A NEW PATHWAY TO DEGRADE CYCLIN B1

by

CASSANDRA SHEILA HEIGHINGTON

(Under the Direction of Edward T. Kipreos)

ABSTRACT

Cyclin B1 is the major regulator of M phase and it is widely conserved in eukaryotes. The anaphase-promoting factor, APC, targets cyclin B1 for degradation during the metaphase-to-anaphase transition. Inactivating another ubiquitin ligase, the $CRL2^{ZYG-11}$ complex has many phenotypes; one of them is increased levels of cyclin B1. Here we used a forward genetic screen for *zyg-11* suppressors and identified a mutation in CYB-2.1. Reducing the overall levels of cyclin B1/2.1/2.2 rescued the *zyg-11* lethality, suggesting that the $CRL2^{ZYG-11}$ complex reduces the level of cyclin B. We tested the interaction between ZYG-11 and CYB-1 to determine if the $CRL2^{ZYG-11}$ is directly targeting CYB-1 for degradation, along with APC. We found that $CRL2^{ZYG-11}$ directly binds and regulates CYB-1 in *C. elegans* meiosis and mitosis. To determine if this level of cyclin B regulation is conserved, we knocked down ZYG11A/B in human cells. We found an increased level of cyclin B1 during late metaphase when ZYG11A/B are knocked down. We also found that ZYG11B directly binds to cyclin B1,

suggesting that the regulation of cyclin B by the $CRL2^{ZYG11A/B}$ complex is conserved in humans.

INDEX WORDS: *C. elegans*, cyclin B1, meiosis, mitosis, ZYG-11, ZYG11A, ZYG11B, APC

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B.S., North Carolina State University, 2006

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

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August 2013

DEDICATION

I dedicate this dissertation to my sister, Kate Heighington. She has provided emotional and scientific support throughout my entire graduate school experience.

ACKNOWLEDGEMENTS

First and foremost I would like to acknowledge my advisor Edward Kipreos. He has spent countless hours discussing not only experimental ideas, but also details about the techniques we should use to address the questions we are addressing. He has provided never-ending enthusiasm for my project and my growth as a scientist. I would like to thank my committee for all their helpful advice over the years.

I would like to also thank all the members of the Kipreos lab, past and present. Mohammad for his guidance on how to communicate with Edward, Madhumati for her help on my project while she was rotating in the lab, as well as her helpful advice throughout my time in the lab, Snehal for her always bright and sunny personality, Mei for her thoughtful advice and kind words, Natalia for help with biochemical assays, Kyoengwoo for his thoughtful discussion and advice on my project. All three of the undergraduates that helped with my project; David Owen, Catherine Trebotte, and Jeff Lane. And last, but not least, Riju for helping me with this project with great enthusiasm and who will be continuing this project after I'm gone.

I would also like to thank my family and friends for their support over the years. My parents, who have emotionally and financially supported me, and the recently graduated Dr. Bernard, who has supported me during the last year and a half of this journey.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The eukaryotic cell cycle division process is broken down into several distinct stages; G1, S, G2, and M-phase. M-phase, when replicated DNA is condensed and segregated, will be the focus of this dissertation. Timely degradation of cell cycle regulators is essential for progression through the cell cycle and the majority of protein degradation in cells occurs by ubiquitin-mediated degradation (Rock et al., 1994). In this dissertation we will focus on the role of an E3 ubiquitin ligase, the $CRL2^{ZYG-11}$ complex, in the progression through meiosis and mitosis. In the literature review we will describe the previously identified events for cyclin B/CDK1 activation and deactivation in meiotic and mitotic progression.

Cyclin B/CDK1 is the major regulator of mitosis and meiosis

The activated cyclin B/CDK1 complex phosphorylates several targets that drives the cell into mitosis. The “trans-acting” component in mitotic cells that is responsible for chromosome condensation was first observed when HeLa cells that were blocked in mitosis were fused to interphase cells from many different species, which resulted in premature chromosome condensation (PCC) (Johnson et al., 1970). The maturation of *Xenopus* G2-arrested oocytes into mature eggs can be induced when the cytoplasm from unfertilized eggs arrested in metaphase of meiosis II was injected into a G2 arrested oocytes. This factor was termed the maturation-promoting factor (MPF) (Masui

and Markert, 1971). MPF activity is not unique to frogs. The cytoplasm from mammalian cells arrested in mitosis injected into G2-arrested frog oocytes also stimulates oocyte maturation, demonstrating that MPF is highly conserved. Cytoplasm from mitotically arrested mammalian somatic cells injected into interphase cells induces entry of the interphase cells into mitosis (Johnson et al., 1970), therefore, MPF controls the entry of mammalian somatic cells into mitosis as well as the entry of frog oocytes into meiosis, so MPF can also stand for mitosis-promoting factor.

Study of MPF continued in frog embryos and a system was developed to study the oscillations of MPF activity during embryonic cleavage cycles. The genes required for MPF activity were cloned in budding and fission yeast by Lee Hartwell (Hartwell et al., 1974) and Paul Nurse (Nurse et al., 1976), respectively. In 1983, Tim Hunt and his group were the first to observe proteins they described as “cyclins”. Using sea urchin embryos, they observed abundant protein synthesis from 3-4 maternally supplied mRNAs in the embryo, not in the unfertilized egg. These proteins were destroyed every time the cell divided (Evans et al., 1983). Years of research have shown that MPF is a heterodimer composed of a catalytic protein kinase subunit and a regulatory subunit that controls which proteins are phosphorylated by the catalytic subunit (Lohka et al., 1988). Nurse found that cdc2 (CDK1) kinase activity is conserved in humans because it could complement fission yeast cdc28 (Lee and Nurse, 1987). And David Beach used antibodies raised against yeast cdc2/cdc28 (CDK1) to show the cyclin B/CDK1 complex was conserved in HeLa cells (Draetta et al., 1987). Another group was finally successful at purifying the proteins required for MPF activity and found that they

consisted of CDK1 and cyclin B (Lohka et al., 1988). We now refer to the MPF as the cyclin B/CDK1 complex.

Several events must take place for a successful mitosis including chromosome condensation, chromosome cohesion and dissolution, assembly of the mitotic spindle, attachment of the chromosomes to the spindle, spindle elongation and separation of chromosomes, mitotic exit, and cytokinesis. MPF, which is the cyclin B/CDK1 complex, plays an important role in the events driving the cell into mitosis by phosphorylating critical targets that regulate these events. So important, in fact, that CDK1 is the only essential CDK for the eukaryotic cell cycle (of the 20 that have been studied so far), and it's sufficient to promote DNA replication and chromosome segregation (Fisher et al., 2012). Cdk1 is sufficient to initiate organogenesis in mouse embryos lacking all other essential cell cycle regulatory Cdks (Cdk2, Cdk4 and Cdk6) and promote development to midgestation (Rahman and Kipreos, 2010; Santamaria et al., 2007).

Introduction to sister chromosome cohesion and condensin

During mitosis, the newly replicated sister chromatids are condensed and pulled to opposite poles of the cell to equally segregate a single copy of genomic DNA into each daughter cell. As genomic DNA is being replicated, the sister chromatids are held together by a process called 'chromosome cohesion' until anaphase (Nasmyth and Haering, 2009). Chromosomes are condensed with help from condensins and attached to the mitotic spindle, which is the scaffold that physically separates the DNA, during mitosis. The cyclin B/CDK1 complex regulates both processes.

The sister chromatids are physically connected to each other as they are being replicated during S-phase. They are weakly connected along the chromosome arms

and more tightly connected at the centromeres. This process is essential for setting up the connections between sister kinetochores and mitotic spindle microtubules so that sisters are pulled in opposite directions during anaphase (Nasmyth and Haering, 2009). Bi-orientation of the sister chromatids during meiosis I is achieved by a process of error correction. The kinetochore-microtubule connections are unstable unless they generate tension, which only occurs when maternal and paternal kinetochores are pulled in opposite directions (Nicklas, 1967, 1997). The same principle probably applies to mitotic cells.

The cohesin complex is made up of a core complex with two Smc proteins, Smc1 and Smc3, and two non-Smc proteins, Scc1 (also known as Mcd1 or Rad21) and Scc3 (known in mammalian cells as SA1 and SA2). All components are essential for maintaining sister chromatid cohesion in post replicative yeast cells (Nasmyth and Haering, 2009). The Smc1 and Smc3 form a heterodimer and each subunit is composed of 50nm long intermolecular antiparallel coiled-coil that forms a rod-shaped protein with a globular “hinge” domain at one end and an ATP nucleotide-binding domain (NBD) at the other (Nasmyth and Haering, 2009). The complex forms a ring when the N-terminal of Scc1 binds to the Smc1 NBD and C-terminal of Scc1 binds to Smc3 NBD. Scc3 binds the complex through Scc1 (Rowland et al., 2009). In addition to the core complex there are other proteins that are associated with the cohesion complex with unknown functions, other proteins that are essential for cohesin’s association with chromosomes, but are not required for cohesion maintenance after DNA replication and some other proteins that are not essential, but improve efficiency of cohesins (Nasmyth and Haering, 2009).

The dissolution of sister chromatid cohesion takes place in two steps: 1) during prophase, cohesin is lost from sister chromatid arms; and 2) at the metaphase-to-anaphase transition Separase cleaves the Scc1 component of the cohesin complex that is associated with the centromere. Lagging chromosomes inhibit the cleavage of Scc1 at the metaphase-to-anaphase transition. Once the bi-orientation of all the sister chromosomes is achieved during metaphase, destruction of sister chromatid cohesion is initiated to allow the separation of sister chromatids during anaphase (Nasmyth and Haering, 2009).

In addition to being attached via sister chromatid cohesion, the DNA must also be condensed for proper segregation during mitosis. There are two main condensin complexes that are responsible for chromosome condensation during mitosis, condensin I and condensin II. Condensin I is essential for proper condensation and segregation of chromosomes in all organisms studied so far; even bacteria and archaea have condensin-like complexes (Hirano, 2012). Many eukaryotic species have a second condensin complex, condensin II (Hirano, 2012). Both condensin I and condensin II have the same pair of SMC2 and SMC4 subunits (Hirano, 2006), but each complex has a unique set of three non-SMC subunits. Condensin I includes CAP-D2, CAP-G and CAP-H, while condensin II is comprised of CAP-D3, CAP-G2, CAP-H2.

Knockdown of condensin I and condensing II in human tissue culture cells results in different defects in mitotic chromosome architecture and segregation, which suggests that both complexes play essential roles in mitosis (Hirota et al., 2004; Ono et al., 2004; Ono et al., 2003). The two condensins are also differentially localized. Condensin I is sequestered in the cytoplasm during interphase and only associates with chromosomes

after the nuclear envelope breaks down in prometaphase. Condensin II localizes to the nucleus throughout the cell cycle and contributes to the early stage of chromosome condensation during prophase. After NEBD, condensins I and II work together to support proper assembly of chromosomes and to promote faithful chromosome segregation during anaphase (Hirano, 2012).

The shape of the metaphase chromosomes might be a result of a balancing act between condensin I and II. In *Xenopus* egg extracts when the relative ratio of condensin I to condensin II is forced to be smaller, 5:1 shifted to 1:1, the embryonic chromosomes became shorter and thicker (Shintomi and Hirano, 2011). When the amount of Condensin II was reduced to make an extract with a 1:0 ratio of condensin I to condensin II, the chromosomes got longer (Shintomi and Hirano, 2011). These observations strongly suggest that condensin II contributes to axial shortening of chromatids, while condensin I is responsible for lateral compaction of chromatids.

CDK1 substrates promoting mitosis

CDK1 plays a role in chromosome cohesion maintenance by working upstream from SCC1 (part of the cohesion complex that holds sister chromatids together)(Heo et al., 1999). Although no molecular target has been identified yet, mutations that reduce CDK1 activity result in chromosome cohesion defects (Brands and Skibbens, 2008; Kitazono et al., 2003). In addition to promoting chromosome cohesion, CDK1 also phosphorylates Securin (which binds to and inhibits Separase, an enzyme that cleaves cohesion rings), to protect it from APC-mediated degradation (see APC section below).

Before the newly replicated chromosomes can be separated they need to be condensed. CDK1 phosphorylates T19 on Cut3/Smc4, which is part of the Smc2-Smc4 complex that mediates chromosome condensation. CDK1 phosphorylation of Smc4 induces chromosome condensation. This CDK1 activity is conserved in vertebrates and *Xenopus* egg extracts (Hirano, 2005; Kimura et al., 1998). Study in HeLa cells demonstrated that CDK1 phosphorylates the CAP-D3 subunit of condensin II (Thr 1415), promoting the early stage of chromosome condensation (Abe et al., 2011).

The mitotic spindle is the scaffold that physically separates the condensed sister chromatids. Proper assembly and alignment of the mitotic spindle is required for successful chromosome separation. CDK1 facilitates the separation of the spindle pole bodies in *S. cerevisiae* by protecting the kinesins Cin8 and Kip1 and the spindle midzone component Ase1 from APC-mediated degradation by directly targeting several APC subunits (Enserink and Kolodner). Through an unknown mechanism, CDK1 also prevents re-duplication of the spindle pole body (Haase et al., 2001; Simmons Kovacs et al., 2008).

CDK1 might also be involved in the attachment of chromosomes to the mitotic spindle. In *S. cerevisiae* CDK1 phosphorylates ASK1; a component of the Dam1 complex that stabilizes the attachment of microtubules to kinetochores. The *ask1-3(ts)* allele genetically interacted with hypomorphic *cdk1* alleles. At the non-permissive temperature, the *ask1-3(ts)* mutant and the *cdc28(ts)*(CDK1) mutants grew, but double mutants, *ask1-3(ts)* and *cdc28(ts),* showed synthetic growth phenotypes. This genetic interaction supports the idea that Ask1 is a positively-regulated target of CDK1 in budding yeast (Li and Elledge, 2003).

CDK1 also has a role in spindle positioning and elongation. In *S. cerevisiae*, CDK1 phosphorylates Kar9 (a protein required for correct positioning of the mitotic spindle) to control the asymmetric loading of Kar9 onto the spindle pole body (Enserink and Kolodner). CDK1 contributes to mitotic spindle stabilization and elongation by phosphorylating Bir1 (a component of the chromosomal passenger complex), which results in the recruitment of Ndc10 (an inner kinetochore protein that re-localizes to the spindle midzone in anaphase and promotes spindle elongation) (Bouck and Bloom, 2005).

CDK1 activity keeps the cell in a mitotic state and this is reversed by the degradation of cyclin B (discussed below), and also by the de-phosphorylation of CDK1 targets. During anaphase, CDC14 dephosphorylates some CDK1 targets. CDK1 phosphorylates and inhibits a key target in mitotic spindle organization, Ase1 (a microtubule bundling factor and core component of the spindle midbody) during metaphase. During anaphase CDC14 dephosphorylates Ase1, promoting the assembly of the spindle midzone (Enserink and Kolodner; Khmelinskii et al., 2007; Khmelinskii and Schiebel, 2008). During metaphase, CDK1 phosphorylates Sli15 (an inner centromere-like protein or INCENP) within its microtubule-binding domain, preventing it from re-localizing to the spindle. CDC14 dephosphorylates Sli15, re-localizing it to the spindle in anaphase and contributing to spindle stabilization (Pereira and Schiebel, 2003).

Cyclin B/CDK1 is regulated before mitosis by inhibitory and activating kinases

Cyclin B synthesis starts in S-phase and accumulates through G2 phase. The cyclin B/CDK1 complex begins forming in S-phase. The cyclin B/CDK1 complex is kept

inactive through S- and G2- phases by inhibitory phosphorylation by protein kinases WEE1 and MYT1 (Archambault and Glover, 2009; Russell and Nurse, 1987). The activation of cyclin B/CDK1 requires Cdc25, which was originally cloned in fission yeast in 1986 (Russell and Nurse, 1986). Cdc25 is a dual-specificity protein phosphatase that removes the inhibitory phosphorylation catalyzed by WEE1 and MYT1 from CDK1 to activate the cyclin B/CDK1 complex. CDK1 is also inhibited when bound to CDK inhibitors (CKIs), including Sic1 (in S. cerevisiae) and members of the $p21^{Cip1}$ family (in vertebrates) (Fisher review 2012).

Upstream of the activating phosphatase Cdc25, and the deactivating kinases WEE1 and MYT1, that regulate the cyclin B/CDK1 complex is Polo kinase. The polo gene was first discovered in *Drosophila* based on a mutation that resulted in spindle formation defects (Sunkel and Glover, 1988). The polo gene encodes a kinase that is highly conserved in mitotic regulation. Polo/Plk/PLK1 both activates CDC25 and inhibits WEE1 and MYT1 to promote mitotic entry via the activation of the cyclin B/CDK1 complex (Fig 1.1). This activation is amplified because CDK1 can also activate CDC25, therefore creating a positive feedback loop, whereby CDK1 that is newly activated increases the activity of CDC25 to create more active CDK1 that will drive the cell into mitosis (Archambault and Glover, 2009). CDK1 also phosphorylates Wee1, inhibiting it as part of the same feedback loop (Glover 2012) (Fig 1.1). The effects of CDK1 activation are reversed by a protein phosphatase 2A (PP2A) that counteracts the action of CDK1 on WEE1 and Cdc25 (Fisher et al., 2012).

Regulation of the Polo kinase seems to be the determining factor for mitotic initiation. In fission yeast, Polo kinase is activated by rapamycin signaling, which acts

through the mitogen-activated protein kinase stress pathway. The timing of mitotic entry is based on stress, nutrient availability, and unperturbed cell cycles. Human Polo kinase, PLK1, is inactivated by response to DNA damage (Archambault and Glover, 2009). After the DNA damage response, PLK1 needs to be activated for the cell cycle to resume, and Aurora A with its activator BORA is responsible for this activation (Macurek et al., 2008; Seki et al., 2008).

Ubiquitin-mediated degradation

Ubiquitin (Ub) is a small, conserved, polypeptide (76 amino acid) that is ubiquitously expressed (Hershko and Ciechanover, 1998). The poly-ubiquitin locus transcribes a poly-ubiquitin protein that is post-translationally cleaved into individual Ub peptides by ubiquitin C-terminal hydrolyses (Johnston et al., 1999). These individual Ub peptides are covalently bound to proteins in poly-ubiquitin chains that can target the proteins for degradation via the 26S proteasome. The 26S proteasome is a conserved multi-subunit protein complex made up of the 20S proteasome, which is a central core with proteolytic subunits, and two 19S caps that bind the target protein, cleave the ubiquitin off, then unfold and translocate the target protein into the 20S core (Pickart and Cohen, 2004).

Ubiquitin (Ub) is covalently attached to the target protein, or substrate, by a cascade of events catalyzed by three classes of enzymes. A ubiquitin-activating enzyme (E1) uses one ATP to bind Ub, activating it for the next step. The activated Ub is transferred to the ubiquitin-conjugating enzyme (E2), and the E2 binds to the ubiquitin-protein ligase (E3), which is also bound to the substrate; the E2 then either directly transfers the Ub to the substrate or the E2 transfers the Ub to the E3 and it transfers the Ub to the substrate (if it is a HECT-domain E3) (Kipreos, 2005).

The attachment of a ubiquitin chain of 4 or more ubiquitins to the substrate is the signal that is recognized by the 26S proteasome to target substrate degradation. Multiple E2s bring the Ubs to the E3s to make the poly-Ub chain and in some cases another enzyme, the ubiquitin chain assembly factor (E4) is needed to make the chain (Koegl et al., 1999).

Ubiquitin is conjugated to target proteins (or other ubiquitin) through a bond between the conserved C-terminus of ubiquitin and a lysine residue on the target protein (or other ubiquitin) (Hershko and Ciechanover, 1998). Ubiquitin has 7 lysine residues and ubiquitin can be conjugated to all of them, resulting in differential signaling. Lys-48 linkages target substrates to the proteasome for degradation, while Lys-63 linkages are associated with the regulation of endocytosis or changes in target protein function (Kipreos, 2005). A single Ub (or less than 4) changes protein activity via transcriptional regulation, protein trafficking, or endocytosis (Schnell and Hicke, 2003).

Ubiquitin-mediated proteolysis is an important and powerful pathway for protein degradation and it induces a number of cell cycle transitions. Proteolysis is a directional and irreversible method of cell cycle regulation because it keeps the cell cycle progressing forward. The ubiquitin-mediated proteolysis pathway is important for the regulation of a wide range of proteins. Inactivating the Ub proteolytic pathway, results in the stabilization of the majority of cellular proteins (Rock et al., 1994).

There are four major classes of E3s; HECT-domain proteins, U-box proteins, monomeric RING finger proteins, and multi-subunit complexes that include a RING finger protein. The rest of this introduction and dissertation will focus on two multimeric RING finger E3s: $CRL2^{ZYG-11}$, a cullin 2-based complex with ZYG-11 as its substrate

recognition subunit; and APC/C (anaphase promoting complex/cyclosome, here referred to as APC), which includes a cullin-like protein APC2 (Vodermaier, 2004).

The SCF complex is the best characterized member of the cullin-RING family, and plays multiple roles in cell division, in part by targeting CDK inhibitors and G1 cyclins for degradation (Willems et al., 2004). While both APC and SCF regulate cell cycle progression, SCF activity is regulated at the substrate level and APC activity is regulated by the activity of the complex itself, i.e. SCF only targets proteins after they are modified, while APC targets proteins after APC has been modified and released from inhibition (Thornton and Toczyski, 2006). As an example, Sic1, a CDK inhibitor, is synthesized in late mitosis, but is only targeted for degradation by SCF at the G1/S transition, after it is phosphorylated on multiple sites by CDK (Nash et al., 2001). It is likely that the activity of the $CRL2^{ZYG-11}$ complex is also regulated by modification of its targets.

$CRL2^{ZYG-11}$, is a cullin-2-RING ubiquitin ligase complex with ZYG-11 as its substrate recognition subunit (SRS). Cullins and conserved members of E3 components were first identified in *C. elegans* and budding yeast (Kipreos et al., 1996; Mathias et al., 1996). The cullin, CUL-2, is the core component of the $CRL2^{ZYG-11}$ complex, which also includes adaptor proteins elongin C and elongin B that attach the SRS to the cullin. The SRS specifically binds substrates, ubiquitylating them, which is the signal for degradation by the proteasome (Kipreos, 2005). The complex also includes the RING-H2 finger protein Rbx1/Roc1. The E2 binds to the complex through Rbx1 when there is a conformational change that allows the Rbx1 to move away from the complex (Duda et al., 2008)(Fig 1.2).

