REGULATION AND REQUIREMENT OF MCPH1 AND ROUGH DEAL DURING *DROSOPHILA* EARLY EMBRYOGENESIS

By

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CHAPTER I

INTRODUCTION

THE CELL CYCLE

The cell cycle is the ordered series of events that lead to eukaryotic cell division, a process by which one parental cell gives rise to two daughter cells with identical genetic information. Cell division is fundamental for the development and function of all organisms. In single-cell organisms, cell division yields an entirely new organism. In multicellular organisms, however, it generates cell populations that form tissues and organs and replaces damaged and dying cells. Despite these differences, the basic machinery of cell division is shared among all eukaryotes. Cell growth and division have been extensively studied a diverse model organisms such as budding yeast (*Saccharomyces cerevisiae*), fission yeast (*Schizosaccharomyces pombe*), nematodes (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), frogs (*Xenopus laevis*), and cultured mammalian cell lines.

Cell division requires the duplication of genetic information in the parental cell and its equal distribution to each daughter cell. Genetic information is encoded in deoxyribonucleic acid (DNA), a polymer of nucleotides which sequence specifies information for the synthesis of ribonucleic acids (RNAs) and proteins that perform cellular functions. Eukaryotic DNA molecules reside in the nucleus and are extensively-folded and compacted into chromosomes. During cell division, chromosomes are duplicated and equally distributed into daughter cells. These events must occur in the proper order and often in coordination with cell growth to ensure accurate transmission of genetic information and cell viability.

Most eukaryotic cells carry out cell division in four phases: Gap 1 (G1), DNA Synthesis (S), Gap 2 (G2), and Mitosis (M). These distinct events are the basis of the canonical cell cycle. During G1, the cell grows in size and produces RNA and protein required for DNA synthesis. It then progresses through S-phase, where DNA replication occurs, followed by another growth interval referred to as G2. The cell-cycle events prior to mitosis, i.e. G1, S, and G2-phases, are collectedly referred to as interphase. Finally, after the second growth interval, the cell undergoes M-phase. This phase is comprised of two processes: mitosis, during which duplicated chromosomes are distributed into two daughter nuclei, and cytokinesis, during which the cell and its cytoplasmic contents are divided in two.

The events of mitosis are described chronologically in a series of stages (Fig 1.1): prophase, prometaphase, metaphase, anaphase, and telophase. In prophase, closely-associated duplicated chromosomes, referred to as sister chromatids, condense into distinct and separable units in the nucleus. At this stage, most cell types also contain two centrosomes, cytoplasmic organelles that nucleate the formation cytoskeletal filaments called microtubules (MTs). These centrosomes migrate to opposing sides of the cell and nucleate MTs that form a mitotic spindle. This structure is a bipolar array of filaments capable of separating sister chromatids to opposing ends of the cell. During prometaphase, nuclear envelope break down allows sister chromatids to attach to the mitotic spindle and undergo active movement. Sister chromatids are aligned at spindle equator in metaphase such that MTs attach sister chromatids to opposite spindle poles. In anaphase, sister chromatids are separated and pulled towards the spindle poles. And finally, during telophase, the separated sets of daughter chromosomes arrive at the spindle poles, decondense, and new nuclear envelopes reassemble around each set. The final stage of M-phase

