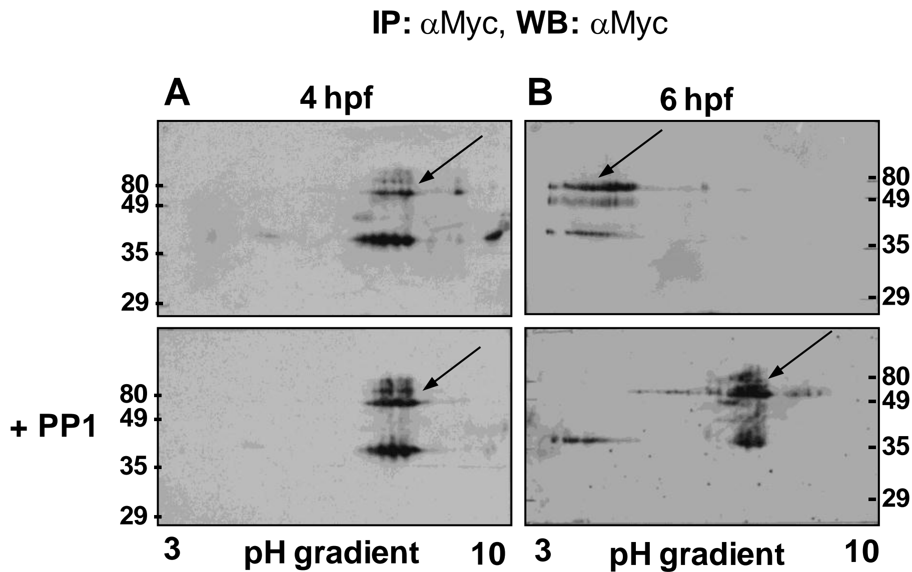


Figure 2. Phosphorylation of cytoplasmic Cyclin E is dramatically increased at the MBT. Two-dimensional gel electrophoresis followed by Western blot analysis of WT Cyclin E immunoprecipitated from pre-MBT (4 hpf) and MBT (6 hpf) embryos. Embryos were injected with WT Cyclin E mRNA and cytoplasmic extracts subjected to immunoprecipitation with Myc antibody. Half of the immunoprecipitate was treated with protein phosphatase 1, (+PP1). Samples were subjected to isoelectric focusing, electrophoresed on 10% SDS-polyacrylamide gels, and transferred to PVDF membrane followed by immunoblotting with Myc antibody. Markers are indicated on the left and right in kDa. Direction of pH gradient (3 to 10) is displayed at bottom. Arrows indicate position of full length Myc-Cyclin E on blots. IP, immunoprecipitation,WB, Western blot. Experiment was repeated 2 times.

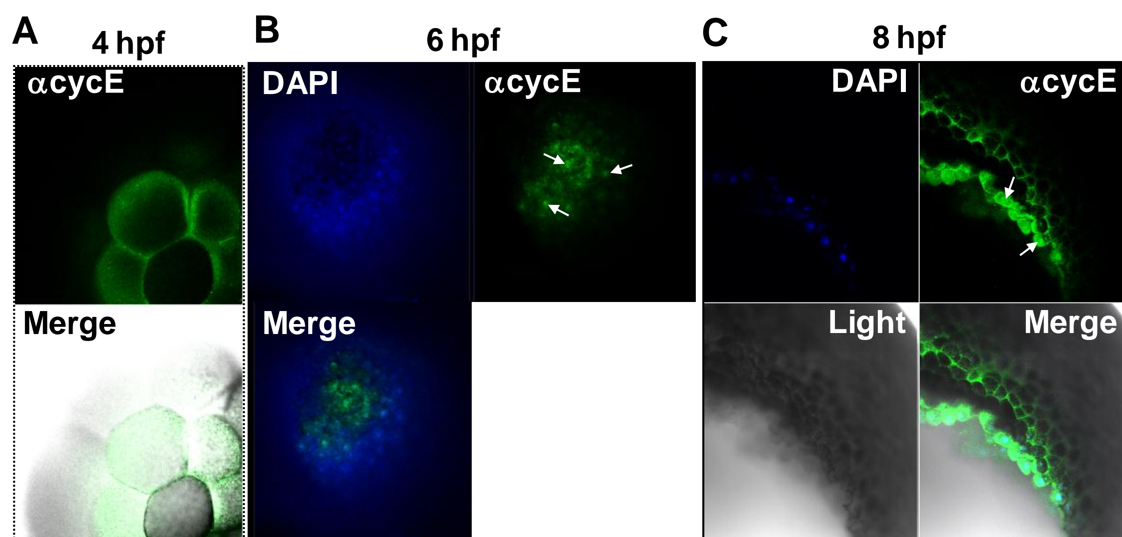


Figure 3. Endogenous Cyclin E accumulates in the nucleus at the MBT. Immunofluorescence analysis of endogenous Cyclin E in A) pre-MBT (4 hpf), B) MBT (6 hpf), and C) post-MBT (8 hpf) embryos. Embryos were fixed and stained with an antibody against Xenopus Cyclin E (αcycE) followed by an Alexa488 conjugated secondary antibody. Merge panel in A is a merged image of Alexa488 and the light channel, in B, Alexa488 and DAPI, and in C, Alexa488, DAPI and light. Magnification is 10X for A and B; 20X for C. White arrows show nuclei stained for Cyclin E. Experiment was performed 3 times.

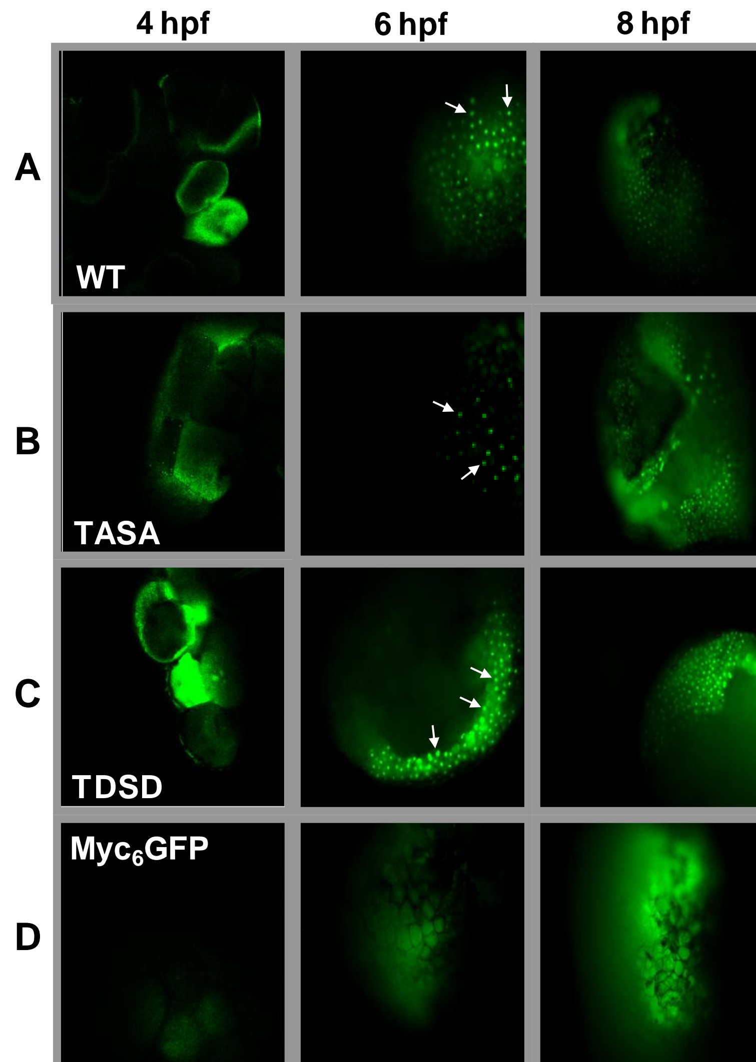


Figure 4. Exogenous Cyclin E and its phosphorylation mutants accumulate in the nucleus at the MBT. Immunofluorescence analysis of WT Cyclin E in embryos. Embryos were injected with the indicated mRNA at the 2-cell stage, fixed at the time indicated at the top of the figure, and stained with anti-Myc followed by an Alexa488-conjugated secondary. A) WT: wild type Cyclin E, B) TASA: T394A S398A, C) TDSD: T394D S398D and D) Myc_6-GFP. White arrows mark individual nuclei. Magnification is 10X. Experiment was performed 3 times.