The anaphase –promoting complex (APC)

APC is a multi-subunit complex with 13 core protein components. The core APC complex is present throughout all cell cycle stages. APC was first identified based on its ability to target both vertebrate and yeast mitotic cyclins for degradation (Thornton and Toczyski, 2006). The second major mitotic target of APC in both yeast and mammals is Securin (discussed below) (Cohen-Fix et al., 1996; Shirayama et al., 1999). APC has other targets including yeast motor proteins, Cin8 and Kip1, yeast spindle protein Ase1, mitotic regulator polo kinase (mentioned above), DNA replication regulators Dbf4 (yeast) and Geminin (metazoa), and its own adaptor subunit Cdc20 (Reed, 2003). While several of these are critical targets, there are other redundant forms of regulation besides APC that also control most of the targets. In yeast, only Securin and the mitotic cyclins must be degraded by APC for cell viability (Thornton and Toczyski, 2003).

APC targets its critical substrates, Securin and cyclin B, for destruction during the metaphase-to-anaphase transition (Peters, 2002). Securin is a protein that binds and inhibits Separase. Separase is an enzyme that cleaves cohesin, which binds sister chromatids together. The cleavage of cohesin allows sister chromatids to separate during anaphase. Targeting Securin for degradation is the central mechanism by which APC promotes chromosome segregation and the metaphase-to-anaphase transition, as the subsequent release of Separase cleaves cohesin to allow sister chromatid separation.

APC recognizes its substrates through specific protein motifs, the most common of which are the conserved 9-amino acid destruction-box (D-box) and the KEN box

motif. There are two other motifs, the A box (Littlepage and Ruderman, 2002) and the GxEN motif (Castro et al., 2003), that are also recognized by APC, but they are less well characterized. Cyclin B1 is targeted for degradation by APC through a destruction-box motif that is located at the N-terminal of cyclin B1 (McLean et al., 2011).

In addition to the destruction box, several different regions of cyclin B1 have been characterized. Work in *Xenopus* led to the predicted site of CDK1 interaction (Goda et al., 2001). A single amino acid point mutation (Y170A) in the N-terminal helix domain of cyclin B1 disrupts binding to CDK1 in human cells (Bentley et al., 2007); and although binding to CDK1 is disrupted, localization of cyclin B1 Y170A from the cytoplasm in interphase to the kinetochores, chromatin, spindle microtubules, and centrosomes during mitosis was not disrupted (Bentley et al., 2007).

APC activators are recruited just before mitosis.

Since the core APC complex is present throughout the cell cycle, additional signals are required for APC activation and the correct timing of target degradation. The cyclin B/Cdk1 complex and the Polo-like kinase 1 (Plk1) (mentioned above) phosphorylates the APC activators, Cdc20 and Cdh1, just before mitosis and the phosphorylated activators are then recruited to the core APC complex (Fang et al., 1998; Kramer et al., 2000; Rudner and Murray, 2000). Based on yeast studies, Cdc20 is the APC activator required for both cyclin B and Securin degradation. However, mammalian cell culture and affinity purifications revealed that unique APC activators are recruited for the degradation of cyclin B and Securin; Cdh1 binds APC in prometaphase and activates the APC complex that degrades Securin during mitosis; and Cdc20, the other APC

activator, binds APC also during prometaphase and activates the APC complex that degrades cyclin B1 (Jeganathan et al., 2005) (Fig 1.3).

APC activators are inhibited until metaphase.

The APC activators, Cdc20 and Cdh1, are also under regulatory control. Emi1 binds to Cdc20 to keep APC inactive throughout the S- and G2-phases (Reimann et al., 2001). Emi1 is degraded before entry into mitosis by $SCF^{\beta TrCP1}$, another ubiquitin ligase (Guardavaccaro et al., 2003). Cdh1 is kept inactive during prometaphase by CDK1 phosphorylation. Cdh1 becomes active when cyclin B1/CDK1 is inactivated (van Leuken et al., 2008). In yeast, Cdh1 activation also requires activation of phosphatase Cdc14, to remove inhibitory phosporylations from Cdh1 (van Leuken et al., 2008). Although the mechanisms to control mitotic exit are not well conserved between yeast and higher eukaryotes, mammalian cells do have orthologs of the proteins that are involved. Human Cdc14, hCdc14a, can dephosphorylate Cdh1 *in vitro* and activate the APC^{Cdh1} complex (Bembenek and Yu, 2001). And activation of Cdc14, by Cdc5 (a Plk ortholog in yeast) also seems to be conserved in human cells, as Plk1 binds and phosphorylates hCdc14a *in vitro* (Yuan et al., 2007). As in *Drosophila*, Cdh1 is not required for mitotic exit in human cells (Engelbert et al., 2008; Jacobs et al., 2002; Keck et al., 2007), but becomes more important in G1 phase. After cyclin B1/CDK1 inactivation in metaphase, a second step may be required to fully activate Cdh1 for G1 functions (Kramer et al., 2000). APC^{Cdh1} also appears to be regulated to degrade mitotic substrates in a defined order, starting with Cdc20 and Plk1 and targeting Aurora A and Aurora B at a later stage in G1, but this regulation is poorly understood (Lindon and Pines, 2004).

APC is inhibited by the Spindle Assembly Checkpoint

APC is negatively regulated prior to metaphase by a group of proteins known as the spindle assembly checkpoint (SAC) proteins. The SAC monitors the presence of unattached kinetochores and other chromosome conditions that would be unfavorable for chromosome segregation and prevents APC activation until all the chromosomes are attached and aligned at the metaphase plate (Chan et al., 2005). Initial studies revealed that the SAC proteins Mad2 and Mad3 directly bind to Cdc20 to reduce APC activity (Hwang et al., 1998; Kim et al., 1998)(Fig 1.3). Bub1 and Bub3 have also been identified as spindle checkpoint proteins, and are localized to meiotic chromosomes (Monen et al., 2005). The SAC proteins Mad2, Bub3, BubR1 are thought to bind and repress the activity of APC^{Cdc20}, while Rae1 and Nup98 bind and repress the activity of APC^{Cdh1} (Jeganathan et al., 2006) (Fig 1.3). More recent studies indicate that Bub1 and Bub3 might be recruiting other checkpoint proteins and that Mad1-Mad2 binding induces a conformational change in Mad2 that results in activation, and the activated Mad2 binds Cdc20 (Stein et al., 2007). This level of regulation couples APC activity to chromosome segregation. Studies on the checkpoint release revealed that poly-ubiquitination by APC is required for the inactivation of the SAC (Reddy et al., 2007). It was thought that SAC release allowed activation of APC, however careful examination of human cell extracts showed SAC dissociation while APC was still inactive, which led to the discovery of the mitotic checkpoint factor 2 (MCF2) complex, which was still associated with APC in its inactive form (Eytan et al., 2008).

APC functions in meiosis.

Studies in mouse, pig, frog, fly and nematode oocytes have revealed a role for APC during meiosis. APC mutants arrest in the metaphase I-to-anaphase I transition during meiosis (Golden et al., 2000). The first meiotic division is different from mitotic divisions because the homologous chromosomes align and are separated, while in mitosis and in meiosis II, it is the sister chromatids that align and are separated. This major difference between the two processes could require a presumably unique mechanism for control. While a previously mentioned study using mammalian cells indicated that Cdc20 and Cdh1 are responsible for degrading cyclin B and Securin, respectively, a study using mouse oocytes suggests that Cdc20 is responsible for both cyclin B and Securin degradation during meiosis (Li et al., 2007). Emi1 and Emi2 also seem to have a role in negatively regulating APC during meiosis (Liu et al., 2007; Tung et al., 2007). Securin with a mutated D-box is degraded through its KEN box during mitosis (Hagting et al., 2002; Zur and Brandeis, 2001), but is stable during meiosis I, which suggests that APC^{Cdh1}, which targets the KEN box, is not active during meiosis I (Herbert et al., 2003). RNAi in *C. elegans* to deplete the levels of seven APC subunits and *fzy-1* (the Cdc20 ortholog), resulted in embryos that arrested at metaphase I but had no effect on meiosis II (Stein et al., 2007). This suggests that APC^{Cdc20} is involved in the metaphase-to-anaphase transition in meiosis I.

Experiments using spindle checkpoint mutants in budding yeast resulted in many problems during meiosis I, indicating that the spindle checkpoint also plays a role during meiosis (Shonn et al., 2000). *C. elegans* have orthologs of 5/6 checkpoint proteins: *mdf-1*(Mad1), *mdf-2*(Mad2), *mdf-3/san-1*(Mad3), *bub-1*(Bub1) and *bub-3*(Bub3), but not

for Mps1(Kitagawa and Rose, 1999; Stein et al., 2007). Deletion or hypomorphic mutants of *mdf-1*, *mdf-2* and *mdf-3/san-1* suppress the meiotic defect of APC mutants, indicating that they regulate APC activity during meiosis (Stein et al., 2007).

There is a meiosis-specific cohesion complex that includes REC-8 instead of Scc1 (see cohesion section above) that holds chromosomes together from S phase until anaphase (Petronczki et al., 2003). In budding and fission yeast, Rec8 is cleaved in non-centromeric regions by Separase during meiosis I to allow separation of the homologous chromosomes (Buonomo et al., 2000; Kitajima et al., 2003), and the centromeric Rec8 is removed (by Separase in fission yeast) during meiosis II to allow sister chromatid separation (Kitajima et al., 2003). In *C. elegans*, Separase is required for chromosome segregation, which suggests that the cohesin degradation mechanism is conserved (Siomos et al., 2001).

C. elegans $CRL2^{ZYG-11}$ *controls meiosis and cyclin B degradation.*

In *Xenopus*, APC is required for the metaphase II-to-anaphase II transition, but is not required for meiosis I (Peter et al., 2001; Taieb et al., 2001). However, in *C. elegans*, APC appears to be required during meiosis I but not during meiosis II (Golden et al., 2000; Shakes et al., 2003; Wallenfang and Seydoux, 2000). Therefore, it is possible that components other than the APC ensure progression through meiosis I in *Xenopus* and meiosis II in *C. elegans*.

ZYG-11 is the substrate recognition subunit in the ubiquitin ligase, $CRL2^{ZYG-11}$ complex. ZYG-11 controls meiotic cell cycle progression and polarity in *C. elegans* embryos. ZYG-11 is required for progression through meiosis II, proper asymmetric division at the one cell stage, and proper localization of P-granules (Kemphues et al.,

1986). When *zyg-11* or *cul-2* are inactivated there is a delayed metaphase II-to-anaphase II transition and meiotic exit, but there is no change in the progression through meiosis I (Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007). The level of cyclin B1 and cyclin B3 are degraded during meiosis and stabilized during meiosis through the first mitotic divisions (Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007). *zyg-11* mutant embryos also have defects in polarity and chromosome condensation (Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007). There are ZYG-11 homologues in other metazoans, including *Drosophila* and humans, suggesting that this family of proteins performs an evolutionary conserved function. The role of this complex in meiotic and mitotic progression will be the focus of this dissertation.

FIGURES AND FIGURE LEGENDS
Figure 1.1

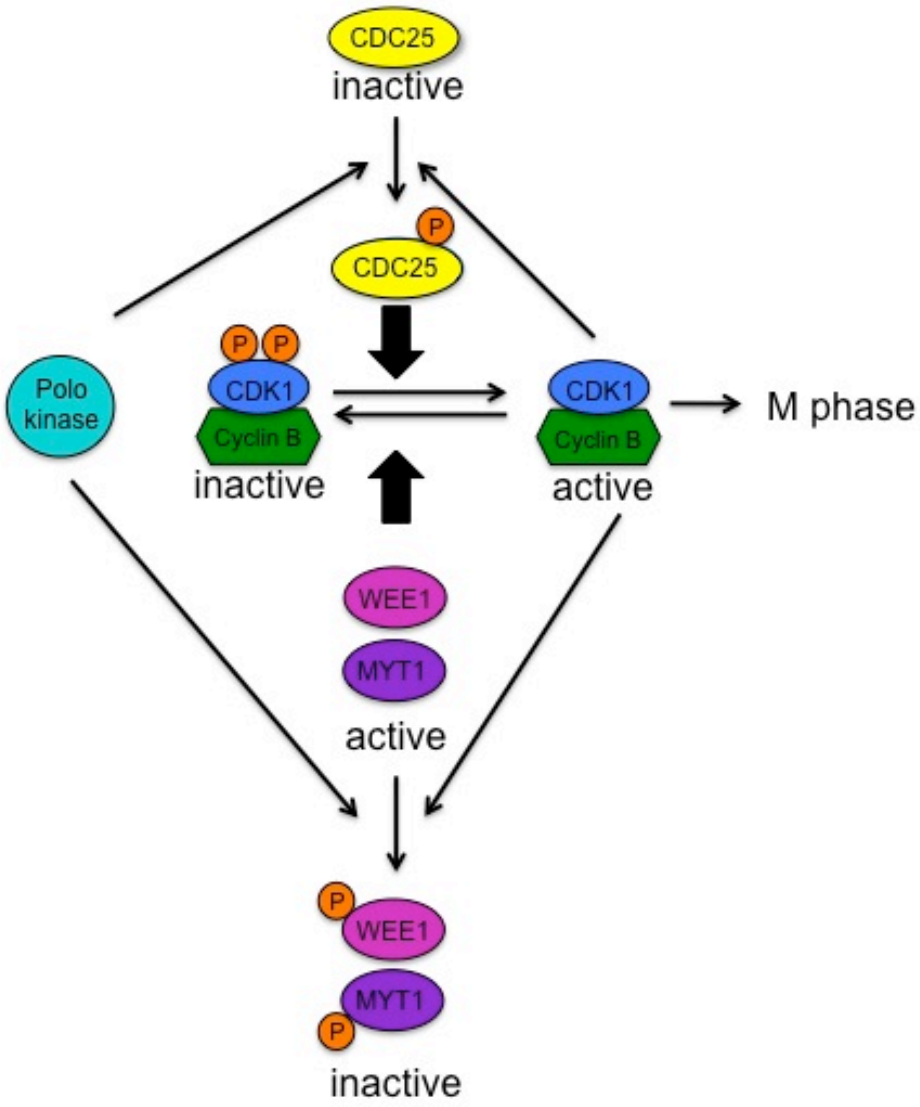

Figure 1.1 Diagram of cyclin B1/CDK1 regulation.

Cyclin B/CDK1 is inhibited by phosphorylation by WEE1 and MYT1. CDC25 removes those inhibitory phosphorylations to activate the cyclin B1/CDK1 complex and allow entry into M-phase. Polo kinase works upstream to inactivate WEE1 and MYT1 and activate CDC25 to further enhance the signal into M-phase. Cyclin B1/CDK1 is involved in a positive feedback loop to activate CDC25 and a negative feedback loop to inactivate WEE1 and MYT1.

Figure 1.2

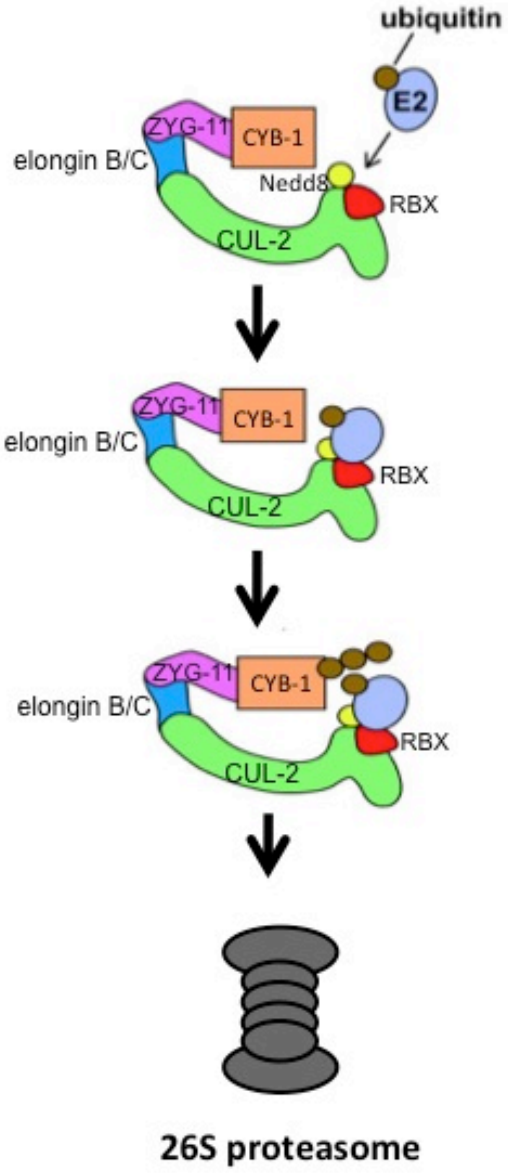
ubiquitin
E2
ZYG-11
CYB-1
elongin B/C
Nedd8
RBX
CUL-2
ZYG-11
CYB-1
elongin B/C
RBX
CUL-2
ZYG-11
CYB-1
elongin B/C
RBX
CUL-2
26S proteasome

Figure 1.2 $CRL2^{ZYG-11}$ complex and ubiquitin-mediated degradation.

The cullin CUL-2 is the core component of the E3 ubiquitin ligase $CRL2^{ZYG-11}$ and ZYG-11 is a substrate recognition subunit (SRS) that specifically binds substrates to allow the complex to ubiquitylate the target protein. The complex also includes the RING-H2 finger protein Rbx1/Roc1. The ubiquitin conjugating enzyme (E2) binds to the E3 complex through Rbx1. The adaptor proteins, elongin B and elongin C, link the SRS to the cullin. This $CRL2^{ZYG-11}$ complex facilitates the covalent attachment of ubiquitin to the substrate, marking it for degradation by the 26S proteasome.

Figure 1.3

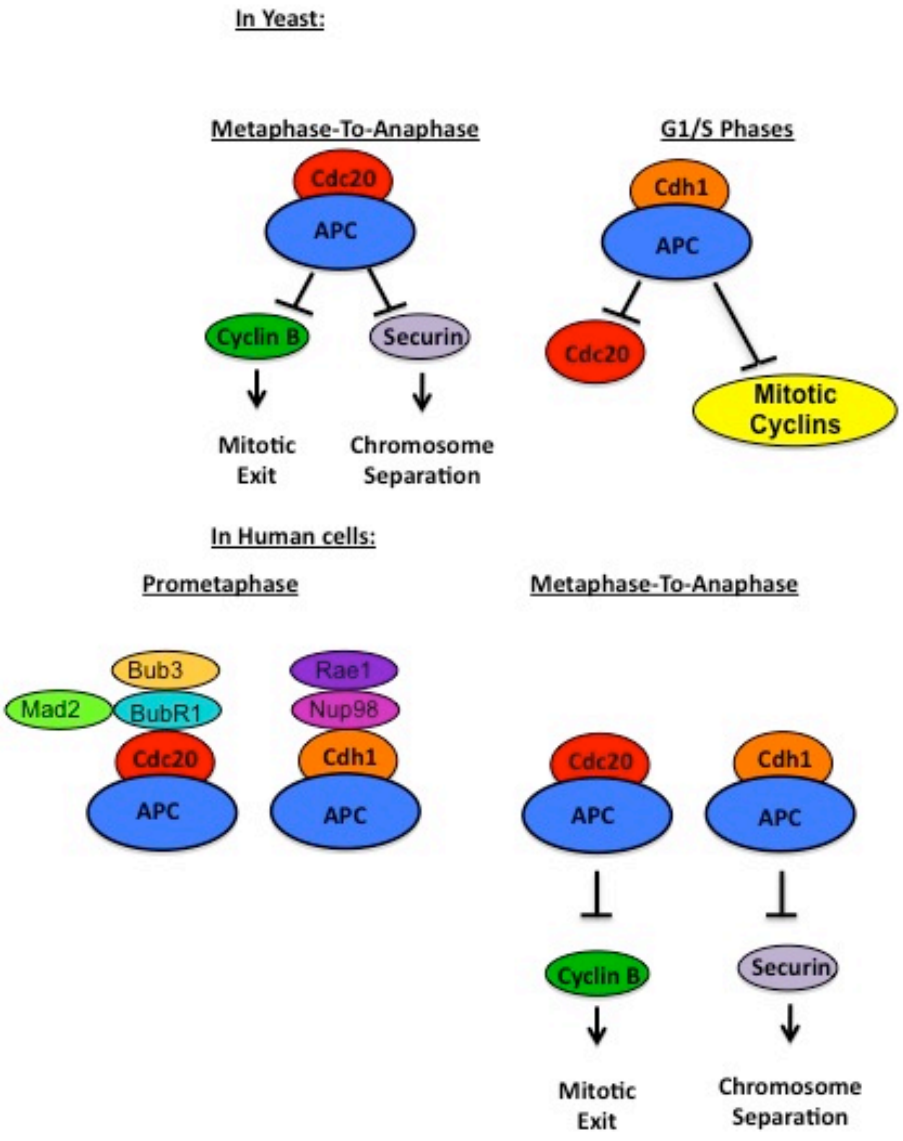
In Yeast:
Metaphase-To-Anaphase
Cdc20
APC
Cyclin B
Securin
Mitotic Exit
Chromosome Separation
G1/S Phases
Cdh1
APC
Cdc20
Mitotic Cyclins
In Human cells:
Prometaphase
Bub3
Mad2
BubR1
Cdc20
APC
Rae1
Nup98
Cdh1
APC
Metaphase-To-Anaphase
Cdc20
APC
Cyclin B
Mitotic Exit
Cdh1
APC
Securin
Chromosome Separation

Figure 1.3 Anaphase promoting complex substrates.

In yeast, the anaphase-promoting complex (APC), with Cdc20 as the adaptor subunit, targets cyclin B and Securin for degradation during the metaphase-to-anaphase transition. The APC with Cdh1 as the adaptor subunit targets Cdc20 and mitotic cyclins after M-phase and keeps the level of Cdc20 and mitotic cyclins low during G1 and into S-phase. In human cells, the APC^{Cdc20} complex is kept inactive by the spindle assembly checkpoint (SAC) proteins during prometaphase and degrades cyclin B1 during the metaphase-to-anaphase transition. The APC^{Cdh1} complex is kept inactive by SAC-like proteins in prometaphase and degrades Securin during the metaphase-to-anaphase transition.

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CHAPTER 2

POSITIONAL CLONING OF ZYG-11 SUPPRESSORS

INTRODUCTION

Proper chromosome segregation is essential to a developing organism. $CRL2^{ZYG-11}$ is an E3 ubiquitin ligase that is required for the meiotic metaphase II-to-anaphase II transition in *C. elegans*. *zyg-11* mutants have many phenotypes including defects in polarity, chromosome condensation, and cytoplasmic organization (Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007). Inactivation of either CUL-2 or ZYG-11 results in increased levels of both CYB-1 and CYB-3 (Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007).