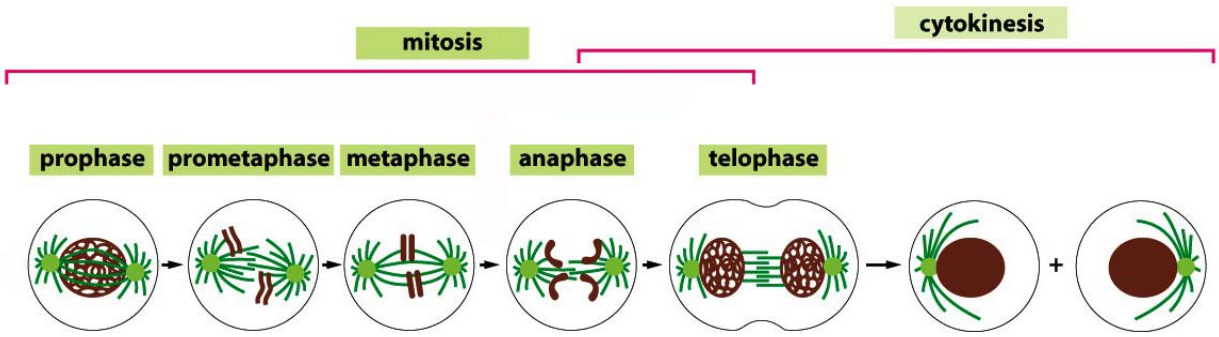

Figure 1.1 Events of Mitotic (M-) phase. After most cells duplicate their chromosomes and undergo sufficient growth, they enter M-phase of the cell cycle. During this phase, they undergo a process of nuclear division, called mitosis, and a process of cytoplasmic division, called cytokinesis. The events of mitosis are described in series of stages. In prophase, duplicated chromosomes condense into separable units and centrosomes nucleate microtubule (MT) filaments that assemble into a bipolar mitotic spindle. In prometaphase, the nuclear envelope breaks down and duplicated chromosomes attach to spindle MTs. Chromosomes are bi-oriented at the spindle equator during metaphase and are segregated to the spindle poles during anaphase. And in telophase, a nuclear envelope reassembles around each set of segregated chromosomes. Finally, cytokinesis starts in late mitosis when a contractile ring forms at the cell membrane and pinches the membrane until the cell divides in two. Figure adapted from (Alberts B. 2008).

is cytokinesis, or the division of cytoplasm (Fig. 1.1). This process begins during anaphase and is completed after telophase. In most eukaryotes, a contractile ring is formed at the cell membrane between the separated sets of daughter chromosomes. As this ring contracts, it pinches the cell membrane inwardly and gradually divides the cell in two.

CELL CYCLES IN DEVELOPMENT

Variations in the order of cell cycle events occur during the development of multicellular organisms to coordinate cell proliferation, growth, and differentiation. These modified cell cycles are specialized and essential to meet the needs of particular organisms and cell types. During the development of *Drosophila melanogaster*, *Xenopus laevis*, and the zebrafish *Danio rerio*, the early embryo undergoes simplified cell cycles consisting of rapid rounds of DNA replication and mitosis with no intervening gap phases (Budirahardja & Gonczy 2009, O'Farrell et al 2004). These cycles are not dependent on cell growth or gene transcription because they are driven by maternally-provided RNA and protein. Gap phases are introduced later in these organisms following the midblastula transition, at which time many maternal proteins are depleted and zygotic transcription begins (Lee & Orr-Weaver 2003, Newport & Kirschner 1984). It has been proposed that the early simplified cycles evolved to promote rapid development and enhanced survival in exposed environments.

Additionally, many organisms have cells that undergo endoreplication. This specialized cell cycle consists of multiple rounds of DNA synthesis and gap phase, with no intervening M-phase (Lee et al 2009). These “endocycles” generate large polyploid cells with numerous copies of the genome. In *Drosophila melanogaster*, most larval tissues and the oocyte-supporting nurse

cells in adult females undergo endocycles (Lee & Orr-Weaver 2003). The resulting increase in gene copy enhances gene transcription and support the rapid growth of these cells types.

Sexually reproducing eukaryotes also employ a modified cell-cycle program called meiosis to generate reproductive cells (reviewed in (Roeder 1997)). Meiosis consists of one round of DNA replication followed by two consecutive rounds of nuclear division. In diploid organisms, homologous chromosomes are segregated during the first meiotic division and sister chromatids are segregated during the second. This process results in specialized reproductive cells with half the normal copies of chromosomes, referred to as gametes, and prevents an inappropriate increase in ploidy when zygotes are formed from the fusion of two parental gametes.

CELL-CYCLE CONTROL

The order and timing of cell-cycle events are controlled by a regulatory network of proteins called the cell cycle control system. The central component of this control system is the cyclin-dependent kinase (Cdk) family of enzymes, which catalyze the covalent attachment of phosphate groups to protein substrates (Morgan 1997). Cdks initiate cell-cycle events by phosphorylating and changing the activation of proteins that control cell-cycle processes. The association of cyclin regulatory subunits via the Cdk PSTAIR domain stimulates Cdk catalytic activity (Jeffrey et al 1995). Cyclin synthesis and degradation also regulates progression through the cell cycle (Glotzer et al 1991, Murray & Kirschner 1989). Oscillations in cyclin levels generate oscillations in Cdk activity during the cell cycle.

Although cyclin-Cdk function is well conserved in eukaryotes, the number of Cdks and cyclins which control cell-cycle events and their pairing vary in different organisms. Unicellular

organisms, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have one Cdk, called Cdk1, which functions with most cyclins (Morgan 1997). Multicellular organisms may have up to four Cdks that associate with different cyclins to regulate specific cell-cycle events (Fig. 1.2). Each cyclin-Cdk complex promotes the activation of the next, ensuring ordered progression through the cell cycle. Generally, Cdk2/cyclin E controls the G1/S transition, Cdk2 or Cdk1 pair with cyclin A to direct S-phase, Cdk1/cyclin B directs mitosis, and Cdk4 or Cdk6 pairs with cyclin D during control cell-cycle entry from a prolonged non-dividing state called G zero (G0). The function of these pairs, however, varies in different organisms and cell types.