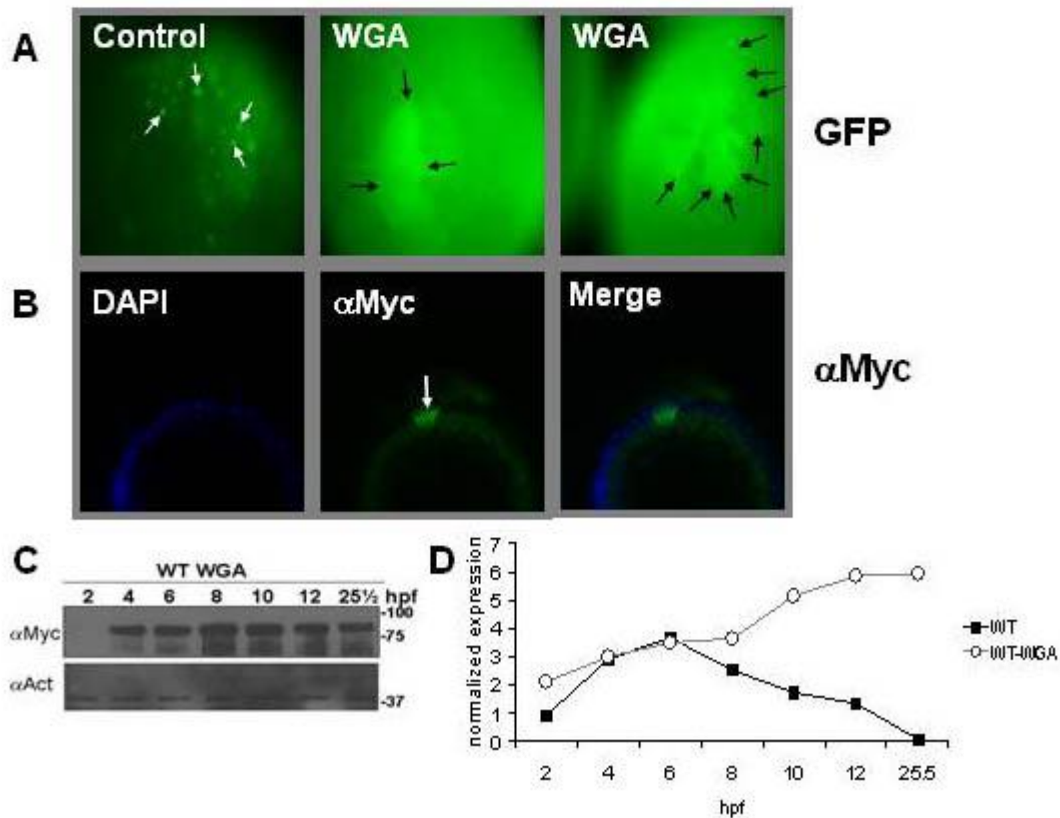
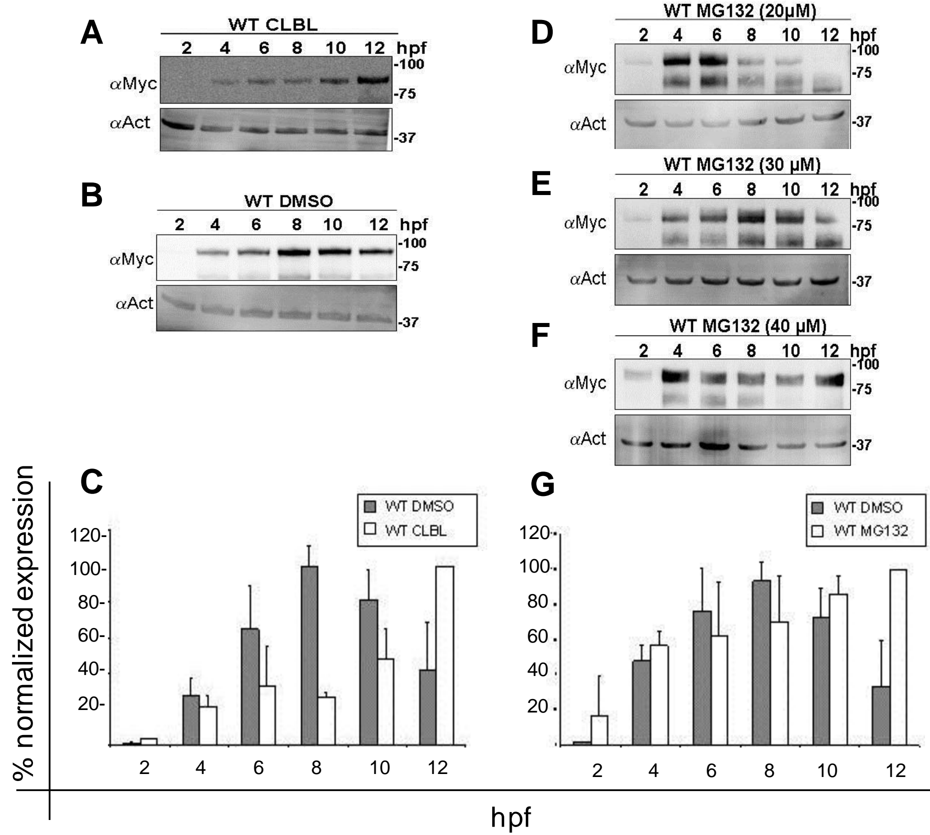


Figure 5. Blocking nuclear import prevents exogenous Cyclin E degradation. 2-cell embryos were microinjected with 0.5 ng of *in vitro* transcribed WT Cyclin E mRNA together with wheat germ agglutinin (235 nM final concentration) and collected at the indicated time points (hpf). A) Live confocal images of embryos showing localization of WT Myc_6-Cyclin E-GFP at the MBT (6 hpf) without WGA (Control) and with WGA (WGA) viewed through a GFP filter. White arrows indicate nuclei, black arrows outline cells within the embryo expressing WT Cyclin E localized in the cytoplasm. B) MBT (6 hpf) embryos injected with WT Cyclin E mRNA in the presence of WGA were fixed and stained with αMyc followed by an Alexa488-conjugated secondary. Nuclei were counterstained with DAPI (DAPI). White arrow shows cells expressing WT Cyclin E in the cytoplasm. Magnification is 10X. C) αMyc Western blot of cytoplasmic lysates from embryos injected with WT Cyclin E mRNA and WGA. Blots were stripped and re-probed for actin (αAct) as a loading control. Markers are on the right in kDa. D) Graph showing mean values (from five experiments) of Cyclin E band intensity normalized to Actin in WGA treated embryos compared to not treated controls.


A
WT CLBL
2 4 6 8 10 12 hpf
αMyc
αAct
-100
-75
-37
B
WT DMSO
D
WT MG132 (20μM)
E
WT MG132 (30 μM)
F
WT MG132 (40 μM)
C
WT DMSO
WT CLBL
G
WT MG132
% normalized expression
hpf

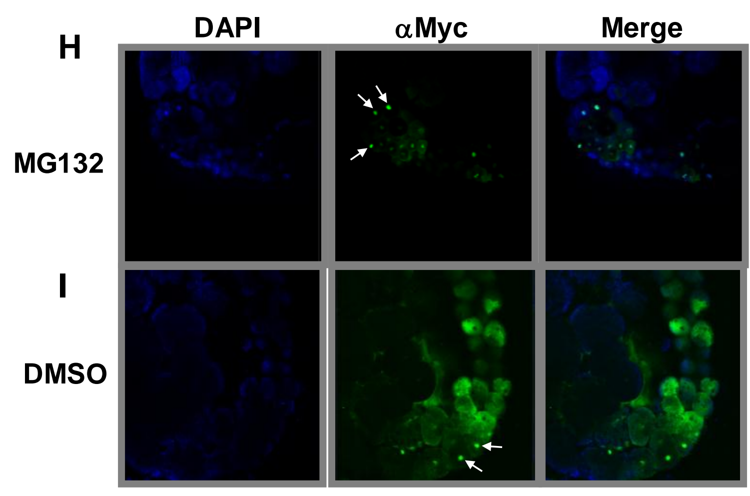

DAPI
αMyc
Merge
H
MG132
I
DMSO

Figure 6. Proteasome inhibition prevents Cyclin E degradation. A, B, D-F: Myc Western blots of cytoplasmic extracts from injected embryos collected at the indicated time (hpf). 2-cell embryos were microinjected with 0.5 ng of *in vitro* transcribed WT Cyclin E mRNA with A) 185 μM of Clasto-Lactocystin β-Lactone (CLBL) in DMSO, B) DMSO, D) 20 μM MG132 in DMSO, E) 30 μM MG132 in DMSO, and F) 40 μM MG132 in DMSO. Blots were stripped and re-probed for actin (αAct) as a loading control. Markers are on the right in kDa. Representative blots from 3 experiments are shown. C and G) Graph of experiments shown in A and F, respectively. Cyclin E levels were normalized to actin and expressed as percent of normalized expression. Values shown are mean ± SEM. Immunofluorescence analysis of WT Cyclin E in 6 hpf (MBT) embryos also injected with H) 40 μM of MG132 or I) DMSO. Embryos were fixed and stained with αMyc followed by an Alexa488-conjugated secondary. Nuclei were counterstained with DAPI. Nuclei containing WT Cyclin E are marked with white arrows.