The multiple *zyg-11* phenotypes suggest that $CRL2^{ZYG-11}$ either targets many substrates involved in early embryogenesis, or targets one major substrate that is upstream of all of these processes. Based on the vast literature that establishes the role of APC for CYB-1 degradation, the increased level of CYB-1 and CYB-3 could be a result of an increase in a ZYG-11 substrate that needs to be degraded to activate APC, or a secondary consequence of a delayed meiosis. We wanted to find the substrates of the $CRL2^{ZYG-11}$ complex. To do that we used a forward genetic screen to find *zyg-11* suppressors, which could be *zyg-11* substrates.

Genetic suppressors are mutations that suppress a mutant phenotype and these suppressor mutations can provide insights into the molecular pathway of the mutant gene that is being suppressed. *zyg-11* temperature sensitive (ts) mutants have a reduction in ZYG-11 function at the non-permissive temperature. A lack of ZYG-11 degradation activity is expected to lead to accumulation of critical substrate(s) and/or other downstream targets resulting in embryo arrest. Suppressors of *zyg-11* can include mutations in ZYG-11 substrate(s) that cause a reduced function of the substrates that compensate for the accumulation of the substrates upon loss of ZYG-11. The existence of multiple *zyg-11* phenotypes suggests that full suppression may require mutations in several genes thereby making this suppressor screen difficult; however the initial screen identified several *zyg-11* suppressors. Here we use SNP mapping and whole genome sequencing to identify the suppressor mutations in the *zyg-11* suppressor strains.

MATERIALS AND METHODS

SNP Mapping

SNP mapping was performed as described (Davis et al., 2005). The *zyg-11(ts)*, +suppressor (in N2 background) was crossed to zyg-11(ts) in a Hawaiian background (HW). 240 F2 progeny were cloned and grown at the non-permissive temperature to allow F3 progeny to grow up. Cloned F3 were sorted into 'suppressed' and 'non-suppressed' individuals. The 30 'most suppressed' and the 30 'least suppressed' worms were separately pooled. PCR was amplified for known SNPs between the N2 and HW strains that were spread evenly throughout the genome, and that contained the DraI restriction site. PCR

products were digested using DraI and the product was run on agarose gels. The amount of HW vs. N2 PCR product was compared between the suppressed and not suppressed worms at each marker to determine the chromosomal location of the suppressor mutation.

DEEP Sequencing and Analysis

Collection of genomic DNA from 30 large plates of ek14 worms. The worms were washed 2x with M9 solution and placed into M9 solution in 15 ml Falcon tubes; incubated 30 minutes at RT on nutator to purge worm guts; washed 2x with M9; 1x with NTE (100mM NaCl, 50 mM Tris, 20 mM EDTA); worm pellet frozen at -80°C for 30 minutes; and incubated at 65°C for 30-60 minutes in 2ml of proteinase K in NTE + 1%SDS (1% SDS, 400ug/ml proK) with periodic agitation. The crude mixture was cleaned up by adding 2 ml of buffered phenol (pH 8), mixing gently then spinning 5-15 minutes at 4000 rpm at room temperature. The aqueous (top) phase was transferred to a new tube; and the phenol extraction was repeated twice more. DNA was precipitated by adding 1/10 volumes NaOAC pH 5.2 and 2.5 volumes of ethanol, mixing gently and spooling DNA using a long pasture pipette with a sealed end. The extracted DNA was dissolved in 400 µl TE in microfuge tube. The DNA was then treated with 20 ug/ml RNAse A for 30 minutes at 37°C; then cleaned up by phenol extraction and ethanol precipitation. DNA was re-suspended in 200 µl TE.

Genomic DNA was sent to CoFactor Genomics for Next-gen sequencing using the Illumina/Solexa genome analyzer. One lane of sequencing with 36bp long reads produced 13X coverage of the *C. elegans* genome. Four different

programs were used analyze the data (MAQ, MAQGene, Galign, Bowtie/IGV). SNPs were verified by PCR amplification and either digestion (if the SNP disrupts a restriction site) or re-sequencing of the SNP PCR product.

RESULTS

Multiple suppressors were mapped to approximate chromosomal locations using traditional SNP mapping

A previous graduate student in the lab, Srividya Vasudevan, used ENU treatment to induce random mutations in *zyg-11(ts)* worms. She screened the F2 generation for viability at the non-permissive temperature to obtain *zyg-11* suppressors.

We used the high-throughput SNP mapping protocol described in to map the suppressor mutations (Davis et al., 2005). There are two common lab strains of *C. elegans* that we used for SNP mapping; the N2 strain, originally from Bristol, Great Britain (which was used to generate the suppressor mutations) and the HW strain, originally from Hawaii. Due to geographic separation these two worm strains have SNPs throughout their genome compared to each other. We used the SNPs to follow the chromosome regions that came from each parent. Using this approach we were able to determine the chromosomal locations of the suppressor mutations in four suppressor strains found in the screen (Table 2.1). A complementation test was performed for the suppressors that mapped to the same approximate chromosomal location to determine if they were the same mutations. Suppressor *ek20* crossed to suppressor *ek18* failed to complement, indicating that these are two separate suppressor mutations. Suppressor *ek23*

crossed to suppressor *ek18* did complement, suggesting that these strains have the same suppressor mutation (Table 2.2).

ek14 DEEP sequencing

Even with a high throughput approach, SNP mapping of several suppressors with multiple regions of interest is labor intensive and time consuming. To expedite the discovery of the *zyg-11* suppressor mutations, we prepared genomic DNA from the strongest *zyg-11* suppressor, *ek14*, and sent it for sequencing on an Illumina/Solexa genome analyzer, which produced 36 bp reads that gave 13x genome coverage.

We used MAQGene (Bigelow et al., 2009), a program designed by a group in the worm community to align the sequence reads (using the program MAQ as the aligner) to the published N2 wild type reference sequence and annotate the SNPs. When we ran our reads through MAQGene we found 1148 SNPs that changed codons between the published N2 genome and the strain containing suppressor *ek14*. Because this number of SNPs was more than we were expecting, we decided to try other sequence alignment software. Galign (Shaham, 2009) gave us 944 codon-changing SNPs, and MAQ (Li et al., 2008) gave us 542 codon-changing SNPs (Table 2.3). To eliminate the incorrect SNPs we compared the lists against each other. We did not find impressive overlap between the alignment programs (Table 2.4). We were not satisfied with any of these programs, so we re-ran our sequence reads through Bowtie and viewed the SNPs using IGV so we could visualize how many reads were mapping to each SNP and thereby use our own judgment to determine if an SNP call was

real or not (data not shown). To check to see which program had more correct SNP calls, we re-sequenced candidate SNPs that were in our region of interest. We found that neither Galign nor MAQGene were able to call reads with certainty: Galign reported 65% accurate SNPs, MAQGene reported 53% (Table 2.5). 13x coverage was reported to be enough coverage to find SNPs; however, the lack of clear SNP calls with multiple different programs is likely due to poor sequence quality.

CYB-2.1 is mutated in suppressor ek14

Although the sequence data was less than optimal, one of the candidates that we were able to confirm was particularly interesting. In the initial whole genome screen of suppressor *ek14* there was enrichment for N2 on chromosomes III, IV, V, and X. We re-crossed *ek14* to HW to isolate each region and determine which regions are responsible for suppression. When attempting to isolate the region on chromosome IV from the other regions with N2 enrichment in *ek14*, we were able to get cloned strains that had N2 and HW regions on chromosome IV and I. Strains with the N2 region on chromsome IV matched the hatching % data, i.e. clones with N2 rescued, while clones with HW on chromosome IV did not rescue; while it did not seem to make a difference whether there was N2 or HW in the chromosome I region (Fig 2.1A). This suggests that the suppressor mutation is located on chromosome IV between map units +1 and +5.9 and that the region on chromosome I is not being selected for, or is being selected for something that is independent of our screen (see *zeel-1*/*peel-1* section of the discussion).

The region that is being selected for on chromosome IV contains both CYB-1 (IV:4.68) and CYB-2.1 (IV:4.78). CYB-2.1 was one of the confirmed SNPs from the DEEP sequencing data. We re-sequenced three of the cloned strains, two that rescued and one that didn't rescue. We found that both clones that rescued, had a mutation in CYB-2.1 and the clone that did not rescue, lacked the SNP in CYB-2.1. We cloned out progeny from one of the suppressed individuals to confirm the rescue and found that all of the clones did rescue, as we expected (Fig 2.1 A). This suggests that the mutation in CYB-2.1 is the suppressor mutation in *ek14*.

We originally predicted that the suppressor mutation would presumably compromise the function of a ZYG-11 substrate. Since ZYG-11 is a member of the E3 ubiquitin ligase family, the *zyg-11(ts)* mutant at the non-permissive temperature, would theoretically have high levels of ZYG-11 substrates. Mutating these substrates would potentially compensate for the increased levels due to ZYG-11 inactivity and restore normal activity levels. The mutation in CYB-2.1 changes amino acid 121 from glutamic acid to a lysine (Fig. 2.1B). It is possible that this mutation reduces CYB-2.1 activity to compensate for the increased level of CYB-2.1 in ZYG-11 mutants, as we predicted. It is also possible that the additional lysine, created by the mutation, provides an additional ubiquitination site for ZYG-11 or another E3 to target CYB-2.1 for degradation, therefore restoring its normal protein level even when ZYG-11 activity is compromised.

Reducing the amount of CYB-2.1 rescues zyg-11(ts) lethality

To test the theory that an excess of CYB-2.1 is leading to *zyg-11(ts)* lethality, we planned to inactivate CYB-2.1 in a *zyg-11(ts)* mutant. The RNAi construct in the Ahringer feeding RNAi library (Fraser et al., 2000; Kamath and Ahringer, 2003) that targets CYB-2.1, also targets CYB-1, so we designed a new RNAi vector that included a sequence that was specific for CYB-2.1/2.2 and not CYB-1.

We grew *zyg-11(ts)* worms on *cyb-1/2.2/2.1* RNAi, *cyb-2.1/2.2* RNAi and *cyb-3* RNAi at the semi-permissive temperature, 20°C, and compared the embryonic lethality to *zyg-11(ts)* worms that were not treated with any RNAi. We found that inactivating CYB-1/2.1/2.2 rescued the hatch percent by 38.7%; inactivating CYB-2.1/2.2 rescued the hatch percent by 26.7%; and inactivating CYB-3 actually reduced the hatch percent by 17.4% (Fig. 2.1C). Both CYB-2.1 and CYB-1 are at abnormally high levels in the *zyg-11(ts)* mutant and by decreasing their level using RNAi we are able to partially rescue the *zyg-11(ts)* embryonic lethality.

Reducing the amount of CYB-2.1 does not rescue all zyg-11 phenotypes

To test the extent of rescue when increased levels of CYB-2.1 are reduced in *zyg-11(ts)* mutants, we treated *zyg-11(ts)* worms with *cyb-2.1/2.2* RNAi and determined if reducing the level of CYB-2.1 had any effect on polar body extrusion, cortical ruffling, and granule-free zones. We found that reducing the level of CYB-2.1 in *zyg-11(ts)* mutants did not rescue the polar body extrusion, cortical ruffling, or granule free zone defects in the *zyg-11(ts)* embryos (Table 2.6).

DISCUSSION AND FUTURE DIRECTIONS

A suppressor screen was used to discover novel ZYG-11 interactors. We successfully mapped the location of one of the suppressors, *ek14*, to a mutation in CYB-2.1. Both CYB-2.1 and CYB-1 are at abnormally high levels in the *zyg-11(ts)* mutant and by decreasing their level using RNAi we were able to partially rescue the *zyg-11(ts)* embryonic lethality. The four *C. elegans* cyclin B genes have partially redundant functions (van der Voet et al., 2009). Our result suggests that a broader reduction of cyclin B levels provides a more effective rescue of the *zyg-11(ts)* mutant than inactivating one cyclin B paralog. The relative ineffectiveness of CYB-3 RNAi rescue could be because *cyb-3* RNAi itself arrests cells in meiosis.

Reducing the amount of CYB-2.1 in a *zyg-11(ts)* mutant with RNAi is consistent with our theory that a ZYG-11 substrate would be at high levels and reducing the level would compensate for loss of ZYG-11. However, we need to confirm that the mutation we found in *cyb-2.1* does indeed reduce the activity of CYB-2.1. *ek14* should be outcrossed so the mutated *cyb-2.1* is the only mutation left in the strain and we should confirm that this mutation is actually suppressing *zyg-11*.

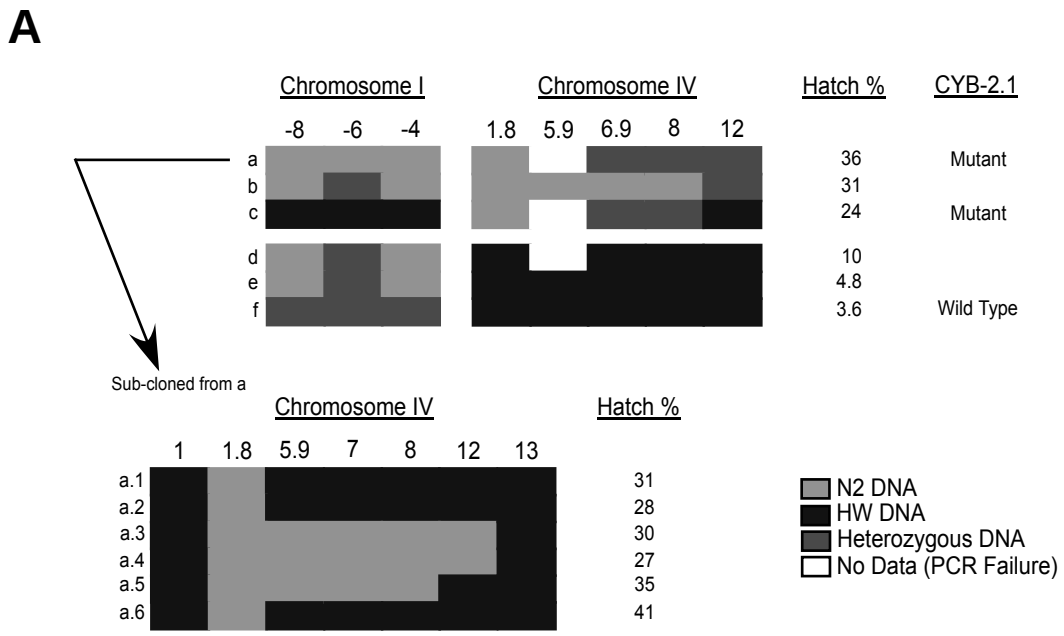
One problem with the SNP mapping approach where HW is crossed into N2 and SNPs between these genetic backgrounds are used as genotype markers to follow selection is that regions of the N2 genome may be under positive selection for reasons that are not related to the screen. While the majority of the SNPs between the HW and N2 strain are not functionally selected

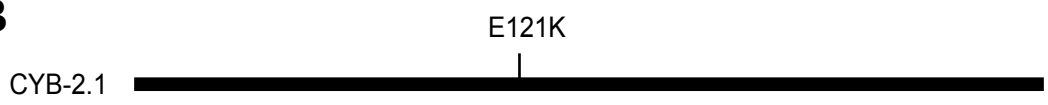
for, one region on chromosome I is always selected for in N2/HW crosses regardless of the selection screen that is being used, purely because of the discrepancy between the N2 and HW genetic background. In the N2 strain, there is a sperm delivered toxin, *peel-1* and an embryo-expressed antidote, *zeel-1* (Seidel et al., 2011). Both *peel-1* and *zeel-1* are located next to each other on chromosome I. The toxin is maternally delivered to all offspring, therefore an N2/HW heterozygous animal would only generate viable offspring that also had the antidote, i.e. at least on copy of the N2 region on chromosome I.

The example of the toxin/anti-toxin between the N2 and HW strains and the time commitment of SNP mapping, combined with dropping sequencing costs, increasing sequencing accuracy, and increasing precision in alignment programs, is making the SNP mapping technique a thing of the past. To find the other *zyg-11* suppressor mutations, the suppressor strains should be sent for whole genome sequencing.

2.6 FIGURES AND FIGURE LEGENDS

Figure 2.1





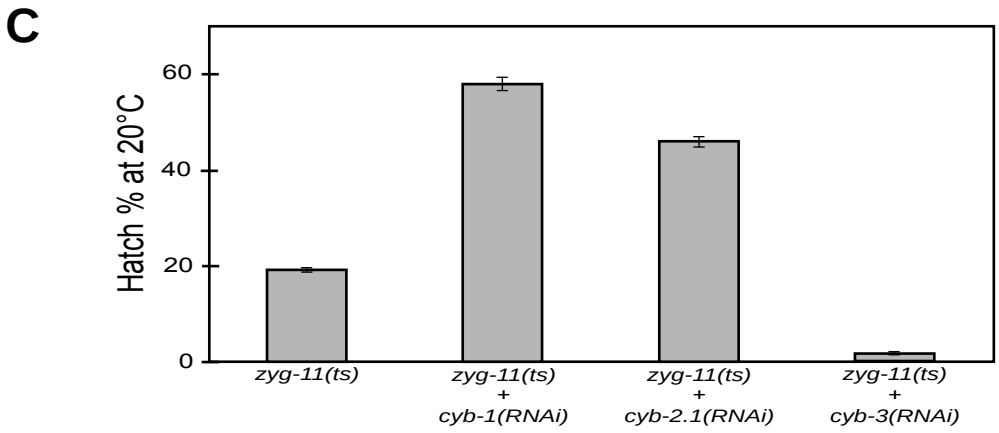


Figure 2.1 CYB-2.1 is mutated in suppressor *ek14*, and reducing the amount of CYB-2.1 resuces *zyg-11(ts)* lethality.

(A) SNP mapping data for suppressor *ek14* on chromosome IV and chromosome II. The suppressor mutation maps between +1 and +5.9 on chromosome IV. **(B)** CYB-2.1 mutation in suppressor *ek14*. **(C)** Hatch% of *zyg-11(ts)* with *cyb-1* RNAi, *cyb-2.1/2* RNAi, *cyb-3* RNAi.

Table 2.1 Chromosomal location of suppressed regions based on pooled DNA from suppressed and non-suppressed worms.

Suppressor Strain	Hatch % (at 26 C)	Chromosome Location
ek 14	45.6	V (-17), III (-1), IV (1), X (-8)
ek 18	25.8	II (-6), I (-6), III (-25)
ek 20	47.7	II (-6)
ek 23	45.3	II (-6)

Table 2.2 Complementation Tests.

	Hatch %
ek 23 X ek 18	43.1
ek 18 X ek 20	0.14

Table 2.3 Summary of SNPs from alignments using three different programs.

Program	# of codon changing SNPs detected
MAQGene	1148
Galign	944
MAQ	542

Table 2.4 The number of SNPs that overlapped between programs.

Program	# of SNPs overlapping
MAQGene vs. MAQ	22
Galign vs. MAQ	67
MAQGene vs. Galign	101

Table 2.5 Re-sequencing results to test accuracy of SNP calls.

Chromosome	Gene	Detected By	Confirmed	Method
V	T01G6.10	both	no	digest
IV	dpy-26	MAQGene	no	digest
IV	C08F8.3	both	no	sequencing
III	brc-1	galign	no	sequencing
V	dpy-11	both	no	sequencing
III	E02H9.2	both	no	sequencing
V	H23N18.4	both	no	sequencing
III	par-3	MAQGene	no	sequencing
V	lin-40	MAQGene	no	sequencing
I	cyb-2.2 M2	MAQGene	no	sequencing
III	cyk-1	both	no (all mut)	digest
III	frm-2	galign	yes	digest
IV	T22D1.5.1	both	yes	digest
V	hcp-1	galign	yes	digest
IV	cyb-2.1	MAQGene	yes	digest
I	cyb-2.2 M1	MAQGene	yes	digest
IV	cpt-2	both	yes	sequencing
V	ftn-1	both	yes	sequencing
X	gap-1	galign	yes	sequencing
IV	rad-26	galign	yes	sequencing
V	Y32F6B.1	both	yes	sequencing
IV	F01D4.5a	both	yes	sequencing
V	unc-61	both	yes	sequencing

Table 2.6 *zyg-11* phenotypes with *cyb-1* RNAi

	zyg-11(ts)	***zyg-11(ts)* + *cyb-2.1* RNAi**	**Wild-type**	**Wild-type + *cyb-2.1* RNAi**
2nd polar body extrusion	0/26	3/9	2/2	3/3
cortical ruffling	2/2 - Severe	1/1 - Severe	1/1 - Mild	3/3 - Absent/Mild
granule free zones	8/9	7/7	2/4	0/5

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CHAPTER 3

CYB-1 REGULATION IN C. ELEGANS

INTRODUCTION

The major regulator of mitotic dynamics is the cyclin B1/CDK1 complex (King et al., 1994). Activation of the cyclin B1/CDK1 complex triggers mitotic entry and the complex phosphorylates multiple targets modifying them to adopt their mitotic function. cyclin B1/CDK1 must be inactivated to exit mitosis. cyclin B1 is degraded at the metaphase-to-anaphase transition allowing the cells to transition back to interphase (King et al., 1994).

CDK1 activity is regulated in part by the association and degradation of cyclins. Eukaryotes express several cyclins, i.e. proteins with a "cyclin box" domain that mediate binding and activation of CDKs. The G1 cyclins, S phase cyclins, and mitotic cyclins are defined by their expression pattern, CDK-binding partners, and cell cycle function. There are two subfamilies of mitotic cyclins, A-type and B-type cyclins, and they are based on sequence similarity. A- type cyclins control S phase and G2/M phase. B-type cyclins with CDK1 control progression through mitosis (Sherr, 1996). While there are some partial overlaps (members of each subfamily share CDKs, and have similar expression patterns, and associated kinase activity), the paradigm in the field was that specific cyclin/CDK combos phosphorylate specific targets promoting distinct cell cycle transitions (van der Voet et al., 2009).

Studies using mice have counteracted the idea that cyclin/CDK complexes are substrate specific because knockout mice embryos lacking D-type cyclins are still able to divide (Satyanarayana and Kaldis, 2009). Also, CDK1 can substitute for all of the other cell cycle regulatory Cdks (Cdk2, Cdk4 and Cdk6) (Santamaria et al., 2007). These studies suggest that it is more important where and when cyclins are expressed, than the specific cyclin/CDK partners, except for CDK1, which cannot be replaced.