Cdk activity is also regulated by phosphorylation. In addition to cyclin binding, phosphorylation by Cdk-activating kinases (CAKs), such as cyclin H-cdk7, is required for Cdk function (Fisher & Morgan 1994, Solomon et al 1993). Additionally, phosphorylation of threonine 14 and/or tyrosine 15 of Cdk inhibits Cdk catalytic activity. Kinases such as Wee1 and Myt1 phosphorylate Cdk1 at one or both of these sites to inhibit mitotic entry (Gould & Nurse 1989, Mueller et al 1995). This inhibitory phosphorylation is removed by the Cdc25 family of phosphatases, which promotes cyclin B-Cdk1 and mitotic entry (Russell & Nurse 1986, Strausfeld et al 1991).

UBIQUITIN-MEDIATED PROTEOLYSIS AND THE CELL-CYCLE

Another important mechanism that controls cell-cycle progression is ubiquitin-mediated proteolysis. This mechanism results in the irreversible destruction of proteins by multi-subunit proteases called proteasomes (Fig 1.3). The destruction of cell-cycle regulators promotes unidirectional transitions through the cell cycle. These proteins are targeted for degradation by ubiquitination, a process that covalently links copies of an 8-kDa protein called ubiquitin.

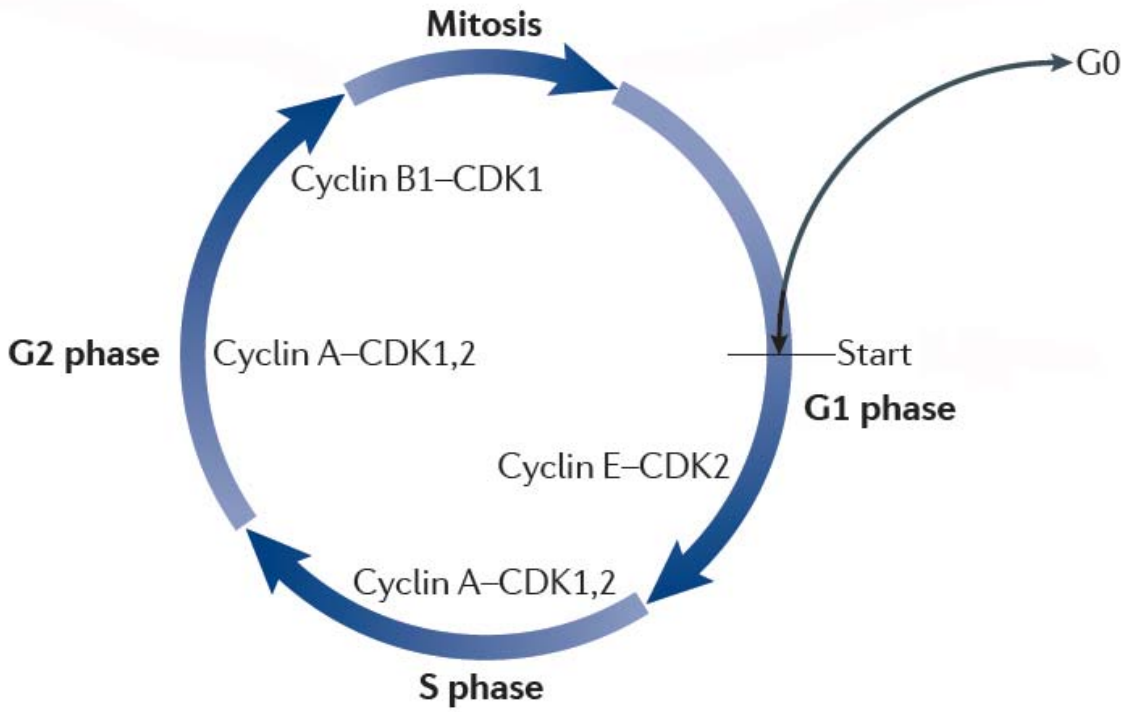

Figure 1.2 Cyclin-Cdk activity during the cell cycle. Cells often enter the cell cycle from a prolonged period of non-division, called G zero (G0). Cdks then direct cell-cycle progression by phosphorylating proteins that control cell-cycle processes. Cdk activity requires association with cyclin subunits, which are synthesized and degraded at specific times during the cell cycle. This generates a series of cyclin-Cdk complexes that govern distinct cell-cycle events. In multicellular organisms, cyclin E-Cdk2 directs the G1/S transition, cyclin A-Cdk2 or -Cdk1 directs progression through S and G2 phase, and cyclin B-Cdk1 directs the G2/M transition and the early events of mitosis. Figure adapted from (Pines 2011).