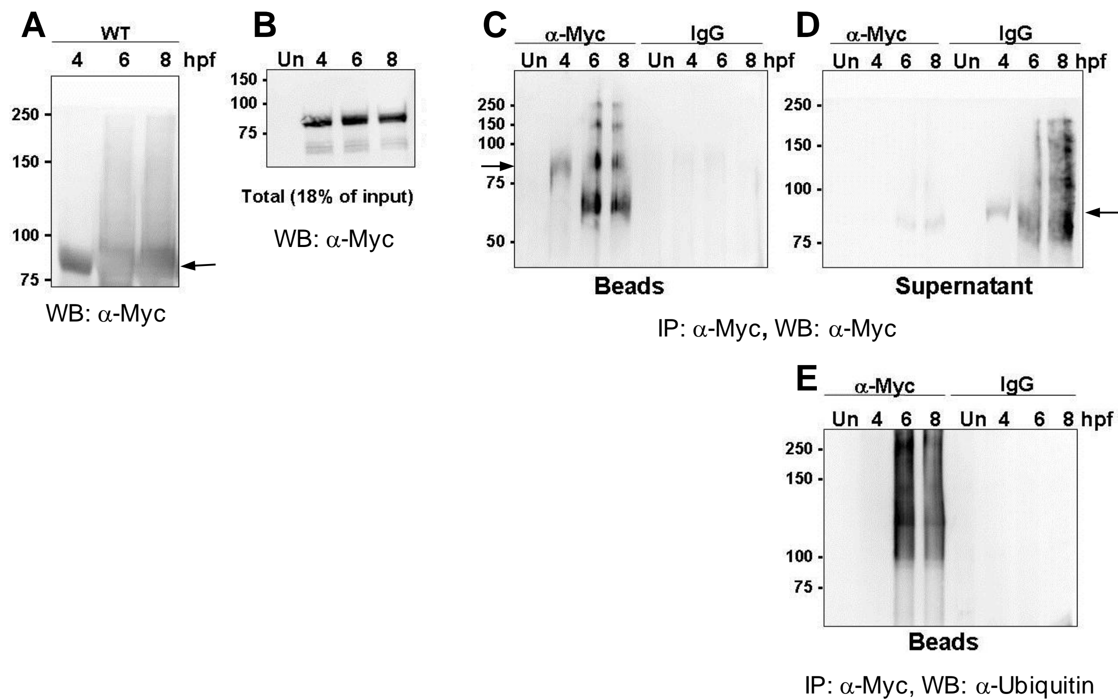


Figure 7. Cyclin E is ubiquitinated at the MBT. Western blots of 2-cell embryos. microinjected with WT Cyclin E mRNA and collected at the indicated times (hpf). Cytoplasmic lysates were prepared in the presence of 20 μM MG132. A) Lysates from four and a half embryo equivalents were immunoblotted with Myc antibody. B) Aliquots of cytoplasmic lysates from embryos that were used for immunoprecipitation (IP) in C were immunoblotted with anti-Myc to show total amount of Cyclin E in the lysates before the IP. C-E) Myc immunoprecipitation of 7.5 embryo equivalents. Precipitation was performed with αMyc or normal mouse IgG as a control. Half of the precipitate was analyzed by Western blotting for Myc (C) and half for ubiquitin (E) D) Supernatants (non-precipitated protein) were also blotted for Myc to determine the amount of Cyclin E remaining in the lysates after IP. Un indicates uninjected 6 hpf embryos. Arrows indicate mobility of nonubiquitinated full length WT Cyclin E. Markers are on the left in kDa. IP, immunoprecipitation, WB, Western blot. Experiment was repeated 3 times.

Chapter 3. Quantum dot labeling of Cyclin E for studying its intracellular localization in *Xenopus laevis* embryos.

Abstract

Luminescent semiconductor nanocrystals, also known as quantum dots (QD), possess highly desirable properties that account for development of a variety of exciting biomedical techniques. To date, QDs have been used visualize the movement of cell surface receptors in real time. We set out to use QD to track the movement of an intracellular protein. We hypothesized that a change in localization of a cell cycle regulator, Cyclin E, contributes to its developmental downregulation at the midblastula transition (MBT) in *Xenopus laevis* embryos. Downregulation of Cyclin E contributes to remodeling of the rapid embryonic cell cycle to the slower adult somatic cell cycle. In order to test our hypothesis we developed a novel method of imaging His_6-tagged Cyclin E in living embryos by attaching it to dihydrolipoic acid (DHLA) capped (CdSe)ZnS quantum dots (QD). Since QD-His_6 protein complexes are stable at physiological pH, they can be microinjected and imaged inside Xenopus embryos *in vivo*. We successfully synthesized and capped the QDs and used them to localize QD-His_6 Cyclin E inside live developing embryos in real time. Consistent with our data from immunofluorescence analysis, QD-His_6 Cyclin E was present in the cytoplasm in early embryos, before the midblastula transition (pre-MBT, 4 hpf) and started to accumulate inside the nucleus at the MBT. Surprisingly, our control QD, uncoupled to His_6 Cyclin E also localized to the nuclei at the MBT, which cannot be attributed to their surface chemistry. Future studies

will use a mutant Cyclin E missing its nuclear localization signal, as well as other known cytoplasmic proteins coupled to QD as controls, to validate that the movement of Cyclin E into the nucleus at the MBT is specific. They will also determine the mechanism directing QD accumulation in the nucleus at the MBT.

Introduction

Luminescent quantum dots (QD) are semiconductor nanocrystals that have unique spectroscopic properties. They have generated a lot of interest in the past two decades, with current and projected applications including use as fluorescent labels for cellular labeling, intracellular sensors, deep-tissue and tumor imaging agents, sensitizers for photodynamic therapy, and as vectors for studying nanoparticle-mediated drug delivery (Delehanty et al., 2009b). The advantages of QDs over dyes and genetically engineered fluorescent proteins for tagging biomolecules are their broad excitation spectra, narrow emissions, brightness, resistance to photobleaching, and the fact that they can be synthesized from the same material to emit a variety of wavelengths (Jaiswal et al., 2004). QD have size tunable fluorescent properties, meaning that increase in QD size results in different colors of the light that they emit. Larger and smaller dots emit redder (lower energy) and bluer (higher energy) light, respectively. Multiple molecules can be labeled with QD of various colors and simultaneously imaged after being excited with a single UV source which prevents overheating of cells, a quality desirable for both *in vitro* and *in vivo* applications.

QD drawbacks include their blinking (intermittency) under continuous excitation and their cytotoxicity attributed to heavy metals in their chemical composition. Inorganic QD containing Cd, Se, Zn, Te, Hg, and Pb can have toxic, neurotoxic and teratogenic effect, depending on their dosage, if they accumulate in the nervous system or liver. Tests on primary hepatocytes showed that exposure of CdSe core QD to an oxidative environment resulted in the formation of reduced Cd on the QD surface and release of free Cd ions that led to cytotoxicity via conventional mechanisms of heavy-meteal toxicity (Derfus et al., 2004). Adding 1-2 monolayers of a ZnS protective shell reduced the oxidation, but some cytotoxicity was still observed followed 8 hrs of photooxidation. If a safe proof sealing of the heavy metal core is achieved for *in vivo* applications, the main question becomes the metabolic clearing of nanoparticles from the body, about which very little is known so far (Colvin, 2003). Other alarming evidence includes cytotoxic effects of various surface modifications (Kirchner et al., 2005) and the DNA damaging potential of QD (Green and Howman, 2005). Therefore, assessment of cytotoxicity and potential adverse effects of chemically distinct QD in living organisms remains very desirable for the overall progress of the nanoscience field.