In *C. elegans*, there are four B-type cyclins (CYB-1, CYB-2.1, CYB-2.2, CYB-3) and one A-type cyclin (CYA-1). Inactivation of CYA-1 or CYB-2.1 and CYB-2.2 has no significant consequences on embryogenesis (van der Voet et al., 2009). Inactivating CYB-1 produces a failure of chromosome congression to the metaphase plate, and inactivation of CYB-3 results in a failure to initiate chromosome segregation at anaphase but does not block chromosome congression. Inactivating both CYB-1 and CYB-3 results in a severe mitotic entry defect, similar to CDK1 inactivation (van der Voet et al., 2009). The distinct phenotypes upon inactivation of CYB-1 and CYB-3, suggests separate functions for the two mitotic cyclins that together are sufficient to promote all of the mitotic cyclin/CDK functions. CYB-1 and CYB-3 have overlapping expression patterns, which suggests that their distinct functions arise not from different timing or location of expression but from differences in substrate specificity (Rahman and Kipreos; van der Voet et al., 2009).

CYB-1 and CYB-3 are also required for normal meiosis II progression. Inactivation of CYB-1 results in a failure of the congression of sister chromatids during meiosis II and inactivation of CYB-3 results in delayed separation of chromosomes during meiosis II. Inactivating CYB-1, CYB-3, or CYB-2.1 and CYB-2.2 does not disrupt

the progression through meiosis I, but inactivating all four cyclins results in a complete block in meiosis I. This suggests that all four B-type cyclins function redundantly in meiosis I (Rahman and Kipreos; van der Voet et al., 2009).

Previous work established that ZYG-11, which is the substrate recognition subunit for the CUL2-based $CRL2^{ZYG-11}$ complex, plays an important role in meiotic progression (Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007). Inactivation of CUL-2 or ZYG-11 leads to a delay in anaphase II and meiosis II exit, and stabilized CYB-1::GFP and CYB-3::GFP in *C. elegans* meiosis II and early mitotic divisions (Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007). It is unclear if the $CRL2^{ZYG-11}$ complex directly targets CYB-1 and CYB-3 for degradation or if $CRL2^{ZYG-11}$ works upstream to activate APC to target CYB-1 for degradation.

Here we work to determine if the $CRL2^{ZYG-11}$ complex directly targets CYB-1 for degradation or if the $CRL2^{ZYG-11}$ works with APC to degrade CYB-1. We found that inactivating both ZYG-11 and APC caused a greater stabilization of CYB-1 than either of the mutants alone. We also found that ZYG-11 physically binds CYB-1 in a region that is distinct from the APC binding domain. Our data suggests that ZYG-11 targets CYB-1 independently from APC to allow complete CYB-1 degradation during *C. elegans* meiosis and mitosis.

MATERIALS AND METHODS

Strains and alleles

C. elegans strains were cultured as previously described (Brenner, 1974). Strains and alleles used were: *zyg-11(ax491ts)* + GFP::CYB-1, *zyg-11(ax491ts)* + *cyb-1::yfp*, *unc-119(+)* + GFP::CYB-1-NTER, *unc-119(+)* + GFP::CYB-1-CBOX1, and *unc-119(+)* +

GFP::CYB-1-CBOX2. *zyg-11(ts)* mutants were maintained at 15°C; all other strains were maintained at 24°C.

RNAi

RNAi was performed by feeding worms with bacteria expressing dsRNA, as described (Feng et al., 1999; Timmons et al., 2001). The effectiveness of *mat-3* RNAi was confirmed by meiotic arrest. Inactivation of *zyg-11* was assessed by embryonic arrest. Inactivation of CYB-1 was confirmed by the elimination of GFP::CYB-1 signal.

Microscopy

Time-lapse movies were made of embryos in utero. Gravid adults were treated with 10 mM levamisole (Sigma) to paralyze striated muscles and mounted on slides with a 2.5% agarose pad covered with a 22x22 mm coverslip. All microscopy was performed with a Zeiss Axioplan microscope, a Hamamatsu ORCA-ER CCD camera, LuDL hardware controller, automated filter wheels and shutters, and an Apple iMac computer running Openlab software (Improvision). Movies were made with pulsed DIC and epifluorescence every 60 seconds.

Immunoprecipitation

Wild-type worms and worms expressing GFP::CYB-1 were grown with and without CUL-2 RNAi. Animals were harvested for immunoprecipitation as described (Kim et al., 2008; Starostina et al., 2007). Immunoprecipitation was performed with GFP-Trap (Chromotek). IP samples and whole-worm lysate were analyzed by Western blot with anti-GFP monoclonal (GF28R, Thermo Scientific), anti-ZYG-11 polyclonal (Vasudevan et al., 2007), and anti-α-tubulin monoclonal (DM1a, Sigma) antibodies.

Protein interaction studies in mammalian cells

Cells were transfected using Liptofectamine (Invitrogen), using the recommended protocol. Immunoprecipitation of proteins ectopically expressed in HEK293T cells was performed as previously described (Kim et al., 2008; Starostina et al., 2007).

Vulva Precursor Cell (VPC) analysis

zyg-11(ts); YFP::*cyb-1* worms were grown on large plates and collected as gravid adults. The adults were treated with sodium hypochlorite solution (30% bleach, 16% NaOH) to harvest the eggs. The eggs were kept in M9 solution at 16°C for 36 hours to synchronize the population at the L1 stage. The L1 larvae were grown on OP50 or *mat-3(RNAi)* plates at the non-permissive temperature, 26°C, and 23 hours later the L2/L3 stage worms were mounted on slides with 2.5% agarose pads in a 50 mM levamisole solution (Sigma). Pictures were taken using a Zeiss Axioplan microscope, with a Hamamatsu ORCA-ER CCD camera, LuDL hardware controller, automated filter wheels and shutters, and an Apple iMac computer running Openlab software (Improvision).

RESULTS

Inactivating both C. elegans ZYG-11 and APC stabilizes CYB-1 in meiosis

CYB-1 is normally degraded during *C. elegans* meiosis. Worms expressing CYB-1::GFP have strong CYB-1 signal in mature oocytes, but after fertilization that signal rapidly disappears during meiosis I and II (Liu et al., 2004). Previous data showed that inactivating APC or ZYG-11 individually resulted in a delay in CYB-1 degradation during meiosis (Liu et al., 2004). To determine if inactivating ZYG-11 in addition to inactivating APC resulted in increased levels of CYB-1, which we would expect if ZYG-11 is working independently from APC, we analyzed the level of CYB-1 when ZYG-11 or APC were

individually inactivated or when ZYG-11 and APC were inactivated together. Worms expressing a GFP::*cyb-1* transgene driven by the *pie-1* promoter (to allow germline expression) were subjected to *zyg-11* RNAi*; mat-3* RNAi (MAT-3 is an APC subunit (Davis et al., 2002)); or *zyg-11* RNAi plus *mat-3* RNAi. We analyzed the level of GFP::CYB-1 in the embryos of gravid adults. Using the position of the embryos in the adult as a marker for how long the embryo has been in meiosis (the one closest to the spermatheca is the youngest), we determined that wild-type embryos were able to exit meiosis and GFP::CYB-1 was efficiently degraded (Fig. 3.1A). Embryos in worms treated with *zyg-11* RNAi were delayed in meiosis when compared to wild-type embryos and the CYB-1 level was not completely degraded, even by the 2-cell stage (Fig. 3.1A). Embryos in worms treated with *mat-3* RNAi never exited meiosis and the level of CYB-1 was also stabilized compared to wild-type embryos (Fig. 3.1A). Embryos in worms treated with both *zyg-11* RNAi and *mat-3* RNAi also never exited meiosis and had stabilized CYB-1 levels compared to the level of CYB-1 in wild-type embryos (Fig 3.1A). We quantified of the amount of CYB-1 in the second embryo in the gravid adult (2-cell stage in wild-type, delayed meiotic embryo in the RNAi treated worms) and normalized the amount of CYB-1 to the amount in the first meiotic embryo. We found that there was a significant increase in the amount of CYB-1 when ZYG-11 and APC were inactivated individually or when they were inactivated together compared to wild-type embryos (Fig 3.1B). While the average amount of CYB-1 level in the second embryo was higher when both ZYG-11 and APC were inactivated compared to each inactivated individually, the variation in the data resulted in no significant difference in CYB-1 levels when ZYG-11 or APC were inactivated individually or when they were inactivated together (Fig

3.1B). This may suggest that ZYG-11 and APC might not work in separate pathways to degrade CYB-1, but ZYG-11 might work upstream to activate APC to degrade CYB-1. RNAi only partially inactivates genes, which could skew these results. Ideally to make a genetic argument that inactivating both ZYG-11 and APC results in increased CYB-1 levels compared to inactivating ZYG-11 or APC individually, as evidence that these E3s work in separate pathways, null mutants that completely inactivate the gene function should be used. The extreme variation in CYB-1 levels is likely due to differences in the meiotic timing of the embryos that were analyzed. While the position of the embryos in the body of the gravid adult are a good rough estimate of meiotic timing, this approach may not be accurate enough to capture the difference in CYB-1 stabilization when both ZYG-11 and APC are inactivated.

To get accurate quantification and timing of CYB-1 degradation during meiosis we followed oocytes as they were fertilized and took time-lapsed pictures capturing the level of CYB-1 every minute as the embryo went through meiosis. Previous data demonstrated that as the embryo progressed through meiosis the level of CYB-1 was reduced (Liu et al., 2004). In the wild-type embryo, the level of CYB-1 was reduced by 80% by the time the embryo had completed meiosis II (Liu et al., 2004). Inactivating APC resulted in a 50% reduction of CYB-1 levels in the same 40 min time period as the wild-type meiosis (APC inactivation leads to arrest in meiosis) and inactivating ZYG-11 resulted in a 62% reduction of CYB-1 in 40 mins (Liu et al., 2004). This result suggests that APC and ZYG-11 can each partially stabilize the level of CYB-1 during meiosis (Liu et al., 2004). We wanted to determine the level of CYB-1 stabilization in meiosis when both ZYG-11 and APC are inactivated to determine if ZYG-11 and APC regulate CYB-1

in parallel pathways. If ZYG-11 and APC work independently to degrade CYB-1, we would expect an increase in CYB-1 levels when both ZYG-11 and APC are inactivated compared to the level of CYB-1 stabilization when ZYG-11 or APC are inactivated separately. We used a *zyg-11(ts)* mutant strain expressing the GFP::*cyb-1* transgene and grew it on *zyg-11* RNAi or *zyg-11* RNAi mixed with *mat-3* RNAi at the non-permissive temperature, 25°C. We analyzed embryos going through meiosis and found that inactivating ZYG-11 alone stabilized CYB-1, however, the overall level from the start of meiosis, until 40 minutes later (which is the normal time to complete meiosis and CYB-1 degradation) still dropped by an average of 22%. Inactivating both ZYG-11 and APC resulted in higher overall levels of CYB-1 and the level of CYB-1 from the start of meiosis, until 40 minutes later only dropped by 15% (Fig. 3.2A). The observation that inactivation of both ZYG-11 and APC leads to further CYB-1 stabilization suggests that ZYG-11 and APC work in independent pathways to degrade CYB-1 during meiosis.

To test if this increase in CYB-1 level when ZYG-11 is inactivated is functionally important, we reduced levels of CYB-1 in *zyg-11(ts)* worms and determined if the embryonic lethality was rescued. We grew *zyg-11(ts)* mutant worms on *cyb-1* RNAi at the semi-permissive temperature, 20°C, and counted the number of offspring that hatched. Reducing the level of CYB-1 in *zyg-11(ts)* mutants increased the average number of offspring from 19% (n=337) to 58% (n=751) (Fig 3.2B). This rescue indicates that the increased level of CYB-1 when ZYG-11 is inactivated has a detrimental impact on the survival of the embryo and that reducing the level of CYB-1 suppresses *zyg-11(ts)* lethality.

ZYG-11 regulates CYB-1 in a proteasome-dependent manner

The $CRL2^{ZYG-11}$ complex is a ubiquitin ligase that targets substrates for degradation by the covalent attachment of ubiquitin, marking them for degradation by the 26S proteasome. If CYB-1 is a direct substrate of the $CRL2^{ZYG-11}$ complex then ectopic co-expression of ZYG-11 should reduce the level of co-expressed CYB-1, and if that reduction is due to ubiquitin-mediated degradation, inactivation of the proteasome should restore the level of CYB-1 when ZYG-11 is present. VSVG-tagged *C. elegans* CYB-1 was expressed with or without ZYG-11 in human 293T cells. We found that the addition of ZYG-11 reduces the level of CYB-1 expression (Fig. 3.3A). The reduction of CYB-1 level is restored when the cells are treated with MG132 (a proteasome inhibitor) (Fig. 3.3A). This suggests that ZYG-11 can target CYB-1 for degradation in mammalian tissue culture cells and that this regulation is proteasome dependent.

C. elegans ZYG-11 physically interacts with C. elegans CYB-1

If ZYG-11 is directly targeting CYB-1 for degradation, ZYG-11 should physically interact with CYB-1. We tested ZYG-11–CYB-1 interaction by expressing FLAG::ZYG-11 and VSVG::CYB-1 as well as FLAG::CUL-4 (as a negative control) in mammalian tissue culture cells and asked if CYB-1 co-immunoprecipitated with ZYG-11 or CUL-4. We found that ZYG-11 and CYB-1 co-immunoprecipitate, while CUL-4 and CYB-1 do not (Fig. 3.3B). This specific physical interaction confirms the hypothesis that ZYG-11 directly binds and targets CYB-1 for proteolytic degradation.

ZYG-11 physically interacts with CYB-1 in a region distinct from APC interaction.

We wanted to know which region of CYB-1 is involved in binding to ZYG-11. The crystal structure for human cyclin B1 was published in 2007 (Petri et al., 2007), and

since cyclin B1 is so well conserved, we could align *C. elegans* CYB-1 with human cyclin B1 to gain insight into the structure of *C. elegans* CYB-1. We used Swiss-Model (Schwede et al., 2003) to align CYB-1 to human cyclin B1 and mapped the alignment onto the human cyclin B1 crystal structure (Fig. 3.4). In order to keep the integrity of the protein structure intact, we used the predicted CYB-1 protein structure as a guide to divide CYB-1 into three pieces being careful to not disrupt any helices; NTER (aa1-110, which contains the APC destruction box), CBOX1 (aa75-209), and CBOX2 (aa203-159). We expressed the pieces of CYB-1 tagged with VSVG in 293T cells and we found that CBOX1 co-immunoprecipitates with ZYG-11, but NTER and CBOX2 do not (Fig. 3.5). The CBOX1 region, which interacts with ZYG-11, does not contain the destruction box motif, which is the region that is targeted by APC. The finding that ZYG-11 binds a region of CYB-1 that does not contain the APC recognition motif, suggests that ZYG-11 has it's own target region for recognition that is distinct from that of APC, thereby providing further evidence that ZYG-11 regulates cyclin B1 independently of APC.

CYB-1-CBOX1 is efficiently degraded in C. elegans in a ZYG-11-dependent manner

To test if the CBOX1 region of CYB-1 is also required for interaction with ZYG-11 in the worm, we constructed worm strains expressing GFP::CYB-1-NTER, GFP::CYB-1-CBOX1, and GFP::CYB-1-CBOX2 and analyzed the level of GFP in the embryos of gravid adults. By comparing the level of GFP in meiosis to the level of GFP in the 2-cell stage, we were able to determine which regions of CYB-1 were necessary for CYB-1 degradation. GFP::CYB-1-NTER had variable levels in the 2-cell stage compared to meiosis, indicating that this construct was not consistently degraded by the 2-cell stage (Fig. 3.6A). GFP::CYB-1-CBOX1 had a significantly lower level of GFP signal in the 2-

cell stage when compared to meiosis, indicating that this construct is efficiently degraded (Fig. 3.6A). GFP::CYB-1-CBOX2 had similar levels of GFP in the 2-cell stage compared to meiosis, indicating that this construct is not efficiently degraded (Fig. 3.6A). This indicates that CYB-1-CBOX1 contains a motif that can function as a degradation signal, in addition to the destruction box motif that was previously discovered in the N-terminus of CYB-1 (see intro).

To determine which E3, APC or ZYG-11, is required to degrade CBOX1. We subjected the worms expressing the GFP::*cyb-1-cbox1* transgene to *zyg-11* RNAi or *mat-3* RNAi and analyzed the level of GFP in the embryos of gravid adults. By comparing the level of GFP in meiosis to the level of GFP in the 2-cell stage, we were able to determine the effect of inactivating ZYG-11 or APC on the degradation of CYB-1-CBOX1. We found that inactivating APC (using *mat-3* RNAi) had no significant effect on the degradation of CBOX1 when compared to wild-type worms, while inactivation of ZYG-11 significantly stabilized CBOX1 compared to wild type (Fig. 3.6B). This demonstrates that ZYG-11 not only interacts specifically with the CBOX1 region of CYB-1, it also uses this region to promote CYB-1 degradation, which is distinct from APC.

ZYG-11 physically interacts with CYB-1 in C. elegans

ZYG-11 is the substrate recognition subunit (SRS) of the $CRL2^{ZYG-11}$ complex and previous work has demonstrated that SRSs are autoubiquitylated when bound to the E3 complex, resulting in the de-stabilization of the SRS when it is bound to the complex (Kipreos, 2005). Inactivating CUL-2 stabilizes the level of ZYG-11 (Sonneville and Gonczy, 2004). We expect ZYG-11 to bind to its substrate and in the context of CUL-2 inactivation, this should allow better capture of the ZYG-11–CYB-1 interaction in vivo.

To determine if ZYG-11 physically binds CYB-1 in vivo, worms expressing GFP::CYB-1 were treated with *cul-2* RNAi, which prevents the ubiquitin-mediated degradation of CYB-1 bound to ZYG-11. We found that with *cul-2* RNAi, ZYG-11 co-immunoprecipitates with GFP::CYB-1, demonstrating that CYB-1 and ZYG-11 physically interact in *C. elegans* (Fig. 3.7).

CYB-1 regulation in C. elegans mitosis

When *zyg-11(ts)* or *apc(ts)* mutants are shifted to the non-permissive temperature, the inactivation of either protein produces a dramatic meiotic phenotype. Young adults of *zyg-11(ts)* or *apc(ts)* mutants, that are shifted to the non-permissive temperature have 100% arrested embryos, however if 2-cell stage eggs (or later) are shifted to the non-permissive temperature for either genotype, the embryos develop to become sterile adults (data not shown). Since both ZYG-11 and APC function redundantly for CYB-1 degradation during meiosis, we wondered whether inactivating both ZYG-11 and APC together would result in increased levels of CYB-1 or mitotic arrest?

To analyze the effect of inactivating both ZYG-11 and APC on mitotic CYB-1 levels, we used worms expressing a *cyb-1*::YFP transgene driven by the *cyb-1* promoter. We synchronized *zyg-11(ts)*; CYB-1::YFP and *emb-30(ts);* CYB-1::YFP worms at the L1 stage at the permissive temperature, 16°C. These L1 larvae were grown in the presence of food (to induce development) with or without *mat-3* RNAi + zyg-11 RNAi at the *zyg-11(ts)* non-permissive temperature, 26°C. We analyzed the cell divisions of vulva precursor cells (VPCs) 23 hours later in L2/L3 stage worms (the stage when the VPCs double from three VPCs to six VPCs). A survey of synchronized worms theoretically should have captured equal number of VPCs in mitosis for each of the

conditions, however when ZYG-11 or ZYG-11 and APC were inactivated the number of cells that happened to be in mitosis when they were analyzed was increased compared to the wild-type worms (Fig. 3.8A). This suggests that there is a delay in the mitotic timing when ZYG-11 or ZYG-11 and APC are inactivated compared to the wild-type situation.

In addition to the increased mitotic index, we looked at situations where one VPC hadn't divided yet, and another VPC had already divided; because VPCs are supposed to divide at the same time, we can infer that the divided cell had just recently divided. In these situations, we observed that when ZYG-11 or APC were each individually inactivated, CYB-1 was degraded in the cells that had just divided. However, in worms where ZYG-11 and APC were inactivated together, CYB-1 was not degraded in the recently divided cell (Fig. 3.8B). This suggests that both ZYG-11 and APC are required for timely CYB-1 degradation in *C. elegans* mitosis. Additionally, this suggests that *C. elegans* somatic cells can exit mitosis without completely degrading CYB-1.

The role of $CRL2^{ZYG-11}$ in non-dividing VPC cells

To determine if ZYG-11 plays a role in non-dividing cells, we analyzed the VPCs in L4 stage worms; at this stage, the VPCs have finished dividing and have terminally differentiated to form the vulva. When we compared the level of CYB-1 in the wild-type vulva cells to the level in the vulva cells of *mat-3(ts)* mutants (mat-3 is an APC subunit) or *zyg-11(ts)* mutants, we found that the CYB-1 level was 2x higher in the *zyg-11(ts)* mutants when compared to wild-type, while the level of CYB-1 in *mat-3(ts)* mutants was slightly lower than in wild-type (Fig 3.8 A, B). This suggests that ZYG-11 also plays a role in regulating CYB-1 levels in non-dividing cells.

DISCUSSION AND FUTURE DIRECTIONS

We have demonstrated that the $CRL2^{ZYG-11}$ complex negatively regulates CYB-1 levels in *C. elegans* meiosis and mitosis. This regulation is likely to result from direct targeting of CYB-1 for degradation via the ubiquitin-mediated proteolytic pathway. It is possible that ZYG-11 works upstream to activate APC, but since ZYG-11 physically associates with CYB-1 it is more likely that the $CRL2^{ZYG-11}$ complex directly targets CYB-1 independently of APC. The combination of the biochemical binding assays, demonstrating that ZYG-11 binds the CBOX1 region of CYB-1, the over-expression of ZYG-11 reducing the amount of CYB-1 in 293T cells, with the level of CYB-1 being restored with the addition of proteasome inhibitor, demonstrates that ZYG-11 is regulating CYB-1 in a proteasome-dependent manner. Together with the complementary phenotypic analysis of CYB-1 levels when ZYG-11 and/or APC are inactivated, the level of CYB-1 stabilization in meiosis or mitosis, all provide evidence for our model of $CRL2^{ZYG-11}$ mediated CYB-1 degradation during *C. elegans* mitosis and meiosis.

Narrowing down the ZYG-11 binding motif to the CBOX1 region of CYB-1 demonstrated that ZYG-11 binds a distinct region of CYB-1 from APC, therefore providing additional evidence for a separate pathway. Further fine mapping of the CBOX1 region is required to determine the ZYG-11 recognition motif. This motif could be used in bioinformatics searches for more ZYG-11 substrates.