The series of enzymatic reactions that results in ubiquitination was first elucidated by Hersko and Ceichanover using the cell-free system of reticulocyte lysates, reviewed in (Hershko 1996, Hershko & Ciechanover 1998) (Fig 1.3). The E1 ubiquitin-activating enzyme initiates the process by forming an ATP-dependent thiol ester linkage to ubiquitin. The activated ubiquitin attached to the E1 is then transferred to an E2 ubiquitin-conjugating enzyme. Finally, the E3 ubiquitin ligase facilitates the transfer of ubiquitin from the E2 to a lysine residue of the substrate. E3 ligases provide substrate specificity by associating with both the E2-conugating enzyme and the target substrate. Additional copies of ubiquitin can be transferred to lysine residues of existing ubiquitin conjugates, generating polyubiquitin chains. These chains are recognized by receptors on the proteasome and target the substrate for destruction (Bedford et al 2010).

Substrate targeting by the SCF complex, a multi-subunit E3 ligase named for its components (Skp1, cullin, and F-box), promotes S-phase and mitotic entry (Cardozo & Pagano 2004). Though the three SCF core subunits are primarily invariable, there are many interchangeable F-box subunits that recruit specific substrates for ubiquitination. For example, the Cdk-inhibitor p27, which binds and inactivates cyclin E-Cdk2, is targeted for degradation by the F-box protein Skp2 and the SCF complex in late G1 (Carrano et al 1999, Nakayama et al 2001). This promotes Cdk activity and results in progression through S-phase. During G2, however, the F-box protein β-Trcp and the SCF complex target the Wee1 kinase for degradation (Watanabe et al 2004). Because Wee1 is a negative regulator of cyclin B-Cdk1, its SCF-mediated degradation during G2 promotes Cdk1 activity and mitotic entry.

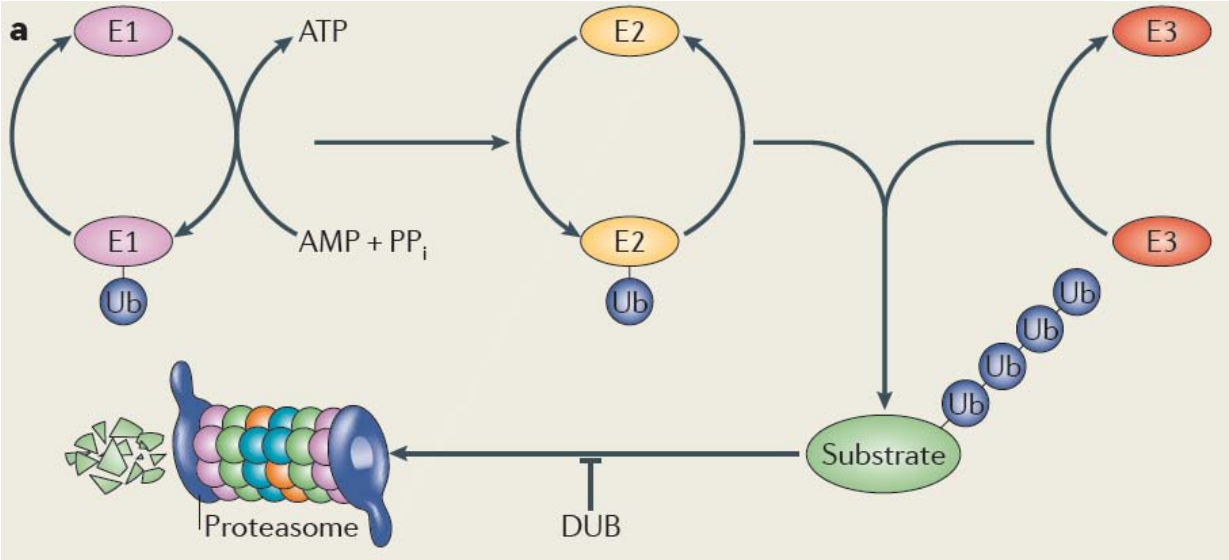

Figure 1.3 Mechanism of ubiquitin-mediated proteolysis. Ubiquitination requires the activity of three enzymes. The first is the E1 ubiquitin activating enzyme (E1), which requires ATP hydrolysis to generate a covalent-linkage to ubiquitin (Ub). The covalently linked ubiquitin is then transferred to the E2 ubiquitin conjugating enzyme (E2). The E3 ubiquitin ligase (E3) interacts with the E2 and the substrate to facilitate the transfer of ubiquitin to a lysine residue on the substrate. Multiple copies of ubiquitin are added to the first to form a polyubiquitin chain. The proteasome, a multi-subunit protease, recognizes and irreversibly degrades the polyubiquinated substrate. Figure adapted from (Pines 2011).