Colloidal nanocrystals most often used in fundamental or applied studies are spherical, with core sizes varying between 15 and 120 A in diameter. CdSe nanoparticles are prepared by reacting organometallic precursors at high temperatures in a coordinating solvent mixture resulting in capping of the inorganic core with an organic layer of trioctyl phosphine/trioctyl phoshine oxide mixture (TOP/TOPO) (Peng and Peng, 2001). Overcoating of the CdSe core with several layers (3-5) of wider band gap semiconducting

material, such as ZnS or CdS permits passivation of the core surface and produces highly luminescent CdSe-ZnS or CdSe-CdS core-shell QD (Clapp et al., 2006). In addition, the shell protects the QD from oxidation and prevents oozing of heavy metal core components out into the environment (Medintz et al., 2005). For biological applications, QD need to be soluble in water. Several methods have been used, such as exchange of native TOP/TOPO organic surface ligand for a water soluble one (cap exchange) (Chan and Nie, 1998), encapsulation within amphiphillic molecule-copolimer micelles (Dubertret et al., 2002), and coating with silica (Bruchez et al., 1998). We utilized the cap exchange strategy, exchanging TOP/TOPO with dihydrolipoic acid (DHLA). DHLA is a bifunctional ligand, containing a bidentate thiol moiety on one end, allowing its stable attachment to the inorganic QD surface, and an opposing hydrophilic end group, which permits its aqueous dispersion (Mattoussi et al., 2002) (Fig. 1A). These DHLA-capped QD still maintain their high photoluminescence and quantum yield.

QD have been extensively used for tagging recombinant proteins *in vitro* (Mattoussi et al., 2000), and for tagging extracellular and cell surface proteins, specifically receptors (Andrews et al., 2008; Gao et al., 2004; Lidke et al., 2004), which can be used as bioactive fluorescent probes in sensing, imaging, immunoassay and other diagnostic applications (Delehanty et al., 2009a). Here we propose to use nanoparticles for imaging of an intracellular protein.

Cyclin E has been localized to nuclei in Xenopus cell-free extracts (Moore et al., 2002) and midblastula (6 hour post-fertilization) embryos (Chevalier et al., 1996). Cyclin E has been shown to shuttle between the nucleus and cytoplasm in mammalian cells

using live cell imaging of GFP-tagged protein (Jackman et al., 2002). We hypothesized that a change in Cyclin E localization contributes to its destabilization at a specific developmental timepoint, the midblastula transition (MBT). Our objectives were first, to synthesize DHLA-capped Cd/Se/ZnS QD, as they are not available commercially and use them to label His_6-tagged recombinant Cyclin E, and second, to visualize intracellular localization of QD labeled Cyclin E in the pre-MBT and MBT embryo. We also wanted to determine if these QD mediate any cytotoxic effects in Xenopus embryos. CdSe/ZnS QD encapsulated in phospholipid micelles have previously been microinjected into one cell of an early cleavage stage *Xenopus* embryo and its progeny followed through to a late tadpole stage (Dubertret et al., 2002), showing the feasibility of these experiments.

In order to accomplish our objectives, we collaborated with Dr. Marek Osinski at the UNM Center for High Technology Materials (CHTM) to develop a novel method of imaging His_6-tagged Cyclin E in living embryos by attaching it to ZnS overcoated CdSe quantum dots capped with dihydrolipoic acid (DHLA). Cap exchange with DHLA not only renders QDs water soluble (Bunge et al., 2003), but also allows direct attachment of His-tagged proteins. Alternatively, a more traditional conjugation approach involves the use of the EDC (1-ethyl-3-(3-dimethylaminopropyl) carbomide) crosslinking agent that reacts carboxy groups on the QD surface to the primary amine groups of the protein. The direct coupling strategy has a few benefits. First, it allows a unidirectional attachment of protein to the QD. Second, QD-protein complexes are less prone to aggregation in neutral and acidic buffers. The attachment of His-tagged proteins to the DHLA capped quantum

dots is proposed to be mediated by a strong metal affinity between the His_6-tag and Zn^{2+} atoms of the QD shell (Medintz et al., 2005), with a dissociation constant (K_d) of 1×10^{-13} (Fig 1A). This interaction is stronger than most antibody binding, which has a K_d of 1×10^{-6}-10^{-9} (Hainfeld et al., 1999). Moreover, a QD based approach could be highly beneficial over GFP-tagged imaging due to increased QD brightness and resistance to photobleaching allowing deeper imaging during prolonged periods of time throughout embryo development. Unlike most mammalian cells, *Xenopus laevis* embryos provide a perfect model for a straightforward and efficient intracellular delivery. Since QD-His_6-protein complexes are stable at physiological pH, they can be microinjected into the 1-1.5 mm in diameter Xenopus embryos and imaged in real time *in vivo*.

Methods

(CdSe)ZnS quantum dot preparation: QDs were prepared using a stepwise approach consisting of core CdSe nanocrystal growth, overcoating with five layers of ZnS, size selective precipitation, and surface ligand exchange and purification.(Bunge et al., 2003) Growth of nanocrystals was monitored by a change in the absorption spectrum by UV-VIS spectroscopy. QDs were made water soluble through exchanging the native capping shell (trioctylphosphine (TOP)/trioctylphosphine oxide(TOPO)/ hexadecylamine) with freshly prepared DHLA (Clapp et al., 2006). DHLA was prepared by ring opening of the DHLA precursor, thioctic acid using $NaBH_4$ as a reducing agent in aqueous solution (Fig. 1A) (Clapp et al., 2006; Gunsalus et al., 1956) followed by DHLA distillation to remove impurities. The QD morphology was assessed by transmission electron microscopy.

Optical characterization: UV-VIS absorption spectra were obtained at room temperature using a Varian Carey 400 Spectrophotometer. Each aliquot was quenched directly in a UV cell containing toluene. The spectra were collected from 300 to 800 nm with a scan rate of 0.5 nm/min.

Transmission electron microscopy: Transmission electron microscopy (TEM) was used to verify size and quality of the cap-exchanged QD. An aliquot of the (CdSe) ZnS dots was placed onto a carbon coated TEM grid (300 mesh) and allowed to dry overnight. The QD were studied using a Phillips CM 30 TEM at 300 kV accelerating voltage.

Recombinant protein purification and conjugation to QDs: His_6-Cyclin E was expressed in *E. coli* (Pepex), grown at 37^0C to an OD_{600} of 0.7 and then induced with a final concentration of 1mM of isopropyl-β-D-thiogalactopyranoside and grown to an OD_{600} of 1. Cells were lysed followed by centrifugation and the supernatant loaded onto a Ni-NTA column for purification (according to the manufacturer's specifications). His_6-Cyclin E was eluted by addition of phosphate buffered saline containing imidazole. Protein quality and quantity was assessed by SDS-PAGE and visualization on Coomassie stained gels. His_6-Cyclin E attachment to DHLA capped QDs was carried out by addition of His_6-Cyclin E (in phosphate buffered saline) to QDs in an aqueous solution of sodium tetraborate buffer (pH 9.5), mixing and incubating for 15 min at room temperature. Conjugation was assessed by measuring quantum yield and photoluminescence of conjugated versus unconjugated QD (Optical characterization).

Intracellular delivery and microscopy: *Xenopus laevis* embryos were prepared as described (Hartley et al., 1996). One cell of a two-cell albino embryo was microinjected

with 27.6 nl of His_6Cyclin E-(QD_{564})DHLA or (QD_{564})DHLA alone. Embryos were allowed to develop to the time post-fertilization indicated and imaged live. An empirically chosen constant exposure time was used for imaging on a Zeiss LSM510 confocal microscope with a GFP filter.

Results

After synthesis and cap exchange, the morphology of DHLA coated ZnS/CdSe (QD_{564}) was assessed by transmission electron microscopy (TEM). As seen in Figure 1B, the QDs were homogeneous, of consistent size (about 6 nm in diameter), shape (spherical) and did not form aggregates. Next, we conjugated His_6-cyclin to QDs and confirmed attachment by measuring photoluminescence and quantum yield. A 30% increase in photoluminescence and 18% increase in quantum yield of conjugated versus unconjugated QDs confirmed attachment (data not shown).