ZYG-11 binds to the CBOX1 region of CYB-1. The CBOX1 region also binds to CDK1. ZYG-11 either binds regions of CBOX1 required for CDK1 binding (which would be competitive binding between ZYG-11 and CDK1), or it binds to regions of CBOX1

that do not compete with CDK1 binding. It will be interesting to determine the context of the ZYG-11–CYB-1 interaction to clarify the role of ZYG-11 in CYB-1 degradation. ZYG-11 could be regulating the active cyclin B/CDK1 complex, or it could target excess CYB-1 that is not bound to CDK1 for degradation.

ZYG-11 is the substrate recognition subunit of the $CRL2^{ZYG-11}$ E3 ubiquitin ligase complex. E3's target proteins for degradation by the covalent attachment of ubiquitin chains onto the substrate, marking them for degradation by the 26S proteasome. Presumably ZYG-11 is targeting CYB-1 for degradation via the ubiquitination pathway. To confirm this hypothesis, it would be helpful in the future to show that the $CRL2^{ZYG-11}$ complex can ubiquitylate ZYG-11 in an *in vitro* ubiquitination assay.

Our results suggest that *C. elegans* VPCs can divide without degrading CYB-1. In the wild-type situation, cyclin B1 is degraded as soon as the spindle assembly checkpoint has been inactivated by the alignment of all chromosomes to the metaphase plate (Clute and Pines, 1999). Introducing a non-destructible cyclin B in *Xenopus* egg extracts or in budding yeast, does not arrest cells in metaphase, indicating that cyclin B degradation is not required for the metaphase-to-anaphase transition (Holloway et al., 1993; Surana et al., 1993). However, expression of the non-destructible cyclin B (or elevation of overall cyclin B levels) causes these cells to arrest in telophase, indicating that cyclin B degradation is required for mitotic exit (Holloway et al., 1993). Here we have observed that larval-stage *C. elegans* cells in which ZYG-11 and APC are co-inactivated with RNAi can exit mitosis with readily detectable levels of CYB-1. This suggests that either CYB-1 degradation is not required for mitotic exit in *C. elegans* or the amount of CYB-1 stabilization with ZYG-11 and APC RNAi is not high enough to

trigger an arrest in telophase. The CRL2ZYG-11 complex likely directly targets CYB-1 for degradation in addition to APC. It will be interesting to see how this level of regulation is controlled and if this level of regulation is conserved.

3.6 FIGURES AND FIGURE LEGENDS

Figure 3.1

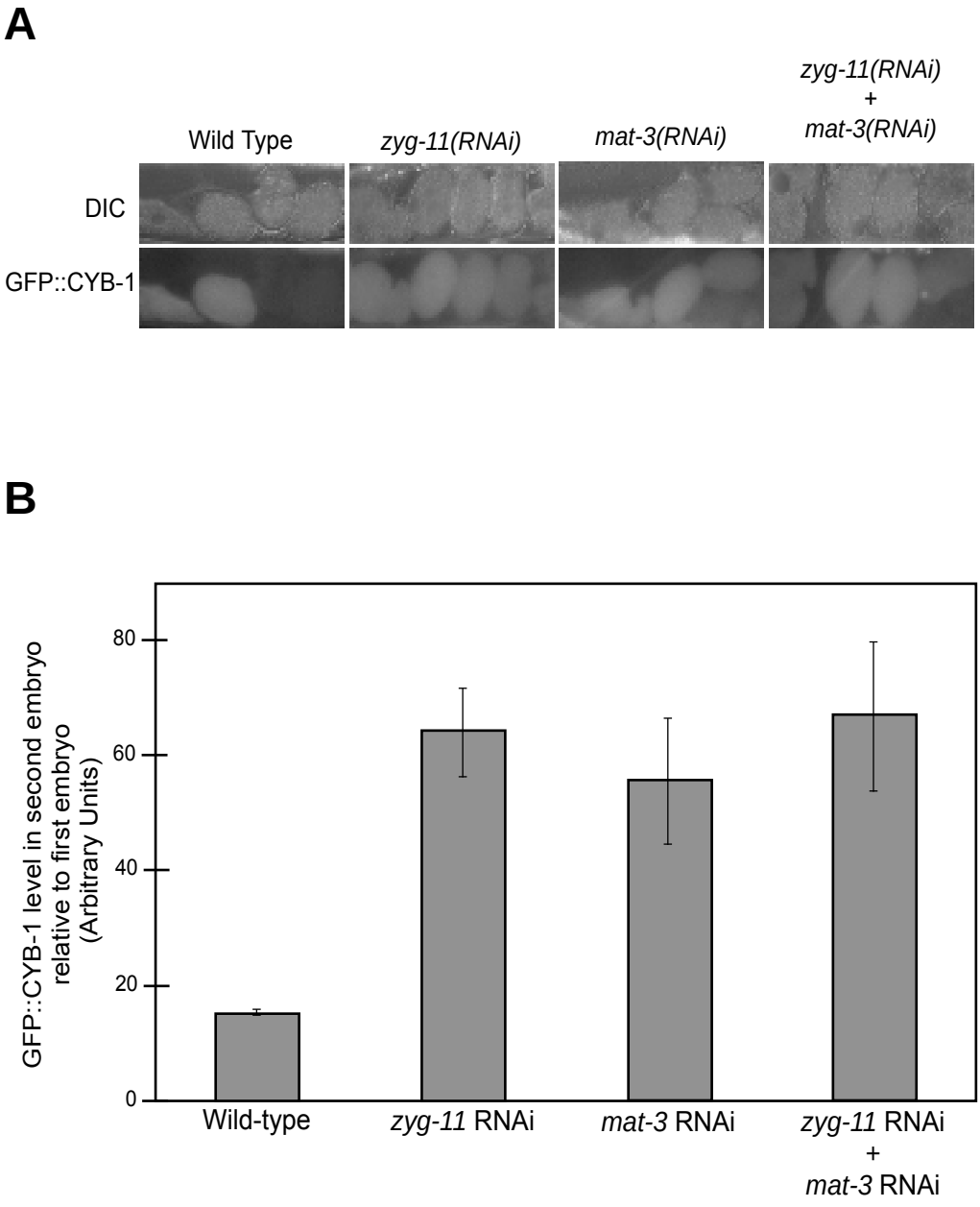

Figure 3.1 Inactivating ZYG-11 and APC stabilizes CYB-1 during *C. elegans* meiosis.

(A) DIC (top) and florescence (bottom) images of gravid adults expressing a *cyb-1*::GFP transgene, treated with *zyg-11* RNAi, *mat-3* RNAi, and *zyg-11* RNAi + *mat-3* RNAi. The level of CYB-1::GFP is dramatically reduced when comparing the 2-cell stage to meiosis in the wild type worm. Inactivating either ZYG-11 or APC or both ZYG-11 and APC stabilizes the level of CYB-1::GFP when comparing the 2-cell stage to meiosis. **(B)** Quantification of 2-cell stage embryos.

Figure 3.2

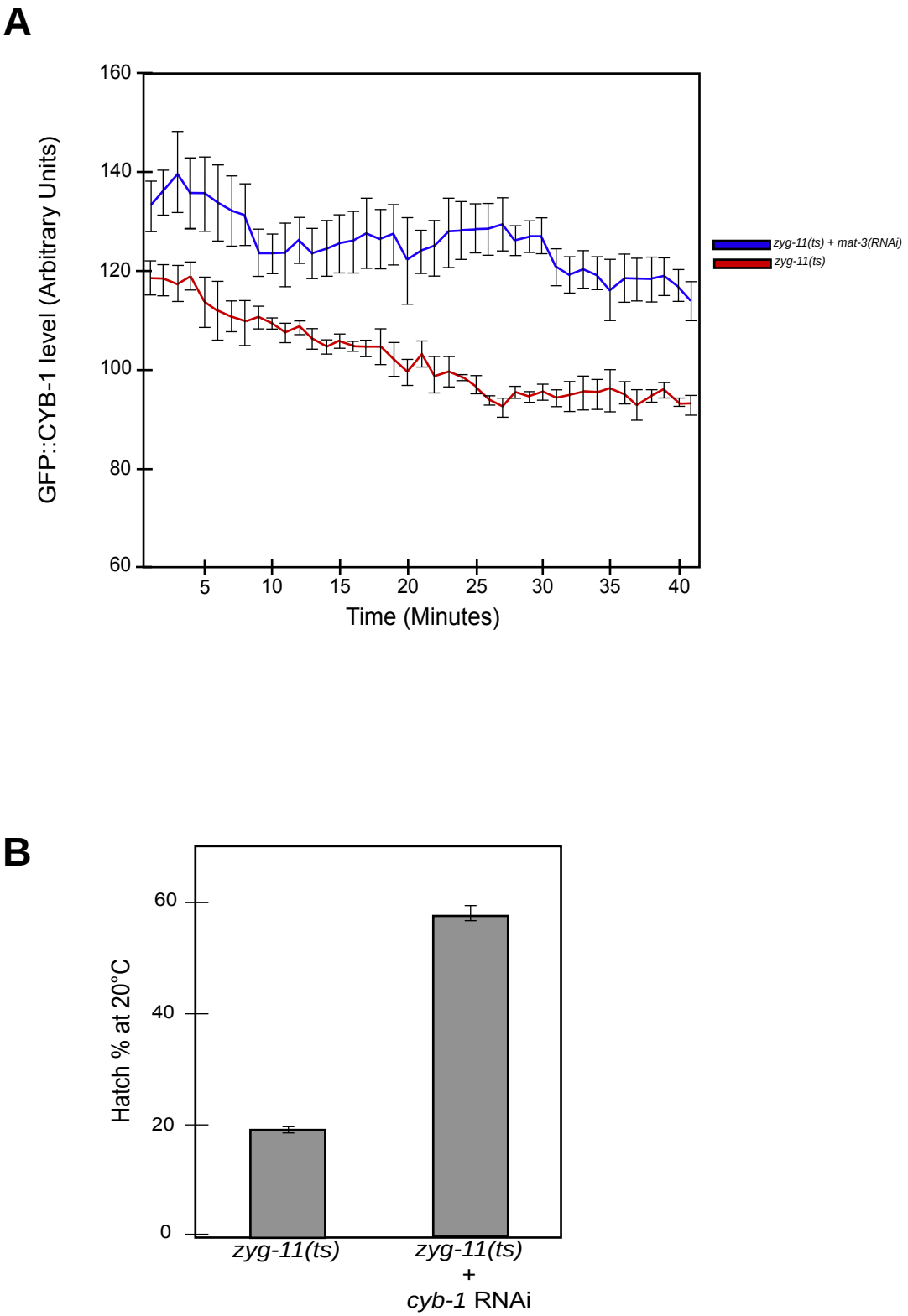
A
160
140
120
100
80
60
GFP::CYB-1 level (Arbitrary Units)
5
10
15
20
25
30
35
40
Time (Minutes)
zyg-11(ts) + mat-3(RNAi)
zyg-11(ts)
B
60
40
20
0
Hatch % at 20°C
zyg-11(ts)
zyg-11(ts)
+
cyb-1 RNAi

Figure 3.2 CYB-1 stabilization during meiosis when ZYG-11 or ZYG-11 and APC are inactivated. High levels of CYB-1 in *zyg-11* mutants is functionally relevant.

(A) Graphical representation of CYB-1::GFP quantification from movies of CYB-1::GFP degradation during meiosis. The error bars represent the error from the three embryos test using each condition. **(B)** Percent of eggs that hatched from *zyg-11(ts)* worms grown at a semi-permissive temperature (20^{o}C) with and without *cyb-2.1* RNAi. Reducing the level of CYB-2.1 with RNAi in *zyg-11(ts)* mutants, increased the viability of *zyg-11(ts)* offspring by 52%.

Figure 3.3

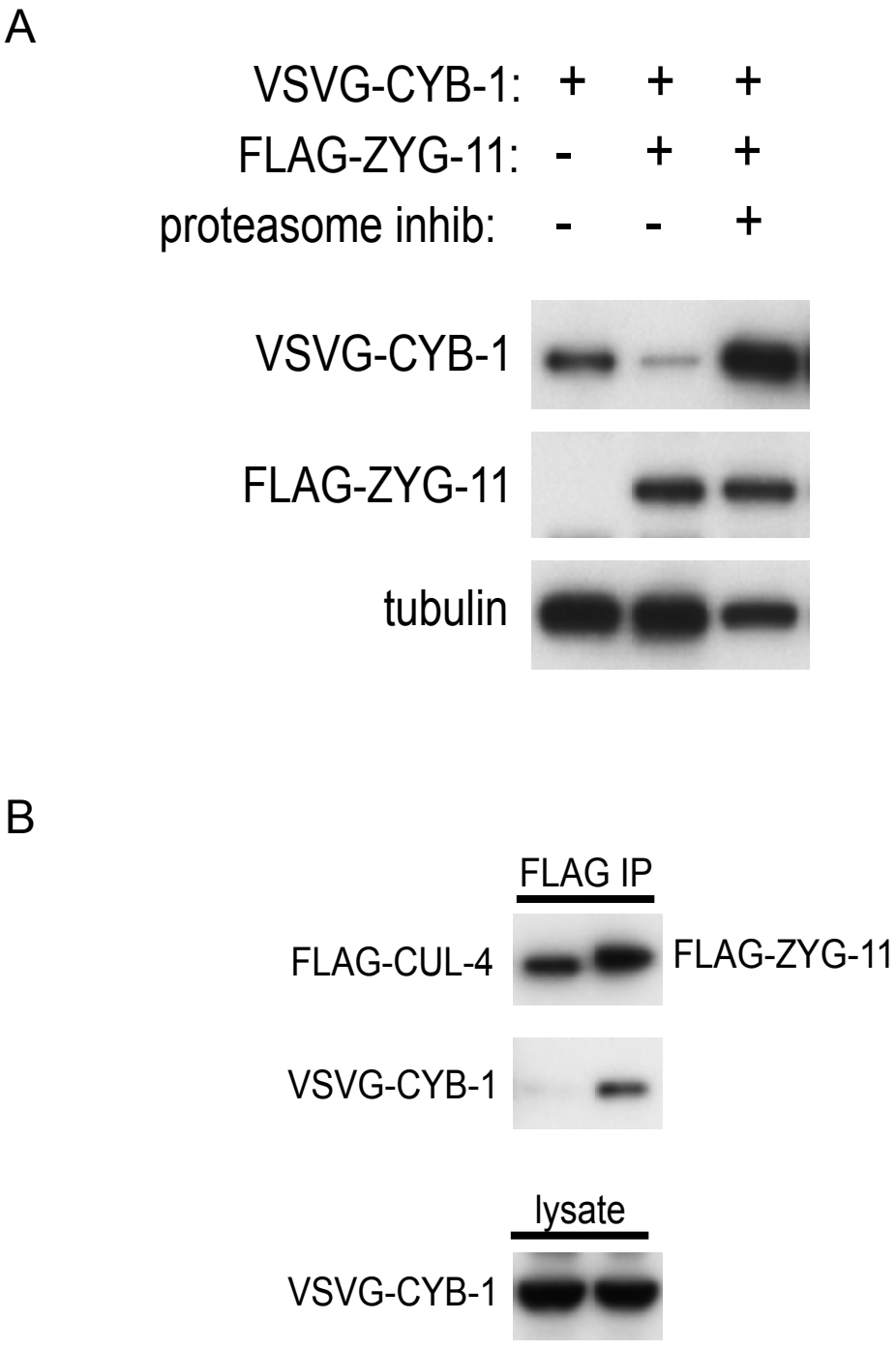
A
VSVG-CYB-1: + + +
FLAG-ZYG-11: - + +
proteasome inhib: - - +
VSVG-CYB-1
FLAG-ZYG-11
tubulin
B
FLAG IP
FLAG-CUL-4
FLAG-ZYG-11
VSVG-CYB-1
lysate
VSVG-CYB-1

Figure 3.3 ZYG-11 physically interacts with CYB-1 and regulates CYB-1 levels in a proteasome-dependent manner.

(A) VSVG-CYB-1 level decreases with the addition of FLAG::ZYG-11 and the level is rescued by the addition of MG132. **(B)** Co-immunoprecipitation of ectopically-expressed FLAG-ZYG-11 and VSVG-CYB-1 in 293T cells.

Figure 3.4

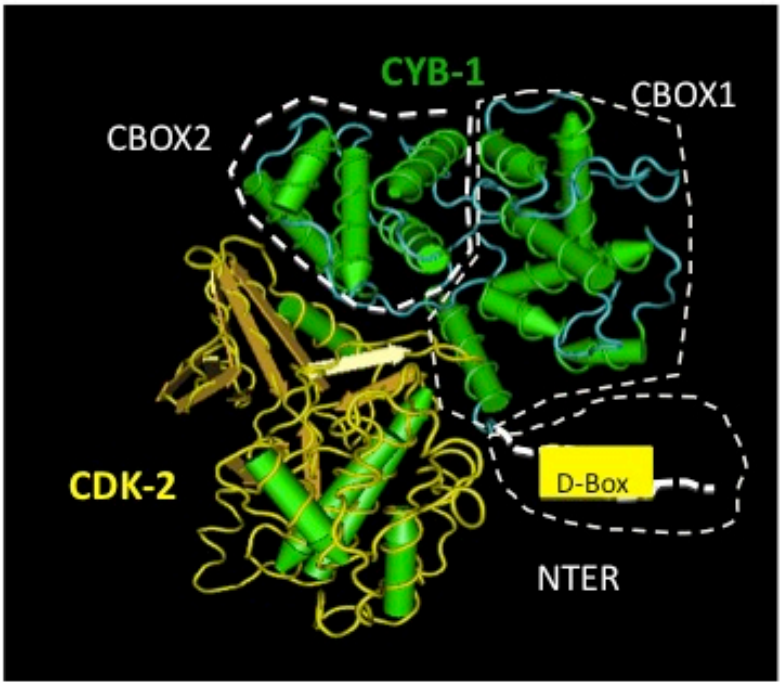

Figure 3.4 Rendition of the CYB-1 structure based on human Cyclin B1 crystal structure.

Rendition of CYB-1 crystal structure when bound to CDK-2 using Swiss-Model based on the human Cyclin B1 crystal structure. CYB-1 NTER, CBOX1, CBOX2 regions are labeled.

Figure 3.5

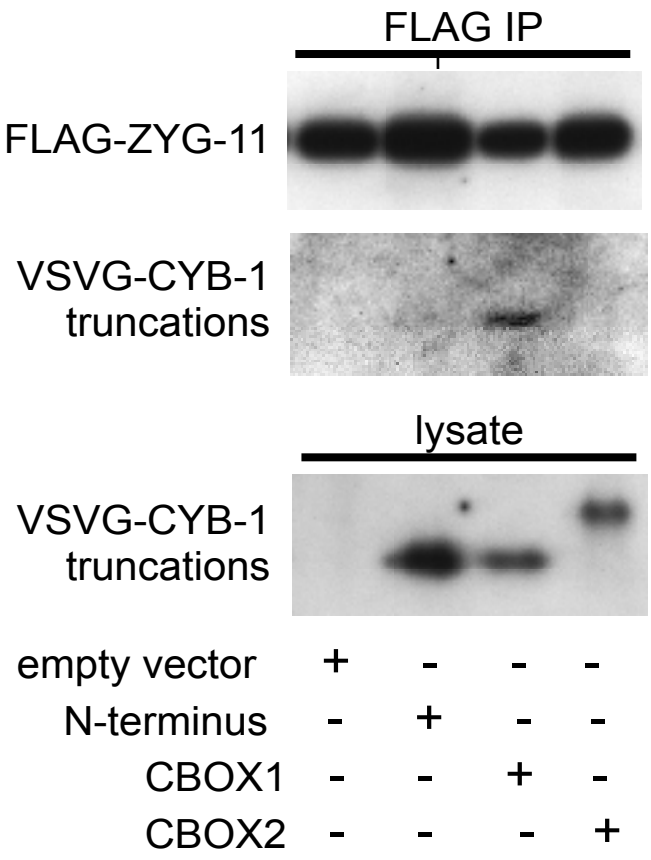
FLAG IP
FLAG-ZYG-11
VSVG-CYB-1
truncations
lysate
VSVG-CYB-1
truncations
empty vector + - - -
N-terminus - + - -
CBOX1 - - + -
CBOX2 - - - +

Figure 3.5 ZYG-11 specifically binds CYB-1-CBOX1.

(A) Co-immunoprecipitation of FLAG::ZYG-11 and VSVG::CYB-1-CBOX1, but no co-immunoprecipitation of FLAG::ZYG-11 and VSVG::CYB-1-NTER or VSVG::CYB-1-CBOX2.