Substrate targeting by another multi-subunit E3 ligase, the Anaphase-Promoting Complex (APC), promotes mitotic exit and ensures low Cdk activity during G1 (reviewed in (Peters 2006, Sullivan & Morgan 2007)). At the metaphase-anaphase transition, the APC-dependent destruction of securin and cyclin B initiates sister chromatid separation and anaphase onset. The resulting inactivation of Cdks during mitosis allows for dephosphorylation of mitotic Cdk substrates and the completion of M-phase. The APC is further discussed later in this chapter.

CELL-CYCLE CHECKPOINTS

The cell-cycle control system directs progression through the cell cycle at three regulatory transitions called checkpoints (Hartwell & Weinert 1989). The first checkpoint commits the cell to cell-cycle entry in late G1, the second initiates mitosis at the G2/M transition, and the third initiates sister chromatid separation at the metaphase-anaphase transition. Surveillance mechanisms that monitor cellular events block cell-cycle progression at these checkpoints if problems are detected.

If problems in DNA replication occur or damaged DNA is detected, for example, cell-cycle progression is arrested at the G2/M transition. This checkpoint ensures that the integrity of the genome is maintained prior to mitotic entry. The DNA damage response (DDR) is the network of proteins responsible for detecting these issues, inhibiting cell-cycle progression, and initiating mechanisms of repair or cell death (reviewed in (Zhou & Elledge 2000)). If the damage is repaired, the cell-cycle resumes; however, in multicellular organisms, if the damage is irreparable, the DDR may initiate cell death.

Additionally, if errors in spindle-chromosome attachment are detected in mitotsis, cell-cycle progression is arrested at metaphase. The spindle-assembly checkpoint (SAC) is the

network of proteins responsible for detecting chromosome attachment to the spindle and preventing sister chromatid separation until all chromosomes are bi-oriented on the spindle (reviewed in (Jia et al 2013)). This ensures accurate chromosome segregation at the metaphase-anaphase transition. The SAC is further described later in this chapter.

CONSEQUENCES OF CELL-CYCLE MISREGULATION

The regulation of cell-cycle events is required to maintain the integrity of the genome. The accumulation of DNA damage and missegregation of chromosomes resulting from cell-cycle dysfunction can lead to disease or development defects. Cancer is a disease caused by uncontrolled cell proliferation in somatic cells and DNA mutagenesis is the driving force behind this abnormal cell growth. Cancer cells accumulate DNA mutations that allow them to proliferate and survive under conditions that normally restrain cell division or induce cell death (reviewed in (Evan & Vousden 2001). Rates of mutation increase in cells that have defects in cell-cycle regulators that govern replication, repair, or segregation of DNA or have been exposed to environmental factors that induce DNA damage, such as ultraviolet radiation and chemical carcinogens. Additionally, chromosome missegregation during meiosis causes miscarriages and genetic disorders associated with mental and physical developmental defects (reviewed in (Hassold & Hunt 2001)). In humans, zygotes with abnormal numbers of most chromosomes fail to survive, resulting in spontaneous abortions. However, embryos with abnormal copies of chromosomes 21, 18, 13, X, or Y may survive. Children born with these chromosomal abnormities develop genetic disorders such as Down, Klinefelter, and Turner Syndrome.

THE ANAPHASE-PROMOTING COMPLEX (APC)

The APC was first biochemically identified from extracts of clam and *Xenopus* oocytes as a large E3 complex (1.5 MDa) capable promoting cyclin A and B ubiquitination (King et al 1995, Sudakin et al 1995). This complex is conserved among eukaryotes and at least 14-15 subunits have been identified in yeast and humans (Pines 2011). Additionally, the APC associates with one of two coactivator subunits, Cdc20 and Cdh1, which regulates its activity during the cell-cycle (Fang et al 1998b) (Fig 1.4). These coactivators also provide substrate specificity by recognizing specific amino acid sequences, called degrons, in targeted substrates. Although there are two canonical APC degrons, the destruction (D)-box (RxxLxxxxN) and the KEN box (Lys-Glu-Asn), some substrates contain distantly related degrons, such as the A-, O-, and G-boxes, or those that are not well-defined (Araki et al 2005, King et al 1996, Ko et al 2007, Littlepage & Ruderman 2002, Pfleger & Kirschner 2000).