(QD_{564})-His_6Cyclin E complexes or QD_{564} alone as a control, were microinjected into 1-cell of a 2-cell embryo between 1.5-2 hours post fertilization (hpf) and visualized in live developing embryos by confocal microscopy. Embryos were viewed from 4 to 7 hpf, in order to assess Cyclin E localization in pre-MBT and MBT embryos. Consistent with our immunofluorescence data (Chapter 2, Fig. 3 and 4), QD-His_6Cyclin E was restricted to the cytoplasm in pre-MBT embryos (Fig. 2A, top and bottom panels; Fig. 2B, top panel) as can be seen by the diffuse staining not bound by intracellular membranes. When viewed at the MBT, QD-His_6Cyclin E began to appear in the nucleus (Fig. 2B bottom). Unconjugated QDs were also cytoplasmic in pre-MBT embryos (Fig.

2C top and bottom panels, Fig. 2D bottom panel). Unexpectedly, at the MBT unconjugated QD also moved into the nucleus (Fig. 2D bottom).

We also assessed potential cytotoxicity of the QD-His_6Cyclin E and QD alone by carefully monitoring embryo morphology and survival for up to 3 days after microinjection. Embryos progressed through the blastula stage, gastrulated normally, and became free-swimming tadpoles. We were able to visualize QD-mediated fluorescence in embryos microinjected with either QD-Cyclin E complexes or QD alone for up to three days after microinjection without any obvious deleterious effects. These results suggest low cytotoxicity of the injected QD, either alone or conjugated to recombinant protein.

Discussion

We accomplished the two objectives we set out to achieve. First, we successfully synthesized functional ((CdSe)ZnS)DHLA QD specifically tailored to our application of attachment of recombinant His_6Cyclin E protein to its surface. Second, we followed the localization of our QD-tagged Cyclin E complexes inside the cells of developing Xenopus embryos in real time.

We found that pre-MBT QD-His_6Cyclin E was cytoplasmic and at the MBT it accumulated in the nucleus of embryo cells, which is consistent with a previous report that Cyclin E is nuclear at the MBT (Chevalier, 1996). Our data analyzing endogenous Cyclin E and exogenous protein translated *in vivo* (Chapter 2) confirmed our results, showing that Myc_6-Cyclin E-GFP moved from the cytoplasm into the nucleus in live embryos at the MBT and in fixed embryos, analyzed by immunofluorescence, both

exogenous and endogenous Cyclin E was cytoplamsic pre-MBT and nuclear at the MBT. However, in the current study our control ((CdSe)ZnS)DHLA QD without attached His_6Cyclin E showed nuclear localization at the MBT as well. Nuclear localization of QD alone was not due to their surface chemistry, as commercial Streptavidin conjugated (CdSe)ZnS QD_{600} (Evident Technologies, Troy, NY) injected into the embryos also localized to the nucleus at the MBT (data not shown). A previous example of nuclear accumulation of (CdSe)ZnS QDs with a different surface modification, encapsulation in phospholipid micelles containing a mixture of *n*-poly(ethylene glycol) phosphatidylethanolamine and phosphatidylcholine hydrophilic lipids (Dubertret et al., 2002) was shown in MBT Xenopus embryos. Taken together, these results show that QD themselves move into the nucleus at this important developmental transition.

In order to validate our result that localization of QD-His_6Cyclin E complexes into the nucleus is mediated by the cyclin molecule and is not due to movement of the QD itself, future experiments will utilize additional controls. These controls will include $His_6\beta$-actin conjugated to DHLA QD. We have previously confirmed by Western blot analysis of nuclear and cytoplasmic fractions that localization of β-actin is strictly cytoplasmic throughout the first 25.5 hours of Xenopus development. We expect that β-actin will mediate the retention of QD in the cytoplasm as it is a cytoplasmic protein. Alternatively, another Xenopus cytoplasmic protein or Cyclin E without its NLS could be used. A separate study needs to be conducted to examine the mechanisms involved in nuclear accumulation of QD at the MBT. These studies would lead to a better

understanding of both the process of nuclear import and potential side effects of QD use in biological systems.

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Figures and Figure Legends

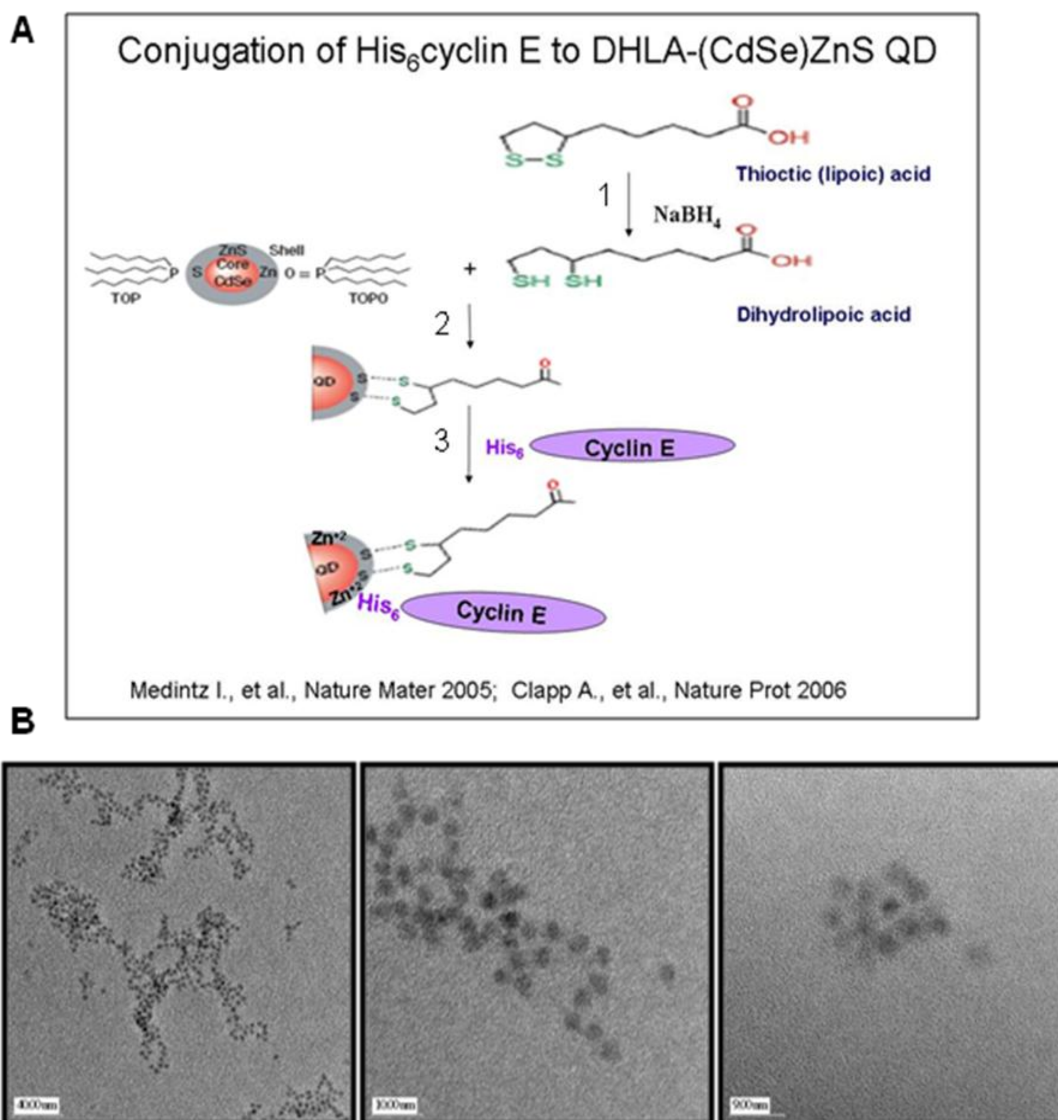


Figure 1. A) Scheme of synthesis of water soluble QD and Cyclin E attachment.

1. Dihydrolipoic acid (DHLA) is produced from its precursor, thioctic acid using $NaBH_4$ as reducing agent in aqueous solution. 2. QD were made water soluble through exchange of native capping shell of TOP/TOPO (trioctyl phosphine/trioctyl phoshine oxide mixture) with freshly prepared DHLA. 3. His_6cyclin E complexes are then attached to QD surface by metal affinity.