Figure 3.6

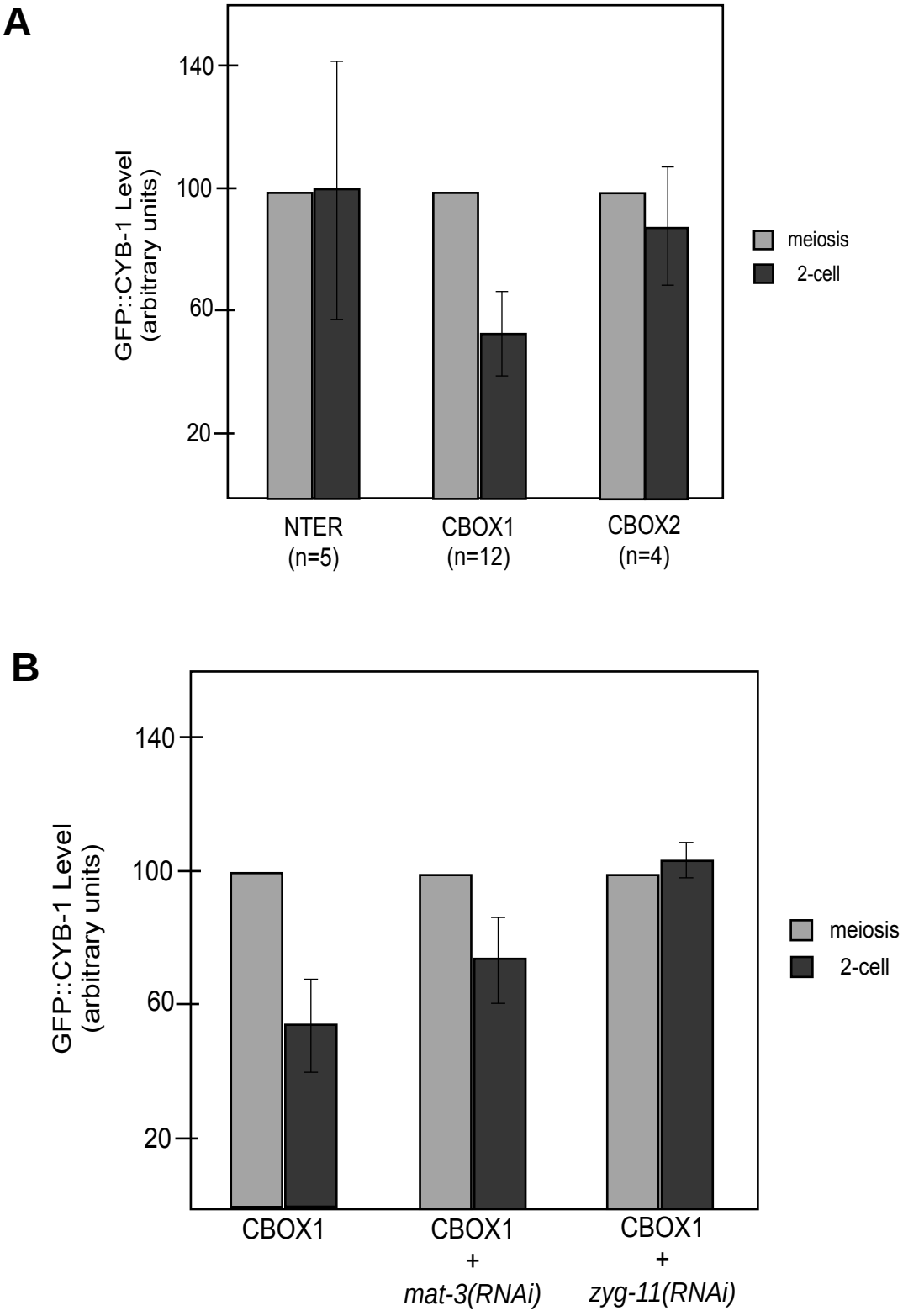
A
140
100
60
20
GFP::CYB-1 Level
(arbitrary units)
meiosis
2-cell
NTER
(n=5)
CBOX1
(n=12)
CBOX2
(n=4)
B
140
100
60
20
GFP::CYB-1 Level
(arbitrary units)
meiosis
2-cell
CBOX1
CBOX1
+
mat-3(RNAi)
CBOX1
+
zyg-11(RNAi)

Figure 3.6 ZYG-11 specifically degrades CYB-1-CBOX1 during *C. elegans* meiosis. **(A)** Quantification of truncated CYB-1::GFP levels in the 2-cell stage relative to the level in meiosis. Note that CBOX1 levels are significantly reduced, indicating normal degradation. **(B)** Quantification of CYB-1-CBOX1::GFP levels in the 2-cell stage relative to the level in meiosis for wild-type, *mat-3(RNAi)*, and *zyg-11(RNAi)* animals.

Figure 3.7

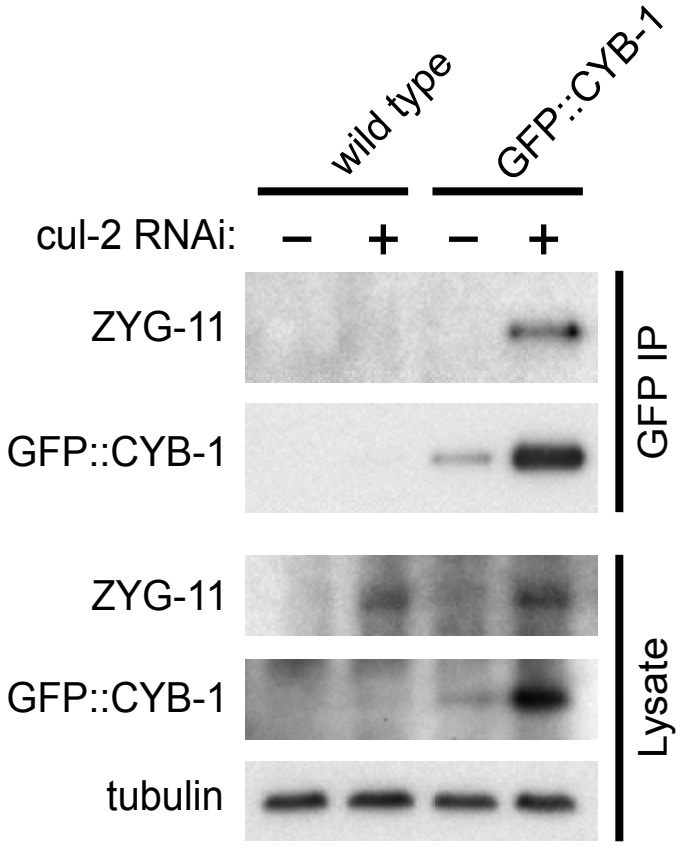
wild type
GFP::CYB-1
cul-2 RNAi:
– + – +
ZYG-11
GFP::CYB-1
GFP IP
ZYG-11
GFP::CYB-1
tubulin
Lysate

Figure 3.7 ZYG-11 physically interacts with CYB-1 in *C. elegans*.

Worms expressing CYB-1::GFP grown on *cul-2* RNAi (to stabilize ZYG-11 and CYB-1), were used for the immunoprecipitation of CYB-1::GFP and endogenous ZYG-11. The immunoprecipitation was performed using GFP-Trap beads and the western blot is probed with for endogenous ZYG-11 using a ZYG-11 antibody as well as GFP antibodies.

Figure 3.8

A

	total # of VPCs	# of VPCs in metaphase	mitotic index
CYB-1::YFP	27	0	0.0
zyg-11(ts); CYB-1::YFP	21	3	14.3
zyg-11(ts); CYB-1::YFP + mat-3 (RNAi)	13	1	7.7

B

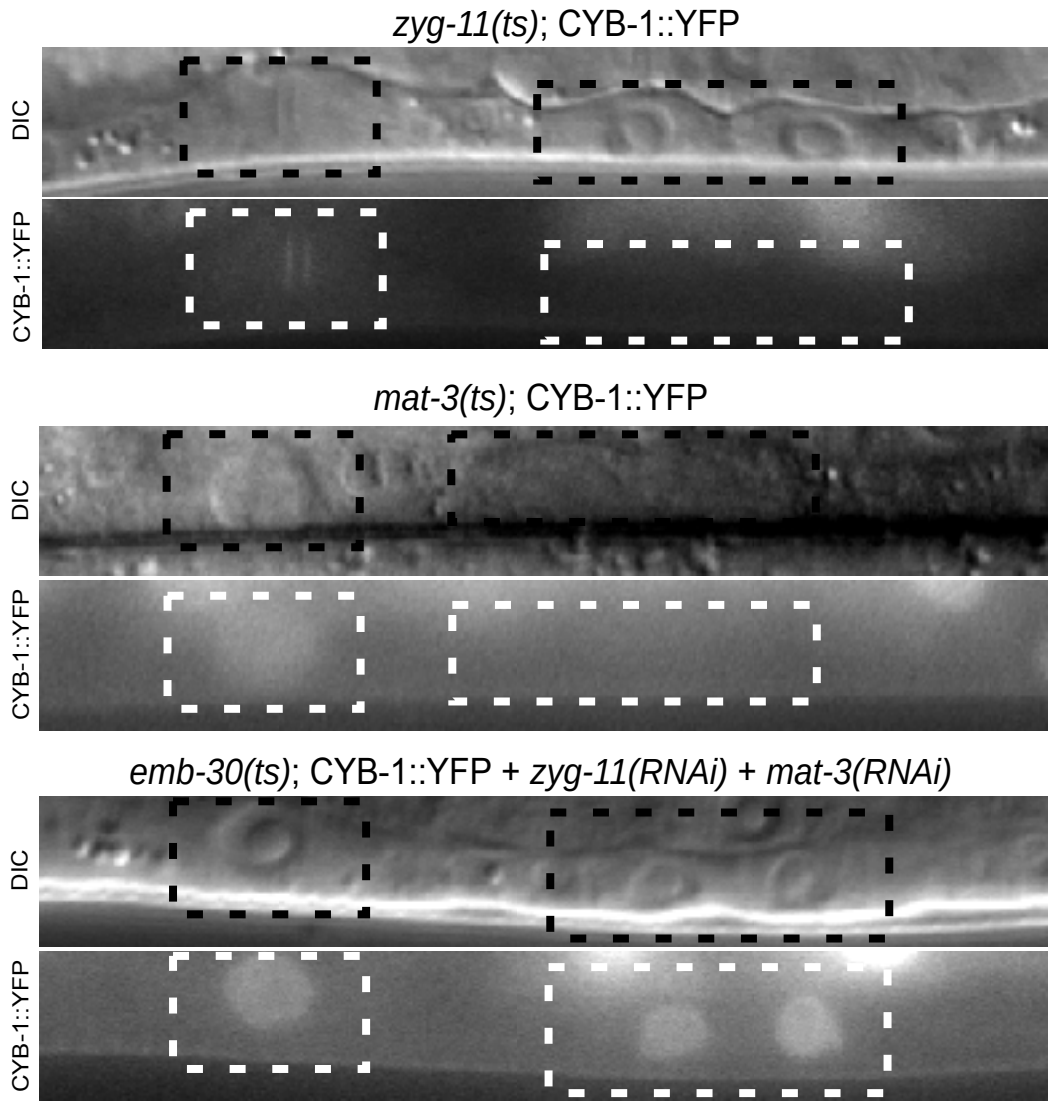

Figure 3.8 *C. elegans* mitosis is delayed and CYB-1 levels are increased when ZYG-11 and APC are inactivated.

(A) Mitotic index of vulva precursor cells (VPCs). **(B)** DIC (upper) and fluorescence (lower) images of dividing vulval precursor cells. In all images, one cell (on the left) has not divided, while one cell (on the right) has recently divided. CYB-1 is stabilized in newly divided VPCs only when ZYG-11 and APC (MAT-3 and EMB-30 are both APC subunits) are inactivated, but not when ZYG-11 or APC are inactivated individually.

Figure 3.9

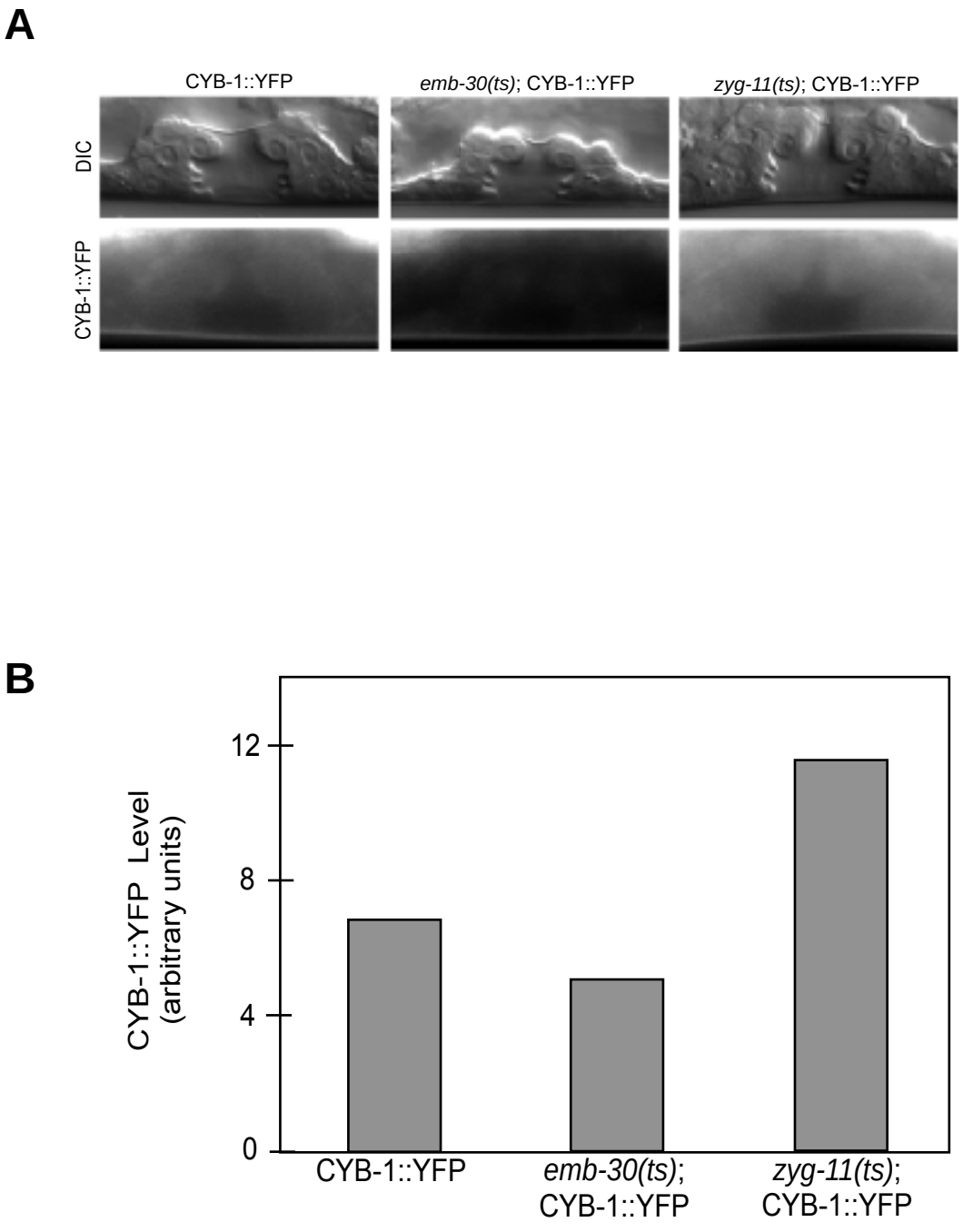
A
CYB-1::YFP
emb-30(ts); CYB-1::YFP
zyg-11(ts); CYB-1::YFP
DIC
CYB-1::YFP
B
CYB-1::YFP Level
(arbitrary units)
12
8
4
0
CYB-1::YFP
emb-30(ts);
CYB-1::YFP
zyg-11(ts);
CYB-1::YFP

Figure 3.9 ZYG-11 negatively regulates CYB-1 in post-mitotic cells.

(A) DIC (upper) and fluorescence of CYB-1::YFP (lower) images of non-dividing vulva cells. Inactivation of ZYG-11 results in increased CYB-1. **(B)** Quantification of A.

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CHAPTER 4

THE ROLE OF CRL2^{ZYG11} IN CYCLIN B1 DEGRADATION

INTRODUCTION

Mitotic progression has been well characterized in *Xenopus* embryonic extracts and yeast, and it has been established that cyclin B1/CDK1 is the main driver of mitosis. The APC E3 ubiquitin ligase was first characterized for its ability to target mitotic cyclins for degradation (Thornton and Toczyski, 2006). The other major target of APC in both yeast and vertebrates is Securin (Cohen-Fix et al., 1996; Shirayama et al., 1999).

Securin binds and inhibits Separase, which is a protease that cleaves cohesin that binds sister chromatids together. The action of Separase allows the separation of sister chromatids during anaphase. Securin needs to be degraded during metaphase to allow sister chromatids to separate during anaphase. Targeting Securin for degradation is the central mechanism by which APC promotes chromosome segregation and the metaphase-to-anaphase transition. However, in vertebrate the majority of cohesins are disassociated from chromosomes in prophase, not in anaphase as in yeast (Darwiche et al., 1999; Losada et al., 1998; Sumara et al., 2000). This prophase event does not depend on the activation of APC, suggesting that Securin destruction and Separase activation are not required to remove the majority of vertebrate cohesin from condensed chromatin (Sumara et al., 2000). While the majority of cohesins are removed from chromosomes during prophase, a small amount of cohesin SCC1 subunit was found to remain associated with human metaphase chromosomes (Waizenegger et al., 2000).

This SCC1 is preferentially localized to the centromeres and is removed by anaphase. The current model for cohesin dissolution in human cells is that in prophase, a cleavage-independent pathway removes the majority of cohesins from the arms of condensing chromosomes, and at the metaphase-anaphase transition an APC and Separase- dependent pathway removes centromere-bound cohesin complexes by cleaving their subunit SCC1.

There is some uncertainty about the essential role of APC in human somatic cells. The strongest possible knockdown (using inducible lentiviral short-hairpin (sh) RNA system) of the APC activating adapter, Cdc20, does not lead to mitotic arrest or stabilization of securin and cyclin B in U2OS cells (Baumgarten et al., 2009). Contrary to the model suggested above, most centromeres are able to separate during anaphase when a non-degradable Securin mutant was expressed in HeLa cells (Zur and Brandeis, 2001). In addition, APC and Securin degradation are not required for the metaphase-anaphase transition in HeLa cells. Using siRNA to knockdown Cdc20 and Apc2 in HeLa cells, one group found that although sister chromatid arm separation is not perfect, centromere separation appeared normal and full sister chromatid separation was observed after a delayed anaphase (Gimenez-Abian et al., 2005). Although Separase activity was not directly tested in this study, Separase was either activated in the presence of Securin, or Separase activity is not needed for centromere separation in mammals. The APC knockdown cells do terminally arrest in telophase with high levels of cyclin B (Gimenez-Abian et al., 2005). These knockdown studies using siRNA to knockdown APC and progression through mitosis continued, suggest that there may be another E3 ubiquitin ligase that can target APC substrates. However, knockdown using

siRNA is generally not a complete loss of function. Another study used a chemical inhibitor that binds to APC and prevents the binding of the APC activators, Cdc20 and Cdh1; the use of this inhibitor arrests cells in metaphase (Zeng et al., 2010).

The other major APC target for degradation in mitosis is cyclin B. The cyclin B localization and degradation pattern through mitosis has been followed by injecting a cyclin B1-GFP fusion protein into HeLa cells and following cyclin B1-GFP through mitosis with time-lapse fluorescence microscopy (Clute and Pines, 1999). Cyclin B1-GFP destruction begins when the last chromosome aligns on the metaphase plate and most of the cyclin B1-GFP is degraded by the end of metaphase. Cyclin B1-GFP associates with spindle poles and chromosomes in prophase and prometaphase, but not in metaphase. When cyclin B1 degradation begins, cyclin B1 disappears immediately from the spindle poles and chromosomes. Re-activating the spindle assembly check point with taxol, after cyclin B1 degradation has begun, results in an immediate return of fluorescence intensity to the spindle poles and chromosomes. Measurement of the change in fluorescence showed an increase in spindle-associated cyclin B1-GFP after taxol treatment, which suggests cyclin B1 moved from the cytoplasm to the spindle.

The localization pattern of a 'non-destructible' cyclin B1 (missing the destruction box) was examined by introducing a vector expressing cyclin B1ΔD-Box-GFP into human cells and following cyclin B1 localization through mitosis by time-lapse fluorescence (Bentley et al., 2007). The cyclin B1ΔD-Box localized normally in interphase, and localized to chromatin, centromeres, spindle microtubules, and kinetochores during mitosis. Surprisingly, the cyclin B1ΔD-Box was delocalized from

kinetochores at metaphase, indicating cyclin B1 disassociation is not dependent on APC. Cyclin B1ΔD-Box failed to be degraded at the metaphase-anaphase transition, as would be expected.

Here we explore the possibility that another E3, $CRL2^{ZYG11B}$ plays a role in cyclin B1 degradation as a redundant pathway for mitotic regulation in human somatic cells.

MATERIALS AND METHODS

Cell Culture

U2OS cells were maintained in DMEM high glucose media, supplemented with L-glutamine and pen-strep, at 37°C, 5% CO_2.

G2 arrest, APC inactivation, and siRNA treatments

Cells were treated with siRNA and analyzed 48 hours after transfection. Dharmacon ON TARGET plus siRNA (a mixture of four siRNAs) were used for knockdowns of ZYG11B and ZYG11A. Both siRNA pools were mixed together and Lipofectamine 2000 (Invitrogen) was used as a transfection reagent using the recommended protocol.

For studies with mitotic entry and APC inactivation, cells were first treated with siRNA, and then split 24 hours later; after a 12 hour recovery period, the cells were treated with the CDK1 inhibitor RO3306, which arrests cells in G2 phase (Vassilev et al., 2006); 16 hours later, they were treated with the APC inhibitor proTAME (Zeng and King); and 4 hours later, they were rinsed 2x with PBS to release the RO3306 arrest and incubated with media containing proTAME prior to the collection time points.

Immunostaining

Cells were grown on glass cover slips (Warner Instruments) or slides with 8-well chambers (LAB-TEK) and cells were fixed and stained on the growth plate. Cells were

permeabilized with a 30 sec wash with –20°C methanol, fixed for 30 minutes in 1xPBS, 0.08 Hepes (pH 6.9), 1.6mM MgSO4. 0.8 mM EGTA, 3.7% formaldehyde, then washed 3x in PBT (1XPBS, 0.1% Triton, 0.1% BSA), and blocked overnight in PBT, as described (Seydoux and Dunn, 1997). Primary antibodies anti-Cyclin B1 (Epitomics, Y106 clone, rabbit monoclonal) used at a 1:50 dilution, and anti-Tubulin (Sigma, DM1A clone, mouse monoclonal) used at a 1:500 dilution, were applied for 2 hours at room temperature. The secondary antibodies (anti-rabbit Dylight Alexa flour 488 used at 1:50 and anti-mouse Rhodamine, from Cappel, used at 1:500) were applied at room temperature for 1 hour. Cells were stained with Hoechst 33258 dye for 10 min and the slide covers were mounted on slides with glycerol for imaging or the chambers were removed from the slide and replaced with glycerol and a slide cover for imaging.

Imaging

All microscopy was performed with a Zeiss Axioplan microscope, and imaged with a Hamamatsu ORCA-ER CCD camera, LuDL hardware controller, automated filter wheels and shutters, and an Apple iMac computer running Openlab software (Improvision).

Immunoprecipitation

Proteins were expressed in 293T cells and immunoprecipitated as described (Kim et al. 2008, Starostina et al. 2007).