The APC is inactive from late G1 until early mitosis, when the phosphorylation of several APC subunits by cyclin B-Cdk1 promotes the association of Cdc20 (Kraft et al 2003, Shteinberg et al 1999) (Fig 1.4). The APC targets substrates such as cyclin A and Nek2A for degradation during prometaphase, although its activity is restrained by the SAC (Geley et al 2001, Hayes et al 2006). Once all the chromosomes are bi-oriented on the spindle and the SAC is inactivated, APC^{Cdc20} targets securin and cyclin B for degradation, which promotes sister-chromatid separation and inhibits Cdk1 activity, respectively (Geley et al 2001, Hagting et al 2002). Reduced Cdk activity leads to dephosphorylation of cyclin B-Cdk1 substrates, such as Cdh1 (Jaspersen et al 1999). Dephosphorylated Cdh1 activates the APC after anaphase onset and targets the destruction of Cdc20 (Fang et al 1998b, Jaspersen et al 1999, Shirayama et al 1998). Furthermore, APC^{Cdh1}–dependent degradation of a broad range of cell-cycle regulators promotes

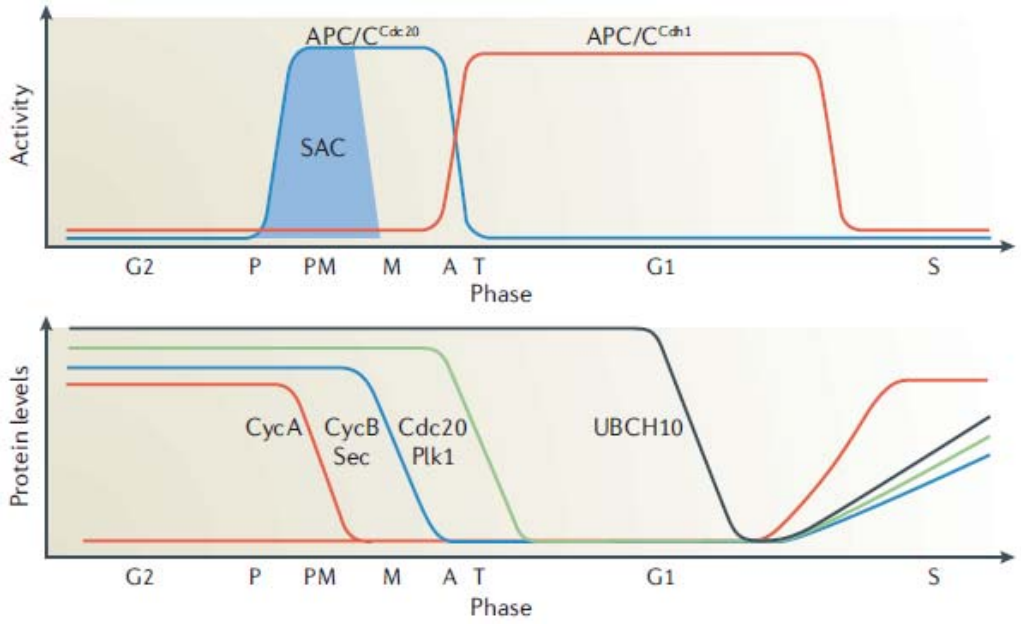

Figure 1.4 Activation of the Anaphase-Promoting Complex by Cdc20 and Cdh1. The APC is activated by phosphorylation during prophase (P). Although APC activity is restrained by the SAC, it targets specific substrates, like cyclin A (CycA) for degradation during prometaphase (PM). During metaphase (M), the SAC is inactivated by bio-orientation of sister chromatids on the mitotic spindle. APC^{Cdc20} then targets cyclin B (CycB) and securin (Sec) for degradation which promotes inactivation of Cdk1 and sister-chromatid separation at the metaphase (M)-anaphase (A) transition. Inactivation of Cdk1 leads to activation of APC^{Cdh1} in late mitosis, which targets Cdc20 and cell-cycle regulators such as polo-like kinase (Plk1) for degradation. APC^{Cdh1} keeps Cdk activity low until the following G1-S transition when it targets the APC specific E2 enzyme, UBCH10, for degradation. Figure adapted from (Peters 2006)

mitotic exit and keeps Cdk activity low until the APC is inactivated at the G1/S transition of the following cell-cycle, reviewed in (Sullivan & Morgan 2007).

MICROCEPHALIN (MCPH1)

Microcephalin was the first causative gene linked to autosomal recessive primary microcephaly (MCPH), a neurodevelopment disorder characterized by reduced brain size (Jackson et al 2002). Currently mutations in 13 genes are known to cause this disease: *MCPH1, WDR62, CDK5RAP2, CASC5, CEP152, ASPM, CENPJ, STIL, CEP135, CEP63, ZNF335, PHC1,* and *MKL2* (Venkatesh & Suresh 2014). It has been proposed that these genes regulate cell-cycle activities, such as spindle organization and DNA damage repair, which are required for proper proliferation and differentiation of neural progenitor cells.