B) TEM images of DHLA-capped QD synthesized by the above described method. Scale bar in left panel, 40nm, middle panel 10 nm, right panel 9 nm.

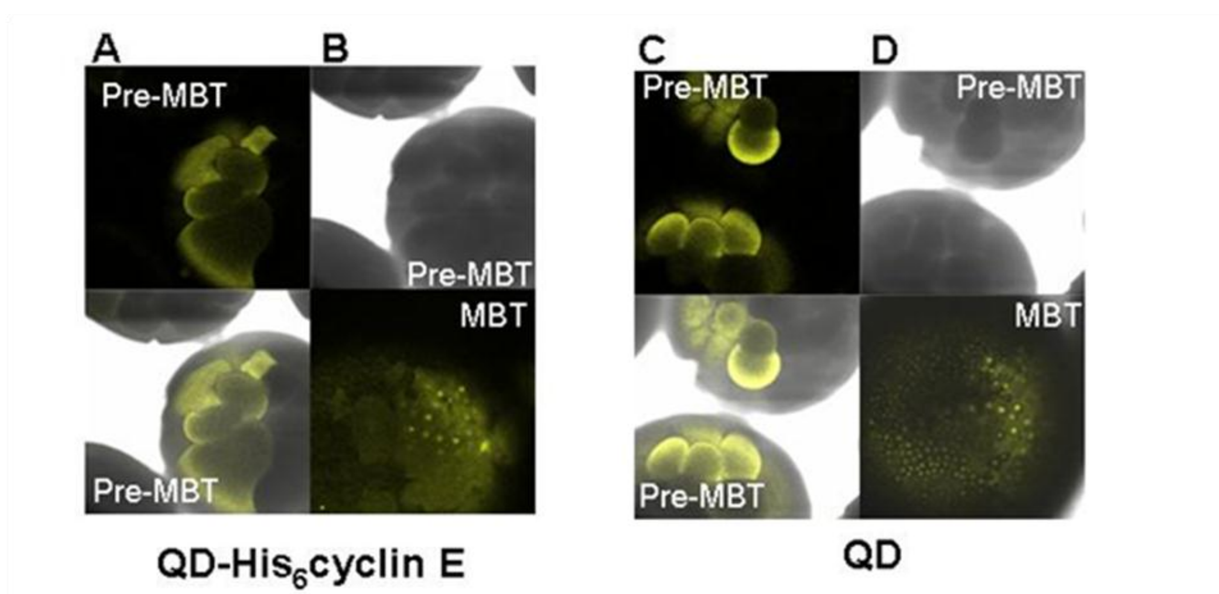


Figure 2. Localization of QD-His6cyclin E inside Xenopus embryos. Embryos were injected with QD-His6cyclin E (A & B) and QD alone (C& D) at 2 cell stage and visualized by confocal microscopy at pre-MBT (4 hpf) and MBT (6 hpf) as indicated. A & C top, B& D bottom, fluorescence channel; B&D top, light image; A & C bottom, merge. Magnification is 10X.

Chapter 4. Final Discussion

In this project, we set out to determine the mechanism of Cyclin E downregulation at the MBT in *Xenopus laevis* embryos. We conclude that it is dependent on Cyclin E phosphorylation and nuclear transfer at the MBT, and is mediated by the 26S proteasome. Support for this conclusion has been obtained by experiments that study the effect of abolishing phosphorylation of the conserved amino acid residues T75, T394 and S398; nuclear import; and proteasomal function on Cyclin E degradation. All three sets of experiments resulted in increased Cyclin E stability, suggestive of the importance of these components in its destabilization. In addition, we showed that Cyclin E is phosphorylated and ubiquitinated at the MBT, corresponding to the time of its disappearance. The similarities between downregulation of human and Xenopus Cyclin E provide strong evidence for conservation of the mechanism of degradation between species and emphasize the importance of prior nuclear presence of Cyclin E for its degradation in Xenopus embryos.

Using a novel approach, we tagged *Xenopus laevis* Cyclin E with QD for tracking its localization in developing embryos in real time. This provided evidence about the feasibility of this approach for prolonged tracking of intracellular molecules inside live cells, which could be of a particular interest for studying tumor metastasis or embryonic differentiation and cell lineages. QD have desirable qualities such as brightness and resistance to photobleaching, which are superior to the commonly used fluorophores. Further advances in the field of microfluidics can perhaps help resolve the inability at this

point to strictly control the ratio of conjugating QD to biomolecules and vise versa. Perhaps the surprising “nuclear-phillic” properties of QD could be further investigated and utilized, for example, for delivery of DNA damaging agents specifically to nuclei of cancer cells. In addition, *Xenopus laevis* embryos provide a very useful system for studying QD mediated cytotoxicity since QD can be delivered inside the cells with a 100 % efficiency by microinjection, and dose-dependency can be assessed as cellular perturbances in embryos result in measurable biological phenotypes. The cell health, cell division and phenotype can be easily monitored using a stereomicroscope.

Based on our results and the literature, the scenario of Cyclin E degradation is complex. The requirement of nuclear import for Cyclin E degradation provides several possible schemes regulated by phosphorylation. These schemes include 1) involvement of phosphorylation in nuclear import; 2) phosphorylation in the nucleus either signaling Cycin E ubiquitination or facilitating its export into cytoplasm; or 3) a separate post-translational modification occurring in the nucleus that results in Cyclin E phosphorylation upon export. Given the multiple potential phosphorylation sites in Cyclin E, it is probable that phosphorylation of a specific combination of sites, or a specific order of phosphorylation triggers Cyclin E relocalization and degradation.

A model based on our data and studies published by others is presented in Figure 1. This figure summarizes what we have learned about Cyclin E degradation at the MBT. At the MBT, Cyclin E is phosphorylated, which may facilitate its NLS-dependent translocation into the nucleus. Results from our 2D gel electrophoresis-Western analysis support this possibility as there is a dramatic increase in Cyclin E phosphorylation at the

MBT. At the same time, none of phosphorylation mutants analyzed were excluded from the nucleus at the MBT, suggesting that if phosphorylation is required for nuclear import, it is likely on other sites, or that it is not required for nuclear import. After its nuclear translocation, Cyclin E is phosphorylated either on T75, T394 or S398 or a combination of these sites, triggering its recognition by Cdc4 for ubiquitination. Single phosphorylation likely results in lower affinity binding to Cdc4 and slower degradation, while phosphorylation on multiple sites (i.e. both T394 and S398), result in higher affinity binding to Cdc4, as shown by structural analysis of human Cyclin E (Hao et al., 2007) and could potentially mediate its rapid "switch-like" removal similarly to another cell cycle regulator, CKI Sic1 in yeast (Orlicky et al., 2003). Thus, the efficiency of Cyclin E degradation at the MBT likely depends on the integration of inputs from multiple signaling pathways. Taking into account the preferred view that nuclear isoforms of Cdc4 play a bigger role in targeting human Cyclin E for ubiquitination than cytoplasmic (Grim et al., 2008; Sangfelt et al., 2008; van Drogen et al., 2006), and the apparent conservation of the degradation mechanism between species shown by our studies, I speculate that ubiquitination of Xenopus Cyclin E mediated by T75, T394 or S398 phosphorylation also occurs in the nucleus. Ubiquitinated Cyclin E is then re-localized into the cytoplasm, where it is degraded by the 26S proteasome.

In this model, several unresolved questions require further investigation. Is Cyclin E phosphorylation necessary for its nuclear import? If it is, the identification of these phosphorylation sites will shed further light on the mechanism of nuclear entrance. The identification of *in vivo* phosphorylation sites that are involved in Cyclin E degradation

will tell us if different sites (or a combination thereof) mediate localization and degradation. The identity of the kinases responsible for MBT phosphorylation of Cyclin E is not yet known, nor are the upstream signaling pathways activating these kinases. Since the two major kinases implicated in phosphorylation of human Cyclin E are Cdk2 and GSK3β (Welcker et al., 2003), and regulatory mechanisms of Cyclin E turnover in human and Xenopus are similar, these kinases remain the top candidates for phosphorylating Xenopus Cyclin E. Moreover, as was mentioned earlier, T75, T394 and S398 fit the consensus sites for these kinases.