RESULTS

Inactivation of ZYG11A & ZYG11B causes a delay in Cyclin B1 degradation in metaphase

Our previous work demonstrated that ZYG-11 directly targets CYB-1 for degradation in *C. elegans*. The ancestral ZYG-11 gene underwent a gene duplication event in

mammals, resulting in ZYG11A and ZYG11B isoforms. To test if the function of *C. elegans* ZYG-11 is conserved in human tissue culture cells, we treated U2OS cells with a mixture of ZYG11A and ZYG11B siRNA and analyzed the level of endogenous cyclin B1. The cells were stained with anti-cyclin B1 antibodies, anti-tubulin antibodies, and Hoechst staining. The images of mitotic cells were grouped into mitotic stages based on the amount of DNA condensation and mitotic spindle structure. When comparing the amount of endogenous cyclin B1 between cells treated with control siRNA and ZYG11A/B siRNA, we found that ZYG11A/B knockdown produced an increase in the amount of cyclin B1 in late metaphase cells (Fig. 4.1 A,B). ZYG11A/B knockdown cells were still able to exit mitosis and cyclin B1 was fully degraded by the time they had gone into anaphase (Fig. 4.1 A). When the cells were ordered based on DNA condensation and the cyclin B1 levels were plotted as a linear regression, the cells treated with control siRNA showed a decreasing amount of cyclin B1 as the cells progressed through metaphase, as would be expected. Cells treated with ZYG11A/B siRNA showed no decrease in the level of cyclin B1 as the cells progressed through metaphase (Fig. 4.1 C), indicating that ZYG11A/B plays a role in cyclin B1 degradation during metaphase.

ZYG11A & B work independently from APC to degrade cyclin B1

While it is well established that APC targets cyclin B1 for degradation, we wanted to determine what role ZYG1A/B has in cyclin B1 degradation. Does ZYG11A/B contribute to cyclin B1 degradation, and if so, is it working in an independent pathway from APC, or upstream to facilitate APC activity? To address this, we analyzed the effect of knocking down ZYG11A/B in cells in which APC was inhibited. To enrich for mitotic

cells and more accurately analyze cells that had recently entered mitosis, we synchronized the cells with a G2 arrest, then released them and collected the cells one hour later, as the cells were going through mitosis. We also treated the cells with an APC inhibitor, proTAME, and either control siRNA or ZYG11A/B siRNA to determine the role of ZYG11A/B when APC is inactive. We stained the cells with anti-cyclin B1 antibodies, anti-tubulin antibodies, and Hoechst staining and compared the level of cyclin B1 in pro-metaphase cells with the level of cyclin B1 in metaphase cells with and without ZYG11A/B. We found that knocking down ZYG11A/B resulted in a significant increase in the level of cyclin B1 associated with the spindle in metaphase when compared to inactivating APC alone (Fig. 4.2 A, B). This indicates that ZYG11A/B works independently from APC and perhaps specifically targets the cyclin B1 that is associated with the spindle for degradation.

Increase in mitotic index when APC and ZYG11A/B are knocked down

To determine the effect of ZYG11A/B knockdown on mitotic progression we used RO3306 to block the cells in G2, then released and collected the cells at 0 hrs, 1 hr, 5 hr, and 10 hr time points. When we analyzed the mitotic index at each of the time points, we found that at 1 hour, cells treated with ZYG11A/B siRNA, proTAME, or both ZYG11A/B + proTAME had increased mitotic indexes compared to cells treated with control siRNA, indicating that cells were delayed from exiting mitosis in all of these conditions (Fig. 4.3 A). At the 5-hour time point, cells treated with ZYG11A/B had a low mitotic index, indicating that the cells had exited mitosis. The mitotic index in cells treated with proTAME or with proTAME + ZYG11A/B remained high at the 5-hour time point, indicating that cells were still delayed in mitosis. When comparing the 1-hour time

point to the 5-hour and 10-hour time points, cells treated with proTAME had a slight reduction in the mitotic index, while cells treated with both proTAME and ZYG11A/B siRNA had an increase in the mitotic index. This indicates that ZYG11A/B knockdown combined with APC inactivation produces a longer mitotic delay (Fig. 4.3 A). Overall this suggests that ZYG11A/B plays a role in mitotic progression in human cells and that this role is independent from the APC pathway.

Mammalian ZYG11B physically interacts with mammalian cyclin B1

Previous work in our lab established that human ZYG11B is able to bind to human Cullin-2, demonstrating that the $CRL2^{ZYG-11}$ complex that was discovered in *C. elegans* is conserved in humans (Vasudevan et al., 2007). To test for interaction of ZYG11B with cyclin B1, we expressed ZYG11B-VSVG and Venus-cyclin B1 as well as GFP-CDT-1 and VSVG-CYE-1 as control proteins in 293T cells. We found that cyclin B1 co-immunoprecipitates with ZYG11B, but CDT-1 did not co-immunoprecipitate with ZYG11B and cyclin B1 did not co-immunoprecipitate with CYE-1 (Fig. 4.4). This demonstrates that ZYG11B physically interacts with cyclin B1, providing additional evidence that ZYG11B directly regulates cyclin B1 in humans.

DISCUSSION AND FUTURE DIRECTIONS

This work demonstrates that the role of $CRL2^{ZYG-11}$ in cyclin B1 regulation is conserved in humans. The observation that knockdown of ZYG11A/B stabilizes cyclin B1 levels in metaphase defines the role of $CRL2^{ZYG11A/B}$ in the regulation of cyclin B1 during metaphase. $CRL2^{ZYG-11}$ appears to target a subpool of cyclin B1 localized at the spindle; this gives us some insight into the specific role of ZYG11-mediated cyclin B1 degradation. The targeting of a subpool of cyclin B1 by ZYG11, as well as the direct

binding of ZYG11B and cyclin B1, solidifies the argument that ZYG11B directly targets cyclin B1 for degradation independently of APC.

This is just the start of our understanding of the role $CRL2^{ZYG11A/B}$ plays in cyclin B1 regulation, and there are still many questions to be addressed. Do both ZYG11A and ZYG11B regulate cyclin B1 levels? Does ZYG11A/B bind the cyclin B1/CDK1 complex or cyclin B1 that is not bound to CDK1? Does ZYG11A/B directly ubiquitiylate cyclin B1?

Previous work reported that ZYG11A is a pseudo-gene based on the presence of mRNAs that lacked significant stretches of ZYG-11 homology (Vasudevan et al., 2007). More recently, a composite mRNA has been proposed by the NIH Mammalian Gene Collection (MGC) program in which genomic DNA is merged with mRNA sequences to produce a larger transcript that contains all genomic regions with ZYG-11 homology. Full-length cDNA are necessary to validate this larger proposed isoform of ZYG11A. Particularly given the question about the ZYG11A transcript, it is important to determine the role that ZYG11A and ZYG11B individually play in cyclin B1 regulation. The knockdown experiments need to be repeated comparing ZYG11A knockdown to ZYG11B knockdown, and to ZYG11A/B double knockdown. If the knockdown of both ZYG11A and ZYG11B has a stronger phenotype than either individual knockdown, this would indicate that both ZYG11A and ZYG11B play a role in cyclin B1 degradation. Alternatively, it is possible that only one of the ZYG11 paralogs regulates cyclin B1 degradation.

The biochemical analysis of ZYG11B and cyclin B1 interaction indicates that they physically interact. Note that this experiment was performed in the presence of the

proteasome inhibitor MG132, which arrests cells in metaphase. Based on the observed accumulation of cyclin B1 on metaphase spindles, it is possible that ZYG11A/B only binds and targets cyclin B1 during metaphase. To determine if the ZYG11B–cyclin B1 interaction is cell cycle regulated it would be useful to determine the level of interaction for cells arrested in different stages of the cell cycle (G1, S, G2, and M). A pertinent question is whether the ZYG11B–cyclin B1 complex includes CDK1, given that *C. elegans* ZYG-11 binds CYB-1 through the CBOX1 motif. If ZYG11B binds cyclin B1/CDK1 it would suggest that it targets the active complex, while the absence of CDK1 in the ZYG11B–cyclin B1 complex would suggest that ZYG11B targets cyclin B1 that is not bound to CDK1.

As a ubiquitin ligase, $CRL2^{ZYG11}$ presumably targets cyclin B1 for degradation by facilitating the addition of a ubiquitin chain to cyclin B1. This needs to be confirmed using *in vitro* or *in vivo* ubiquitylation assays or both. It is also possible that ZYG11 is not targeting cyclin B1 for degradation, but instead acts to re-localize it off the spindle.

The siRNAs that were used were a pool of four different targets, and while it is likely that this pool is only knocking down ZYG11A/B, this needs to be confirmed by testing individual siRNAs to show that at least two of them produce the same effect. Antibodies against ZYG11A and ZYG11B can be used to confirm the effectiveness of the knockdown. Unfortunately, three commercially available antibodies against ZYG11A and ZYG11B that we tested were not able to specifically detect the proteins on a western blot, making this approach unfeasible. RT-PCR was performed on the cells and we found that the level of ZYG11A and ZYG11B was reduced (data not shown). To test the knockdown efficiency at the protein level we plan to use a tagged version of

ZYG11A and ZYG11B. Once this method has been established, each of the individual siRNAs in the pool will need to be tested for knockdown efficiency and the best one or combination can be used for future experiments.

While using proTAME to inactivate APC was useful to arrest the cells in metaphase, inactivating APC has an effect on its other substrates not just cyclin B1. Perhaps a more direct test of the regulation of cyclin B1 in the absence of APC activity would be to use a cyclin B1 with a mutated destruction box, which theoretically APC would be unable to degrade.

As discussed in the literature review section of this dissertation, the cyclin B/CDK1 complex plays multiple roles in spindle alignment, spindle pole separation, the attachment of chromosomes to the mitotic spindle, and spindle positioning and elongation, all directing the cell to maintain a metaphase state. The inability of cyclin B/CDK1 to disassociate from the spindle with ZYG11A/B knockdown, therefore contributes to increased cyclin B1 levels in metaphase. Anaphase lacks all cyclin B1 even in ZYG11A/B knockdown, suggesting that either the targeting of subpools of cyclin B1 by APC and ZYG11 overlap, or there is dynamic localization of cyclin B1 so that degradation in one or more locations will eventually degrade all cyclin B1 as it relocates to that location.

4.6 FIGURES AND FIGURE LEGENDS

Figure 4.1

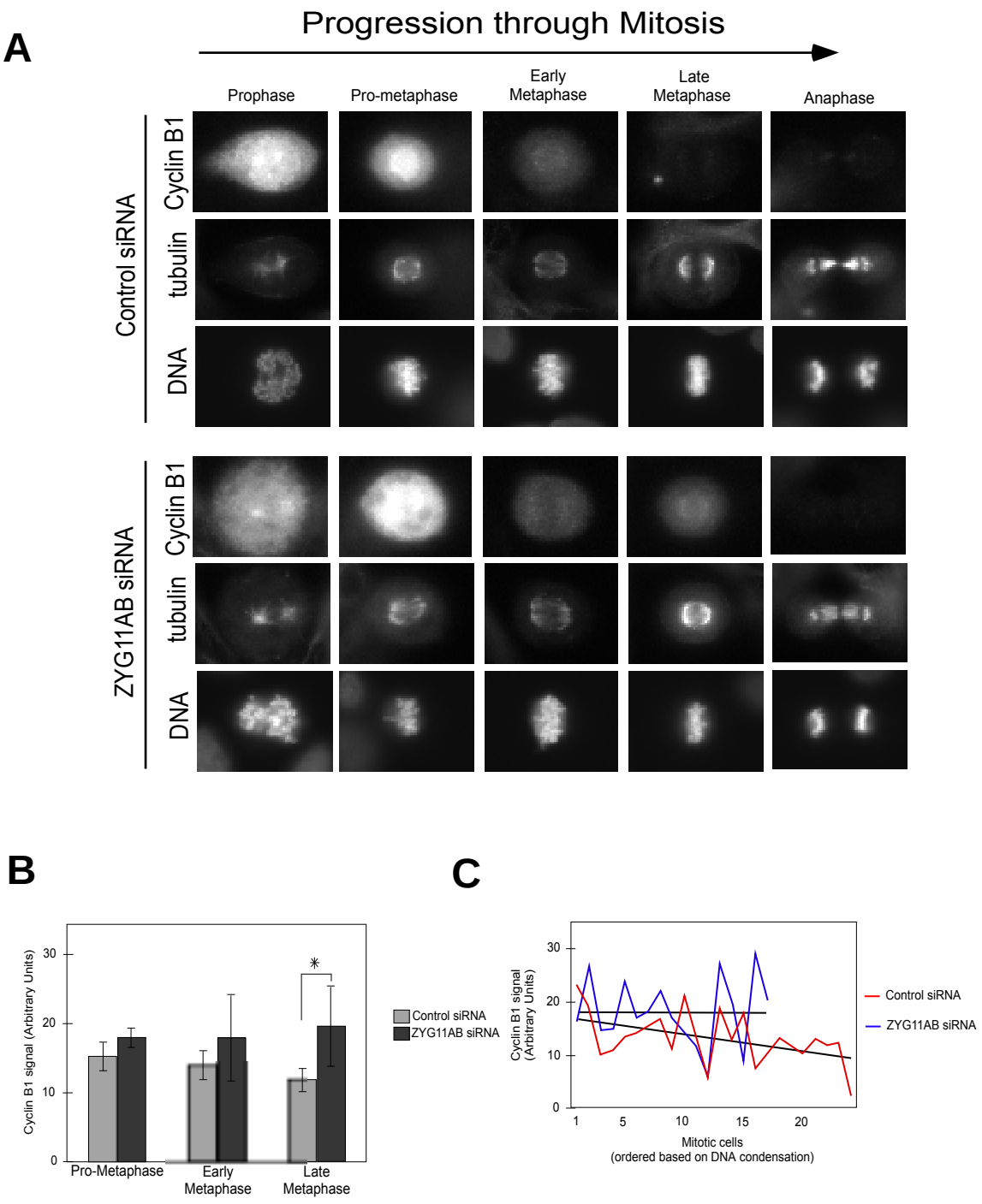

Figure 4.1 Cyclin B1 is stabilized in metaphase with ZYG11A/B knockdown in U2OS cells.

(A) Immuno-staining with anti-Cyclin B1, anti-tubulin, and Hoechst dye of U2OS cells treated with Control or ZYG11A/B siRNA. **(B)** Quantification of Cyclin B1 levels shown in (A). **(C)** Linear regression of Cyclin B1 levels of mitotic cells treated with either Control or ZYG11A/B siRNA.

Figure 4.2

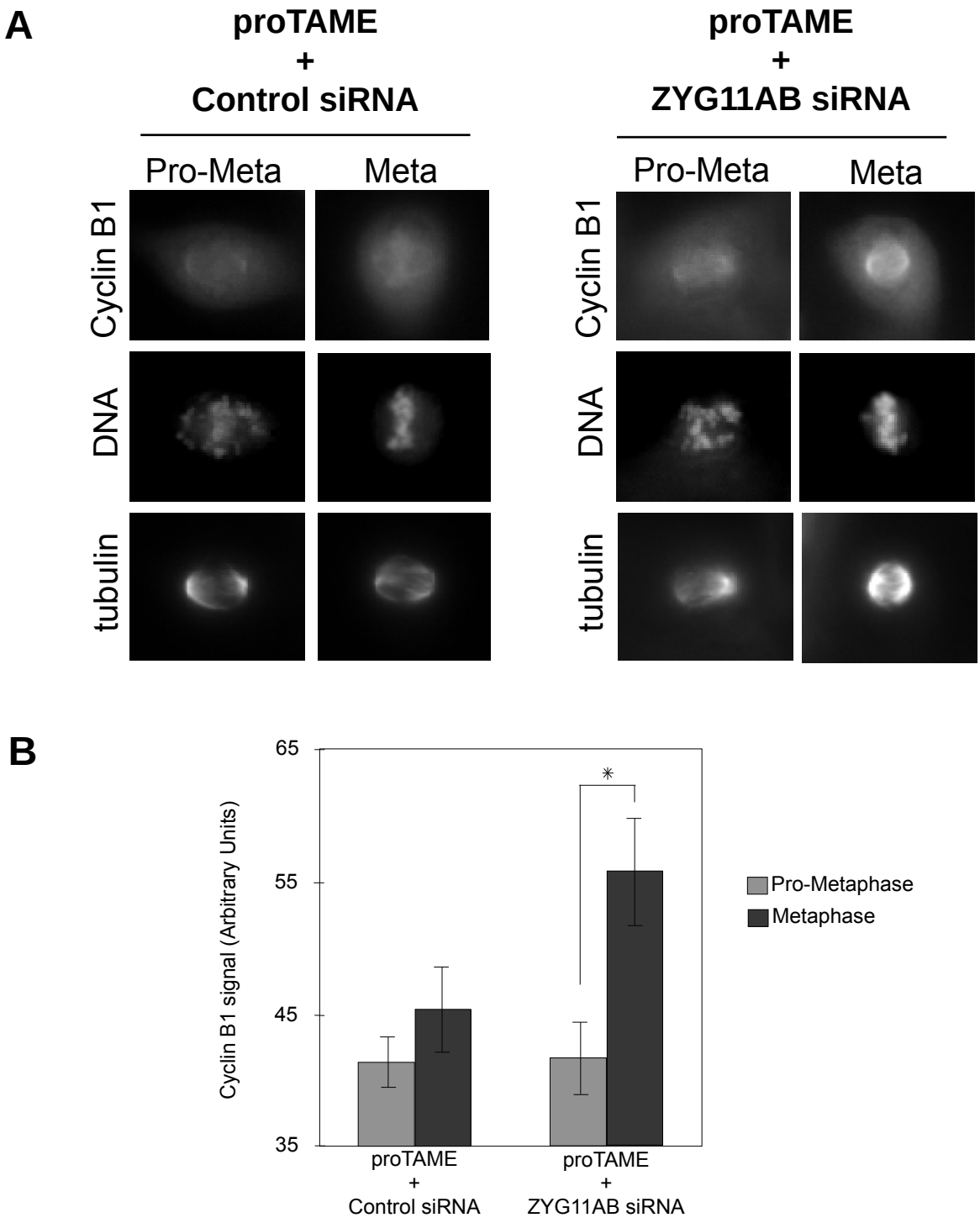
A
proTAME
+
Control siRNA
proTAME
+
ZYG11AB siRNA
Pro-Meta
Meta
Pro-Meta
Meta
Cyclin B1
DNA
tubulin
Cyclin B1
DNA
tubulin
B
Cyclin B1 signal (Arbitrary Units)
65
55
45
35
*
proTAME
+
Control siRNA
proTAME
+
ZYG11AB siRNA
Pro-Metaphase
Metaphase

Figure 4.2 Cyclin B1 is further stabilized on metaphase spindles when U2OS cells are treated with ZYG11A/B siRNA and an APC inhibitor, compared to APC inhibitor alone.

(A) Immuno-staining with anti-Cyclin B1, anti-tubulin, and Hoechst dye of U2OS cells treated with Control or ZYG11A/B siRNA and proTAME. **(B)** Quantification of Cyclin B1 localized to the spindle shown in (A).

Figure 4.3

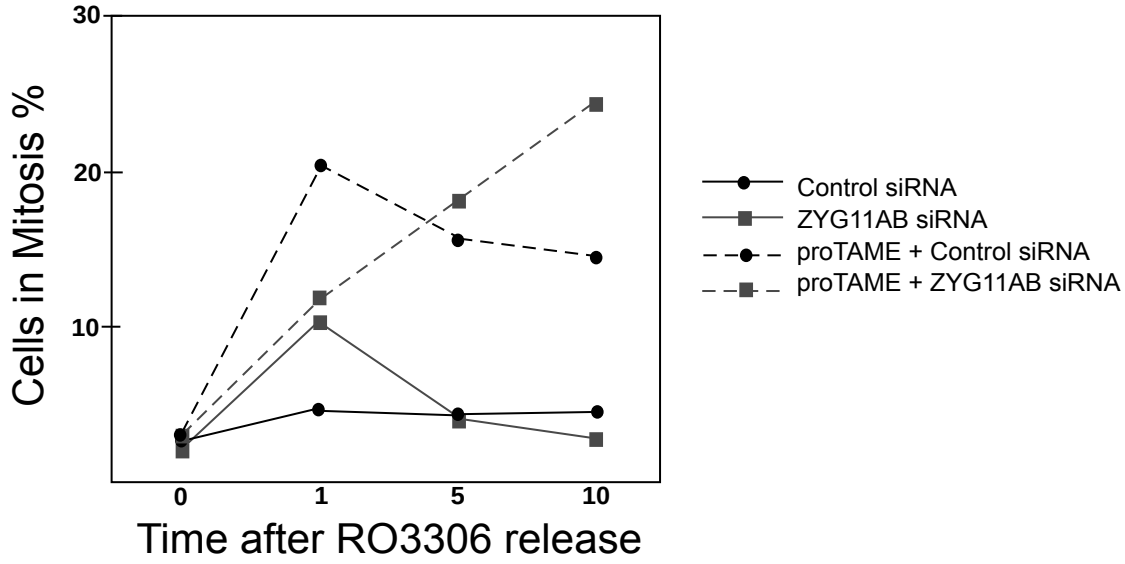
30
20
10
Cells in Mitosis %
0
1
5
10
Time after RO3306 release
Control siRNA
ZYG11AB siRNA
proTAME + Control siRNA
proTAME + ZYG11AB siRNA

Figure 4.3 ZYG11A/B and APC inactivation blocks cells in mitosis.

(A) Mitotic index at time points after release from a G2 block with RO3306. Cells treated with Control siRNA, ZYG11A/B siRNA, proTAME (APC inhibitor) + Control siRNA and proTAME + ZYG11A/B siRNA. Note that ZYG-11 and APC knockdown results in mitotic delay.

Figure 4.4

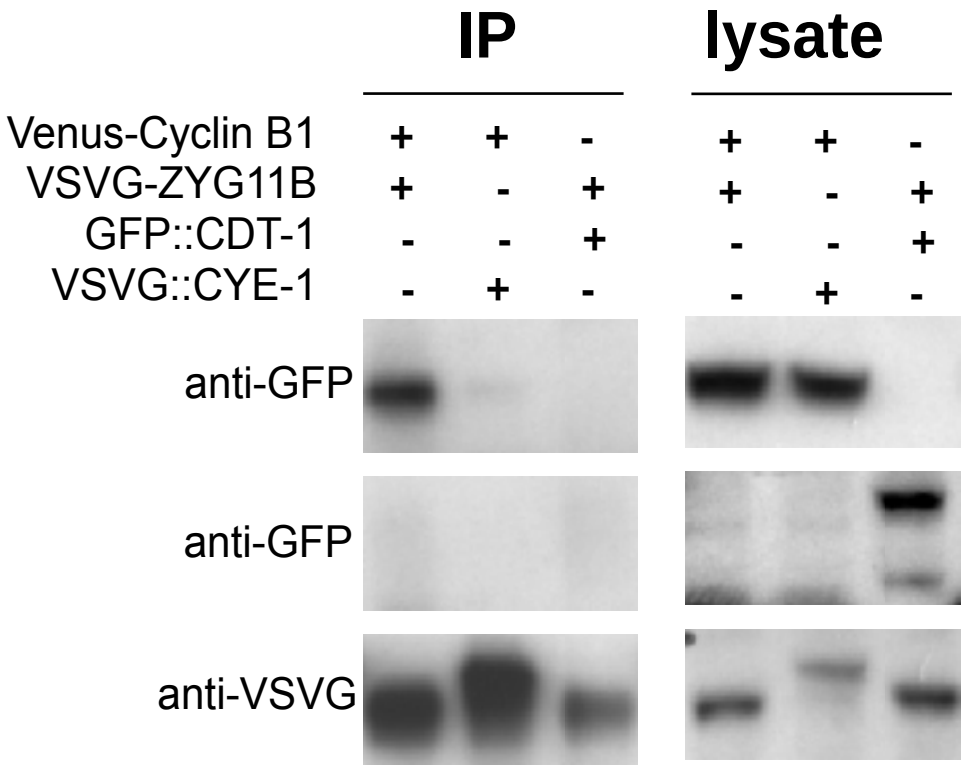
IP
lysate
Venus-Cyclin B1 + + - + + -
VSVG-ZYG11B + - + + - +
GFP::CDT-1 - - + - - +
VSVG::CYE-1 - + - - + -
anti-GFP
anti-GFP
anti-VSVG

Figure 4.4 ZYG11B physically interacts with Cyclin B1.