Full length human MCPH1 is 90 kDa and contains one N-terminal and two C-terminal BRCT domains (Jackson et al 2002). These domains are phospho-peptide binding motifs that are commonly found in mediators of the DNA-damage response. A shorter isoform is also produced by alternative-splicing that lacks the two C-terminal BRCT domains (Gavvovidis et al 2012). Although MCPH1 is conserved among multicellular organisms, it is a rapidly evolving gene with low sequence similarity between homologs (Ponting & Jackson 2005).

MCPH1 is reported to have several functions in the DNA damage response. It promotes the expression of BRCA1 and Chk1, which are required for activation of the intra-S and G2/M checkpoints (Lin et al 2005, Xu et al 2004). Additionally, it localizes to DNA damage repair foci and recruits BRCA2, a protein required for DNA damage repair (Wu et al 2009). It also recruits components of the SWI-SNF complex, which promotes chromatin relaxation and the recruitment of additional DNA repair proteins (Peng et al 2009).

MCPH1 also has several roles outside the DNA damage response. It inhibits premature chromosome condensation by negatively regulating the Condensin II complex during mitotsis and by preventing premature mitotic entry (Alderton et al 2006, Tibelius et al 2009, Trimborn et al 2006, Yamashita et al 2011). MCPH1 is primarily a nuclear protein, but has been shown to localize to centrosomes during interphase in some cell types (Gruber et al 2011, Jeffers et al 2008, Tibelius et al 2009). In these cells, MCPH1 is required for the centrosomal recruitment of Pericentrin and Chk1 during G2, which results in inhibition of cyclin B-Cdk1 and mitotic entry. Additionally, MCPH1 inhibits expression of telomerase and interacts with the E2F1 to promote expression of its target genes (Shi et al 2012, Yang et al 2008).

THE SPINDLE-ASSEMBLY CHECKPOINT (SAC)

The SAC is a surveillance mechanism that detects chromosomes that are not attached to MTs of the mitotic spindle and delays anaphase onset until all attachments are made. Large protein networks, called kinetochores, assemble on specialized chromosomal regions during mitosis and mediate the attachment of chromosomes to spindle MTs (Foley & Kapoor 2013). Proteins which mediate SAC activation are recruited specifically to kinetochores that are not attached to spindle MTs and produce diffusible inhibitors of APC^{Cdc20}, which activity is required for cell-cycle progression through the metaphase-anaphase transition (Jia et al 2013).

The core components of the SAC were first identified in yeast as proteins required for cell-cycle arrest induced by MT depolymerization (Hoyt et al 1991, Li & Murray 1991). These proteins, known as Mad1, Mad2, Bub1, Bub3, and BubR1, are conserved among eukaryotes and promote the inhibition of the APC coactivator Cdc20. BubR1, Bub3, and Mad2 form a complex, called the mitotic checkpoint complex (MCC), which binds and inhibits Cdc20 from activating

the APC (Sudakin et al 2001) (Fig. 1.5). It has been proposed that the MCC inhibits Cdc20 by acting as a pseudo-APC substrate, thereby blocking substrate recruitment, and/or altering the binding of Cdc20 to the APC (Burton & Solomon 2007, Herzog et al 2009, Izawa & Pines 2011, Lara-Gonzalez et al 2011).

Recruitment of the Mad1-Mad2 and the BubR1-Bub3 complexes to unattached kinetochores promotes MCC assembly (Fig 1.5). Kinetochore-bound Mad1-Mad2 promotes a conformational change in free, cytosolic Mad2 that allows it bind Cdc20 (De Antoni et al 2005). Additionally, the interaction of Bub3 and BubR1 is required for the recruitment of BubR1 to kinetochores and promotes SAC activation (Elowe et al 2010). The Mad2-Cdc20 complex interacts with the BubR1-Bub3 complex to inhibit Cdc20 (Chao et al 2012). Although it is unclear how kinetochore recruitment of Bub3-BubR1 results in SAC activation, it has been proposed that it locally increases the concentration of BubR1 and facilitates MCC assembly (Elowe et al 2010). Notably, additional upstream components of the SAC response are required to recruit Mad1-Mad2 and BubR1-Bub3 complexes to unattached kinetochores, which are reviewed in (Jia et al 2013).

The MCC is silenced by destruction of MCC-APC^{Cdc20} complexes and inhibition of MCC formation. MCC-APC^{Cdc20} turnover results from auto-ubiquitination that targets it for proteasomal degradation (Foster & Morgan 2012, Uzunova et al 2012). Additionally, once a kinetochore attaches to a MT, kinetochore-associated dynein-dynactin removes SAC mediators, such as Mad1-Mad2, from the kinetochore and transports them towards the spindle poles (Gassmann et al 2010, Howell et al 2001). This shedding of kinetochore SAC components inhibits the formation of new MCC complexes once kinetochore-MT attachments are made.