Cdk2 is present throughout early embryonic development and its activity fluctuates twice per embryonic cell cycle despite elevated levels of Cyclin E (Hartley et al., 1996). Upon completion of the 12th cell cycle at the MBT, Cyclin E is rapidly downregulated. At this time Cdc25A phosphatase, the activator of Cyclin E-Cdk2 is destabilized as well (Kim et al., 1999) and an inhibitory phosphorylation on Cdk2 increases (Hartley et al., 1996; Kim et al., 1999). It will be interesting to investigate whether Cdk2 first functions to phosphorylate Cyclin E, rendering it susceptible to degradation before being inactivated. GSK3β is also present during Xenopus development, where its phosphorylation of substrates alters their subcellular localization (Ferkey and Kimelman, 2000; Yost et al., 1996) but its role in regulating Cyclin E has not been studied. Understanding Cyclin E constitutive expression pre-MBT and its instability post-MBT awaits information about the regulatory pathways that in some way affect the culprit kinases to hold off their phosphorylation of Cyclin E until the appropriate developmental time.

Given the phosphorylation of Xenopus Cyclin E on residues known to signal Cdc4 recognition of human Cyclin E, the involvement of Cdc4 in ubiquitination of Xenopus Cyclin E at the MBT remains to be determined. The α and β isoforms of Xenopus Cdc4 have been cloned (Almeida et al., 2010)and the γ isoform is present in the Xenopus tropicalis genome database (unpublished observation), but has not yet been cloned. We have shown that Cdc4 α and β are capable of degrading Cyclin E and are present at the MBT (Almeida et al., 2010) but their localization and specific role in Cyclin E degradation at the MBT have not been examined.

Xenopus embryos are only one of several models that have been successfully utilized to study the cell cycle and the regulation of its components, including Cyclin E. Embryos provide an *in vivo* environment where Cyclin E is naturally expressed at high levels throughout the cell cycle and are well suited to investigate more complicated cellular interactions than cell extracts or cells in culture. For example, it has been shown the cell-cell interactions are necessary for cell cycle remodeling at the MBT (Andrews et al., 2008). It would be interesting to determine in future experiments if these interactions are necessary for Cyclin E downregulation, and to delineate the signaling pathways involved.

Although using a relatively simple model system, our study directly answered an important question about how Cyclin E is downregulated during development, and in cells where it is expressed similar to cancer cell expression. Our studies focused on regulation of Cyclin E1, a G1/S regulator often perturbed in breast cancer and involved in its etiology. Our results show a potential network of regulation involving phosphorylation

dependent ubiquitination as well as nuclear import. A number of promising future directions and possible signaling pathways merit continued study and exploration. Other questions that can be answered with the Xenopus system that may contribute to understanding Cyclin E regulation in human cancer cells include determining how Cdk2 activity is regulated in the presence of elevated levels of Cyclin E and how mislocalization of Cyclin E in cancer relates to defects in degradation. Moreover, better understanding of the connection between Cyclin E localization, its phosphorylation status and degradation using Xenopus as a model, will perhaps be relevant to understanding of their perturbation in malignancies. One future goal of our experiments is to use the Xenopus system to identify new targets to interfere with Cyclin E overexpression and/or to regulate Cdk2 in cells with abnormal protein stability.

Figures and Figure Legends

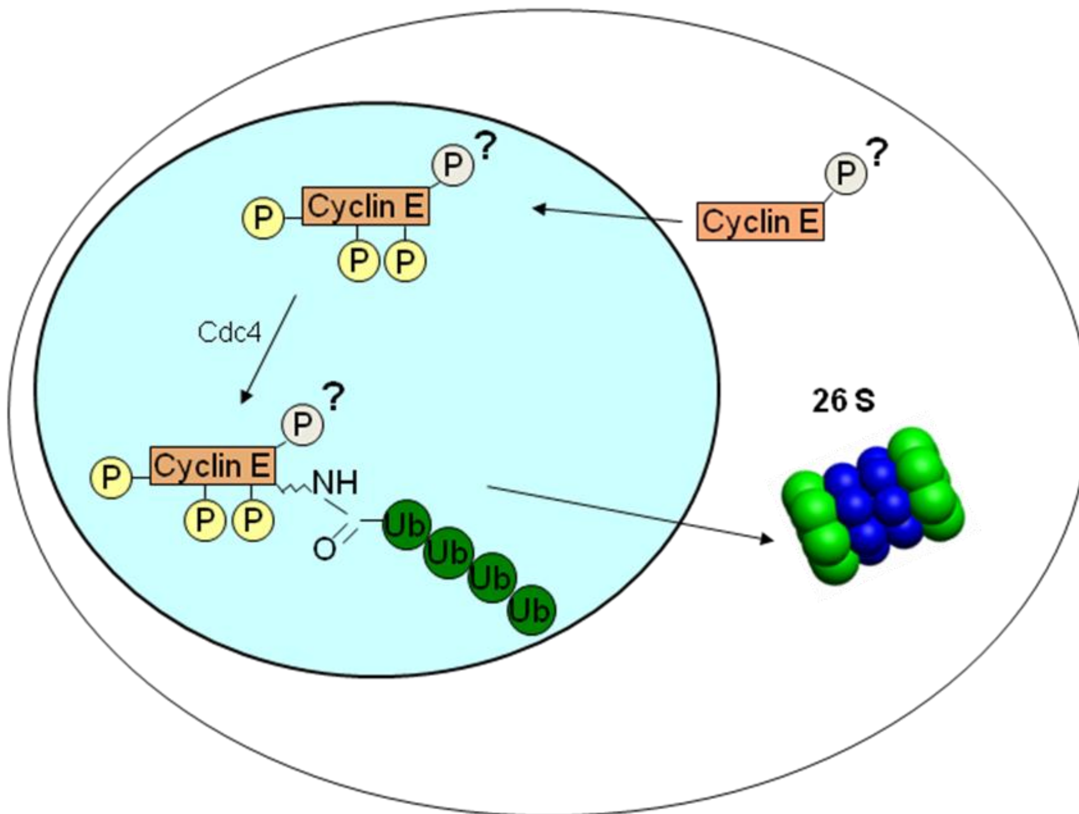
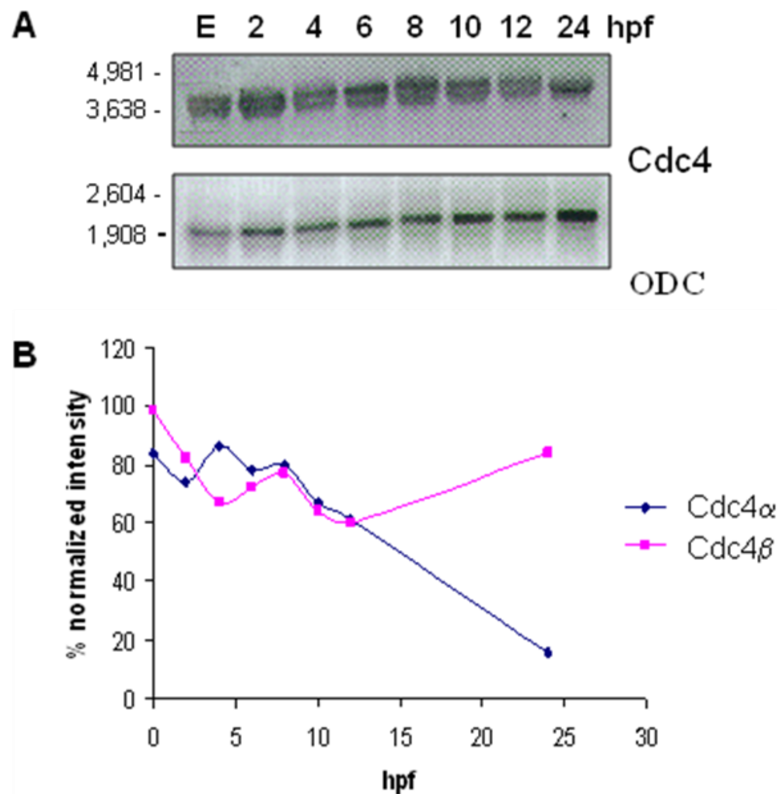


Figure 1. Model of Xenopus Cyclin E degradation. Cyclin E is potentially phosphorylated on the unknown residue in the cytoplasm. (Gray encircled P with a question mark next to it). It is then imported intp the nucleus where it is phosphorylated on either T75, T394, or T398 (Yellow encircled Ps). This targets it for ubiquitination by Cdc4 ubiquitin ligase. Ubiquitinated Cyclin E is exported into cytoplasm for degradation by the 26S proteasome.