(A) Co-immunoprecipitation of ZYG11B-VSVG and Venus-Cyclin B1 expressed in 293T cells. Note that ZYG11B-VSVG does not co-immunoprecipitate with GFP-CDT-1, and VSVG::CYE-1 does not co-immunoprecipitate with Venus-Cyclin B1.

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CHAPTER 5

DISCUSSION AND BROADER IMPACTS

The anaphase-promoting complex (APC) has been well studied in multiple different systems, and its role in cyclin B degradation is well established. Here we provide evidence for an alternate pathway for cyclin B degradation by another E3 ubiquitin ligase, $CRL2^{ZYG\text{-}11/ZYG11A/B}$. We examined the role of the $CRL2^{ZYG\text{-}11/ZYG11A/B}$ complex in both *C. elegans* and human cell culture systems and determined that the $CRL2^{ZYG\text{-}11/ZYG11A/B}$ complex plays a conserved role in cyclin regulation. We have therefore identified $CRL2^{ZYG\text{-}11/ZYG11A/B}$ as a conserved mitotic regulator.

CYB-1 regulation in C. elegans

The work presented in Chapter 3 defined a role for $CRL2^{ZYG\text{-}11}$ in the direct regulation of CYB-1 in both *C. elegans* meiosis and mitosis. Direct binding of ZYG-11 to CYB-1 in a region that is distinct from APC is the most compelling evidence that ZYG-11 works independently from APC to directly regulate CYB-1. The increased CYB-1 stabilization with the inactivation of both APC and ZYG-11 in both meiosis and mitosis is a also supports our model that ZYG-11 degrades CYB-1 independently of APC.

Another distinct E3 was recently shown to target APC substrates for degradation in *C. elegans*. A study of IFY-1 (Securin) regulation in *C. elegans* meiosis, led to the discovery of a *C. elegans* homolog of a HECT- E3 ligase, UBE3C, designated ETC-1 (Wang et al., 2013). Securin IFY-1 is expressed in the cytoplasm of germ cells and declines after meiosis I and stays low in meiosis II. RNAi of ETC-1 stabilized

cytoplasmic IFY-1 and CYB-1 in post-meiosis embryos, but did not affect IFY-1 that was localized to the chromosomes. The authors suggested that ETC-1 specifically targets cytoplasm-localized APC substrates. In a background of reduced APC activity, the additional inactivation of ETC-1 results in an embryonic lethal phenotype, suggesting that ETC-1 collaborates with APC to regulate *C. elegans* meiosis. This study also provided evidence for the direct ubiquitination of IFY-1 in an *in vitro* ubiquitination assay with ETC-1 and the E2 UBC-18.

zyg-11 mutants have several embryonic defects. Inactivating the level of CYB-1 rescues some, but not all of these zyg-11 phenotypes. Inactivating CYB-1 rescues the meiotic timing delay of zyg-11 mutants (Liu et al., 2004) and partially rescues the embryonic lethality (hatch% at 20°C, see Chapter 3 for details); however the granule-free zones and cortical ruffling phenotypes are not rescued with reduced levels of CYB-1 (Vasudevan et al., 2007)(Chapter 2). This suggests that there are additional substrates for the $CRL2^{ZYG-11}$ complex other than CYB-1 that regulate early embryonic events. Biochemical methods, such as ZYG-11 immunoprecipitation followed by mass spectrometry analysis, could be used to identify these additional ZYG-11 substrates.

Inactivation of ZYG-11 and APC resulted in stabilized CYB-1 levels in recently divided larval cells. In the wild-type situation, cyclin B1 is degraded as soon as the spindle assembly checkpoint has been inactivated by the alignment of all chromosomes to the metaphase plate (Clute and Pines, 1999). Introducing a non-destructible cyclin B in *Xenopus* egg extracts or in budding yeast, does not arrest cells in metaphase, indicating that cyclin B degradation is not required for the metaphase-to-anaphase transition (Holloway et al., 1993; Surana et al., 1993). However, expression of the non-

destructible cyclin B (or elevation of overall cyclin B levels) causes these cells to arrest in telophase, indicating that cyclin B degradation is required for mitotic exit (Holloway et al., 1993). Inactivating ZYG-11, APC or ETC-1 individually does not result in CYB-1 stabilization in larval cells, CYB-1 stabilization is only seen during meiosis and early embryonic divisions when the E3s are inactivated individually. Perhaps these E3s have overlapping functions in larval cells. Inactivation of both ZYG-11 and APC results in CYB-1 stabilization in recently divided larval cells. This suggests that ETC-1 is not required for CYB-1 stabilization in larval cells. This also suggests that *C. elegans* larval cells can divide without completely degrading CYB-1, or perhaps the CYB-1 that is stabilized is not active, either not bound to CDK1 or not bound to its substrates.

Function of CRL2^{ZYG11} in human cells

Our work has shown that the role of *C. elegans* CRL2^{ZYG-11} for CYB-1 regulation is conserved in human cells. Knocking down CRL2$^{ZYG11A/B}$ stabilizes cyclin B1 associated with the spindle in metaphase, which is the same mitotic period in which APC regulates cyclin B1. The SAC regulates APC, but it is unclear how the regulation of cyclin B1 by ZYG11A/B is controlled. Since ZYG11A/B is acting at the same timepoint as SAC release, it possible that ZYG11 activity is also regulated by the SAC, in addition to the SAC regulating APC.

APC^{Cdc20} complex is only active during mitosis, while the APC^{Cdh1} complex is active in the late stage of mitosis, and G1 and S phases. During mitotic exit there is a switch from APC^{Cdc20} activity to APC^{Cdh1} activity and this switch is facilitated by a negative feedback loop where the APC^{Cdh1} complex targets the degradation of Cdc20 (Peters, 2006). After CDK1 de-activation in metaphase, the APC activating subunit

Cdh1 is de-phosphorylated and the newly activated APC^{Cdh1} complex promotes mitotic exit by targeting polo kinase, Aurora kinases, and the remaining mitotic cyclins, as well as the other APC activating subunit Cdc20. The regulatory pathway for APC has been well established, but mechanisms for ZYG11 activation and inactivation are still yet to be determined.

Cyclin B1 disappears from the spindle and the chromosomes before it is completely degraded from the cytoplasm (Clute and Pines, 1999). Perhaps ZYG11 and APC each target a sub-localized population of cyclin B1. The inactivation of cyclin B1 is critical for mitotic exit, and so from this perspective it is not surprising that there are two evolutionarily conserved E3 pathways to degrade it.

Significance and Broader Impacts

Proper chromosome duplication and division during cell replication is an essential developmental process. Defects in chromosome segregation result in aneuploidy, which can lead to severe problems for the cell and ultimately for the entire organism. In the case of chromosome missegregation during meiosis, these early problems either lead to abortion of the developing embryo or produce severe problems for the resulting offspring. Problems during meiosis are the leading cause of pregnancy loss in humans and are the cause of many birth defects, developmental disabilities, and metal retardation (Hassold and Hunt, 2001). Due to the critical importance of having the correct number of chromosomes in each daughter cell, the cell has developed a tight regulation of the events that take place during meiosis and mitosis to ensure accurate chromosome segregation. Aging results in increased inaccuracy of the cell cycle both during mitosis (cancer) and during meiosis (increased risk for aneuploid offspring),

indicating that perhaps members of these regulatory pathways are targets for the effects of aging (Baker et al., 2004; Duncan et al., 2009). For these reasons it is important to understand the players that control mitotic and meiotic events, as well as how those key players are regulated. Proper cyclin B regulation is crucial for normal cell division; in fact, high levels of cyclin B have been reported in several cancers. A complete understanding of how cyclin B is regulated is the first step in understanding what potential problems could arise when cells are not dividing normally due to increased cyclin B levels and lead to cancer. Our work in identifying ZYG-11 as an alternative pathway for cyclin B degradation provides critical fundamental understanding of the basic cyclin B control. This work lays the foundation for future drug discovery and diagnosis.

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APPENDIX A

EMBRYOGENESIS: DEGENERATE PHOSPHATASES IN CHARGE OF THE OOCYTE-TO-EMBRYO TRANSITION[1]

[1] Heighington, C.H. and Kipreos, E.T. 2009. *Current Biology.* 3;19(20):R939-41. Reprinted here with permission of the publisher.

The oocyte-to-embryo transition requires drastic reorganizations within a short timeframe. Recent studies show that, in the nematode *Caenorhabditis elegans*, phosphotyrosine-binding pseudo-phosphatases are key regulators of this critical developmental transition.

The transition from an oocyte to an embryo is a profound one, involving major physiological changes. In the nematode *Caenorhabditis elegans*, the two meiotic divisions that segregate maternal chromosomes are completed within 30 minutes following fertilization. During this period, multiple changes convert the cell from an oocyte-like state to a mitotically-dividing embryo (Stitzel and Seydoux, 2007). The kinase MiniBrain Kinase homolog-2 (MBK-2) is a major driver of many of these changes (Greenstein and Lee, 2006).

MBK-2 is known to phosphorylate five targets to promote the oocyte-to-embryo transition. It promotes the formation of the mitotic spindle by phosphorylating the katanin MEI-1, thereby inducing its degradation (Pang et al., 2004; Pellettieri et al., 2003; Quintin et al., 2003). The microtubule-severing activity of MEI-1 is initially required to properly form the two small meiotic spindles; however, MEI-1 must be degraded so that it does not prevent the formation of the larger mitotic spindle (Srayko et al., 2000). MBK-2 also promotes transcriptional silencing in the zygote by phosphorylating the zinc-finger proteins OMA-1 and OMA-2, activating them to sequester a general transcription factor component (Guven-Ozkan et al., 2008). Lastly, MBK-2 contributes to the polarization of the embryo by phosphorylating MEX-5 and MEX-6 to activate their polarity functions (Nishi et al., 2008).

MBK-2 must be kept inactive in oocytes, as its premature activation would promote a shift away from the oocyte-like state. Even after fertilization, MBK-2 is tightly regulated so that it is only activated at the end of the first meiotic division. One question that has vexed the field is how MBK-2 activity is regulated. Two new papers by the Singson and Seydoux labs, one in this issue of *Current Biology* [9] and one in *Cell* [10], report evidence that pseudo-phosphatases control the activity of MBK-2 using a strategy that may have widespread implications for other signal transduction pathways.

Pseudo-phosphatases are proteins that contain a protein tyrosine phosphatase (PTP) domain but that lack at least one critical residue in the catalytic site. These proteins are unable to act as phosphatases, but critically, they are still able to bind to phosphotyrosine residues in proteins (Hunter, 1998; Wishart and Dixon, 1998). Interestingly, a large percentage of PTP-domain proteins are predicted to be pseudo-phosphatases: 62% of all PTP-domain proteins in *C. elegans* (57 of 91), and 40% in humans (20 of 51) (Pils and Schultz, 2004). Animal genomes therefore contain significant numbers of PTP domain proteins that cannot act as phosphatases. The function of these proteins has been largely unexplored. Initially, these proteins were predicted to act as ‘anti-phosphatases’ that bind phosphotyrosine residues to prevent phosphatases from dephosphorylating these sites (Hunter, 1998; Wishart and Dixon, 1998). But, as described below, the analysis of three *C. elegans* pseudo-phosphatases, EGG-3, EGG-4 and EGG-5, extend the molecular functions of this class of proteins beyond the role of anti-phosphatase.

The pseudo-phosphatase EGG-3 has been linked to the localization of MBK-2 (Maruyama et al., 2007; Stitzel et al., 2007). In oocytes, MBK-2 is located on the cortex

in an inactive state. MBK-2 remains on the cortex until anaphase of meiosis I, when it moves to discrete cytoplasmic foci, the structures of which have not yet been determined. After the meiotic divisions, these foci disappear resulting in diffuse cytoplasmic localization of the now active MBK-2 (Figure 1). EGG-3 co-localizes with MBK-2 both at the cortex of oocytes and in the cytoplasmic foci present after meiosis I (Maruyama et al., 2007; Stitzel et al., 2007). EGG-3 physically binds to MBK-2 and acts as an anchor to hold MBK-2 at the cortex. In the absence of *egg-3*, MBK-2 is cytoplasmic in oocytes, but nevertheless remains inactive (although MBK-2 does become activated slightly sooner after fertilization) (Stitzel et al., 2007). Therefore, EGG-3 is a major determinant of MBK-2 localization, but is not a significant inhibitor of MBK-2 activity.

Parry *et al.* [9] screened for *egg-3*-related genes that are expressed in the germline, and discovered two closely related paralogs, *egg-4* and *egg-5*, which encode proteins that also lack a critical PTP catalytic site residue. EGG-4 and EGG-5 are 99% identical and have fully redundant functions; they will be referred to here as ‘EGG-4/5’. EGG-4/5 co-localizes with EGG-3 and MBK-2 on the oocyte cortex. EGG-3 is required to anchor EGG-4/5 to the cortex. In turn, both EGG-3 and EGG-4/5 are required to anchor MBK-2 to the cortex. This localization dependence occurs through direct physical interaction: EGG-3 binds to both EGG-4/5 and MBK-2; and EGG-4/5 binds to MBK-2 (Jean M. Parry, 2009; Ken Chiih-Chien Cheng, 2009) (Figure 1).

Like most kinases, MBK-2 has an ‘activation loop’ that is phosphorylated (Ken Chiih-Chien Cheng, 2009). The phosphorylation of the activation loop induces a conformational change in most kinases to allow substrate binding (Huse and Kuriyan,

2002). Many kinases are controlled by the regulated phosphorylation of their activation loop by other kinases (Huse and Kuriyan, 2002). In contrast, dual-specificity DYRK kinases, which include MBK-2, are known to autophosphorylate their own activation loop on a tyrosine residue, but only when they are undergoing their initial protein folding (Lochhead et al., 2005). Interestingly, when DYRK kinases are fully folded, they lose the ability to phosphorylate tyrosine residues, and can only phosphorylate serine and threonine residues (Lochhead et al., 2005). Therefore, DYRK activation loops are only autophosphorylated as the kinases are created.

Cheng *et al.* [10] found that EGG-4 uses its PTP domain to bind the phosphotyrosine in the activation loop of MBK-2. Biochemical experiments showed that EGG-4 binding inhibits MBK-2 activity by both blocking access to substrates and reducing catalytic activity. Inactivation of *egg-4/5* allowed the ectopic activation of MBK-2 in oocytes in 13% of animals (Ken Chiih-Chien Cheng, 2009). However, the observation that MBK-2 remained inactive in the oocytes of 87% of *egg-4/5* RNAi animals, pointed to an additional level of MBK-2 regulation.

Cheng *et al.* [10] uncovered this second, independent mechanism to control MBK-2. They showed that MBK-2 activation requires phosphorylation on a cyclin dependent kinase-1 (CDK-1) phosphorylation site (Figure 1). If the CDK-1 site is mutated so that it cannot be phosphorylated, MBK-2 never becomes fully active. It is likely that CDK-1 directly phosphorylates MBK-2 *in vivo*, as purified human CDK-1 can phosphorylate MBK-2 *in vitro*, and CDK-1 activity is required for the phosphorylation *in vivo*. To confirm that MBK-2 is redundantly regulated by EGG-4/5 inhibition and CDK-1-dependent phosphorylation, the authors expressed an MBK-2 phospho-mimic mutant

that is active without CDK-1 phosphorylation and observed that upon inactivating *egg-4/5*, the phospho-mimic MBK-2 was now active in the oocytes of 100% of animals. CDK-1 normally becomes active at oocyte maturation (Boxem et al., 1999), and EGG-4/5 disappears from the cell at the end of the first meiotic division (Jean M. Parry, 2009; Ken Chiih-Chien Cheng, 2009). Therefore, the activation of MBK-2 is dually controlled in response to two cell-cycle events: oocyte maturation and the completion of the first meiotic division (Figure 1).

A pertinent question is how MBK-2 is released from the inhibition by EGG-4/5. EGG-4/5 disappears from cells at the same time that MBK-2 becomes active, but what mediates its disappearance is not known. A likely scenario involves the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that becomes active at meiotic anaphase I (Pesin and Orr-Weaver, 2008; Shakes et al., 2003) (Figure 1). APC/C targets the degradation of proteins with a destruction box motif; and both EGG-3 and EGG-4/5 have multiple destruction-box motifs. EGG-3 degradation appears to be mediated by APC/C as it depends on the destruction box motifs and APC/C activity (Ken Chiih-Chien Cheng, 2009). It remains to be determined whether EGG-4/5 is similarly regulated by APC/C.

These new studies provide the first concrete evidence that the binding of a pseudo-phosphatase directly regulates the activity of a target protein. Interestingly, the phenotypes of *egg-4/5* mutants suggest additional functions beyond the regulation of MBK-2. Parry *et al*. [9] show that inactivation of *egg-4/5* produces severe defects in eggshell formation; defects in the polarized actin cap that forms upon sperm entry; a failure to extrude polar bodies during the meiotic divisions; and polyspermy. This latter

phenotype is particularly exciting, as it provides a genetic handle with which to study how *C. elegans* oocytes block the entry of multiple sperm. The block to polyspermy is particularly important for nematodes, where large numbers of sperm surround the oocyte as it enters the spermatheca. The study of these additional EGG-4/5 functions may provide further insights into the molecular roles of pseudo-phosphatases. Given the large numbers of pseudo-phosphatases in animals, we suspect that we are just beginning to appreciate the functions of these proteins in dynamic cellular pathways.

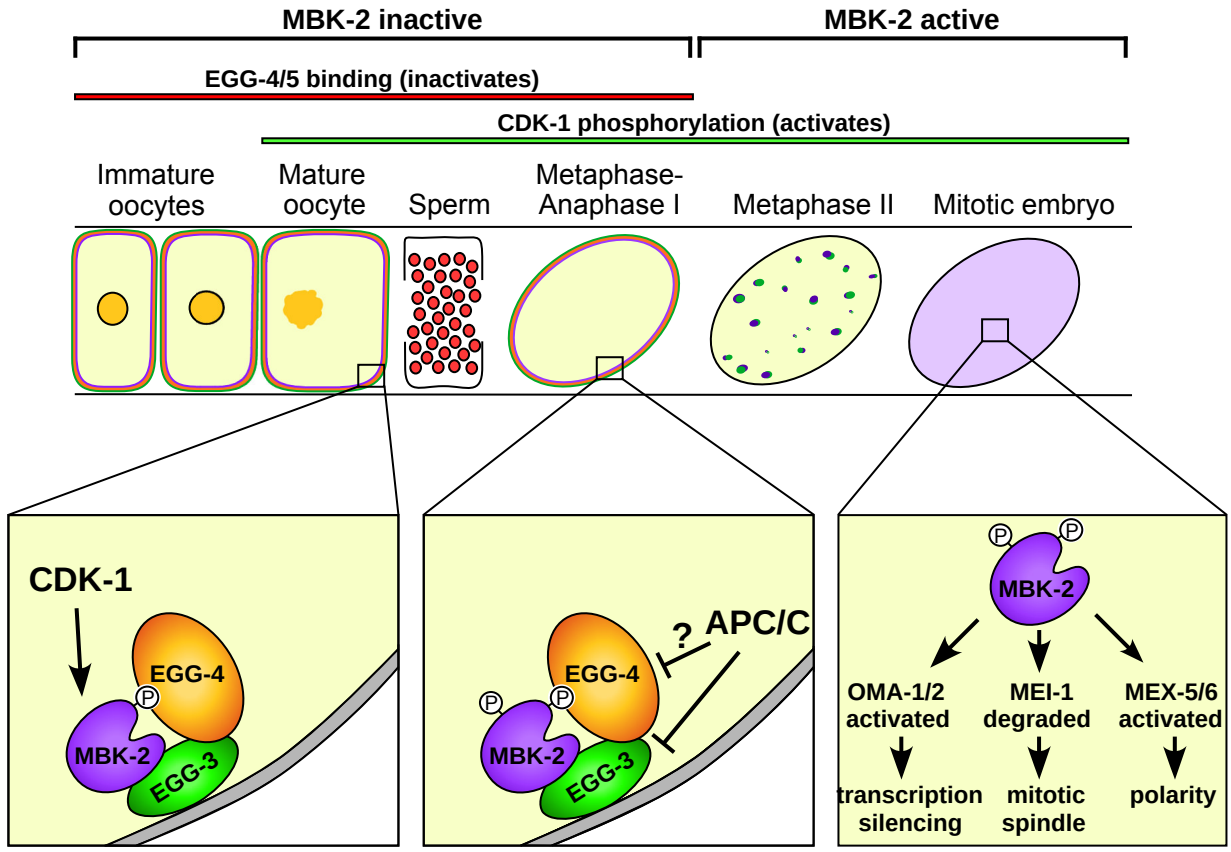
MBK-2 inactive
MBK-2 active
EGG-4/5 binding (inactivates)
CDK-1 phosphorylation (activates)
Immature oocytes
Mature oocyte
Sperm
Metaphase-Anaphase I
Metaphase II
Mitotic embryo
CDK-1
EGG-4
MBK-2
EGG-3
P
?
APC/C
OMA-1/2 activated
MEI-1 degraded
MEX-5/6 activated
transcription silencing
mitotic spindle
polarity

Figure 1. MBK-2 regulation during the oocyte to embryo transition.

The middle section is a representation of the proximal gonad with oocytes and embryos at different stages. The top lines present an overview of MBK-2 activation. The bottom panels show diagrams of MBK-2 regulation and activity at three stages. See text for details.

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