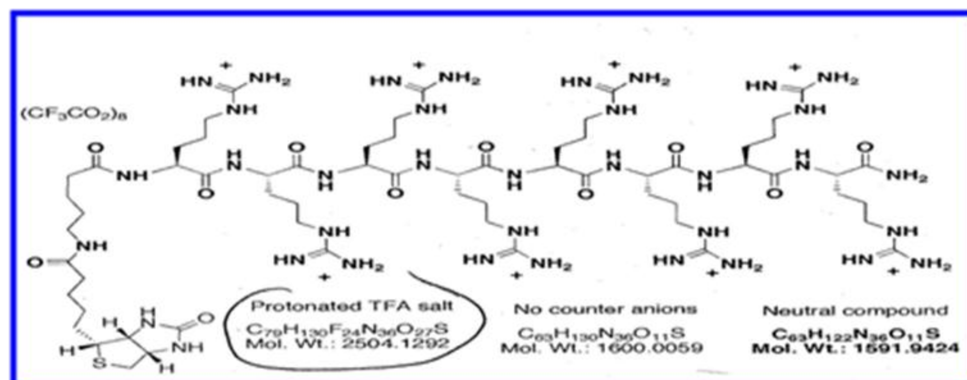
Appendix-Pilot Studies



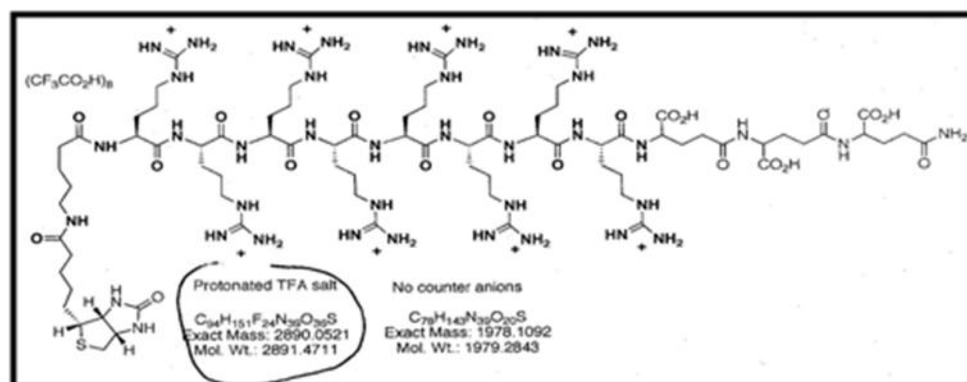
Appendix Figure 1. A) Northern blot of Cdc4 mRNA throughout the first 24 hrs of Xenopus development. Total RNA was extracted from embryos at the time points indicated using Trizol reagent (Invitrogen, Carlsbad, CA) and 7-10 μg were resolved on a MOPS-formaldehyde gel followed by transfer to nitrocellulose membrane. RNA was cross-linked to the membrane using UV-cross linker. Cdc4α cloned into pGEM T Easy vector was kindly provided by Dr. M. Slevin. Probe was prepared by linearizing the Cdc4α with Nco I enzyme. 25 ng of linearized Cdc4α and ornithine decarboxylase (ODC) (loading control) cDNA was used to prepare hybridization probes according to the Prime-a Gene labeling System protocol (Promega, Madison, WI) using P^{32} labeled dCTP (Perkin-Elmer, Boston, MA). Hybridization was performed using QuikHyb hybridisation solution and protocol (Stratagene, La Jolla, CA). Blots were scanned on Storm 860 Phosphorimager. Experiment was repeated 3 times. E, egg.

B). Quantitation of data from experiments shown in A. Means of % of normalized Cdc4 expression for both isoforms α and β from 3 independent experiments are shown. Cdc4α is represented by the upper band in panel A, and Cdc4β by the lower band.

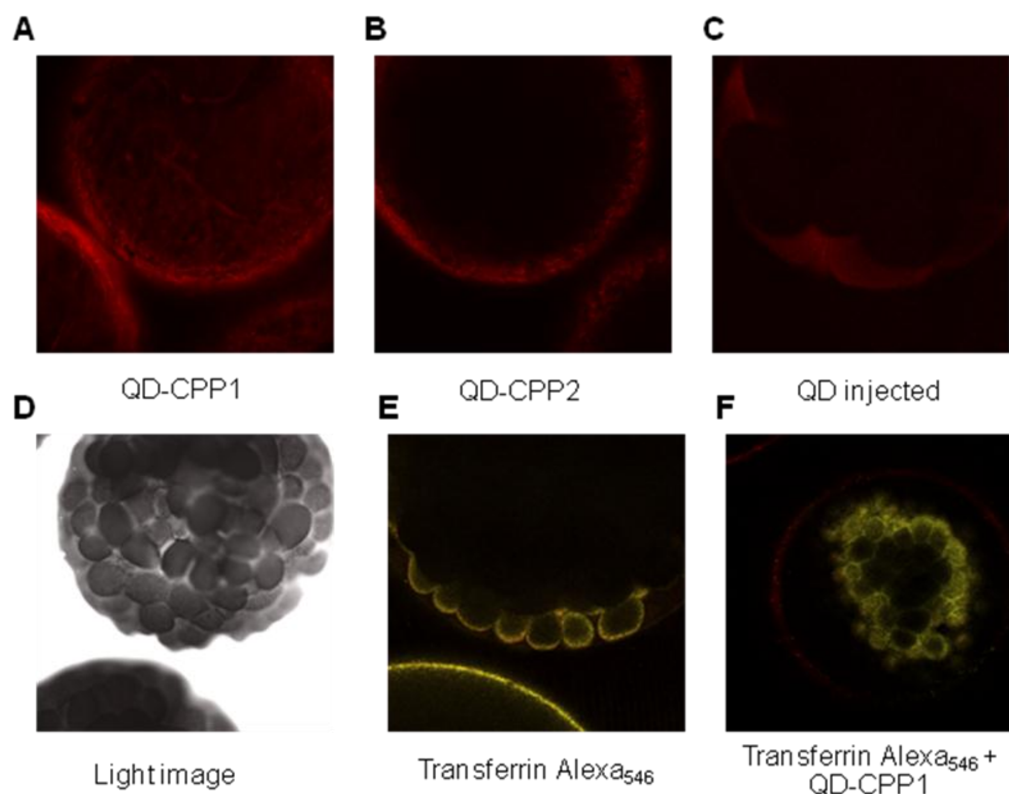
A



B



Appendix Figure 2. Chemical structure of the cell penetrating peptides (CPPs) A) CPP1 is composed of 8 Arginines attached to biotin and is more efficient in intracellular cargo delivery. B) CPP2, composed of 6 Arginines and 2 Glutamic acids attached to biotin is less efficient in intracellular delivery. Both CPPs were made and kindly provided by Dr. T. Lambert, Advanced Materials Lab, Sandia National Labs.



Appendix Figure 3. Intracellular delivery of QD attached to the cell penetrating peptides (CPP) into Xenopus laevis embryos is impeded by the vitelline membrane.
Commercial Streptavidin-QD_{600} (Evident Technologies, Troy, NY) were conjugated to the CPPs by mixing them at a 1:1 molar ratio in PBS and allowing them to incubate at room temperature for 20 min. Developing embryos were incubated in QD-CPP solutions for 1hr. They were then washed gently in 4 changes of 0.1X MMR (Malter's modified Ringer solution, 0.1M NaCl, 2mM KCl, 1mM $MgSO_4$, 2mM $CaCl_2$, 5mM Hepes, pH 7.8) and imaged *in vivo* by confocal microscopy in depression slides in 0.1X MMR.
Confocal images of embryos incubated with A) QD_{600}-CPP1, B) QD_{600}-CPP2, C) embryos injected with QD_{600} (positive control), D) light image of embryo in panel in E, E) transferrin-$Alexa_{546}$ (Molecular Probes, Eugene; a fluorescent marker for receptor mediated endocytosis), F) QD_{600}-CPP1 and transferrin-$Alexa_{546}$ together. Embryos microinjected with QD_{600} and incubated with transferrin conjugated to Alexa-$Fluor_{546}$ served as controls of delivery into cells. In E yellow is transferrin-$Alexa_{546}$ and red, QD CPP1.

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