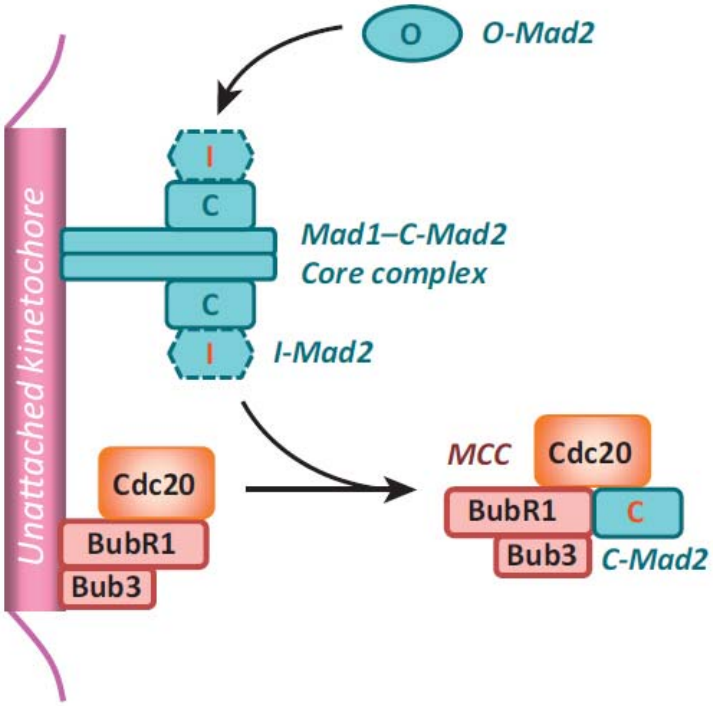

Figure 1.5 Activation of the Mitotic Checkpoint Complex (MCC). The Mad1-Mad2 core complex and the BubR1-Bub3-Cdc20 complex are recruited to kinetochores that are not attached the microtubules of the mitotic spindle. Free, cytosolic Mad2, which is in an open and inactive conformation (O-Mad2) undergoes a conformational change when associated with the Mad1-Mad2 core complex, generating intermediate Mad2 (I-Mad2). When I-Mad2 completes the conformational change to closed, active Mad2 (C-Mad2), it associates with Cdc20, BuBR1, and Bub3, generating the mitotic checkpoint complex that inhibits Cdc20. Figure adapted from (Jia et al 2013).

THE ROD-ZW10-ZWILCH (RZZ) COMPLEX

The genes *rough deal* (*rod*), *zeste-white10* (*zw10*), and *zwilch* were originally identified in *Drosophila melanogaster* and are conserved in all multicellular organisms. Loss of function of these genes in *Drosophila*, *C.elegans*, and human cultured cells results in inactivation of the SAC, lagging chromosomes at anaphase, and missegregation of whole chromosomes, called aneuploidy (Karess 2005).

Column affinity purification identified Rod, Zw10, and Zwilch as the main components of the 800 kDa "RZZ" complex (Kops et al 2005, Williams et al 2003) . This complex localizes to unattached kinetochores during mitosis and is required for the recruitment of Mad1-Mad2 and dynein-dynactin to unattached kinetochores (Basto et al 2004, Buffin et al 2005, Kops et al 2005, Starr et al 1998). While recruitment of Mad1-Mad2 promotes SAC activation, recruitment of dynein-dynactin is required for the shedding of SAC mediators (including RZZ) from the kinetochore after MT attachment (Jia et al 2013).

RZZ associates with dynein-dynactin by direct and indirect interactions. The Zw10 subunit of RZZ interacts directly with dynein-light-intermediate chain (DLIC) and the p50 subunit of dynactin (Starr et al 1998, Whyte et al 2008). Additionally, RZZ recruits Spindly to unattached kinetochores, which promotes kinetochore targeting of dynein-dynactin as well (Chan et al 2009, Gassmann et al 2008, Griffis et al 2007). Affinity chromatography and yeast-hybrid studies performed on RZZ subunits, however, have not revealed the nature of the interaction between RZZ and Mad1-Mad2 (Kops et al 2005, Starr et al 2000, Williams et al 2003).

The mechanism by which RZZ assembles and is recruited to kinetochores is not well understood. Because the combined molecular weight of Rod, Zw10, and Zwilch, is roughly half of that of the whole complex, it has been suggested that RZZ either contains two copies of each

subunit or forms a stable dimer (Karess 2005). Furthermore, studies in vertebrate cells and *Drosophila* have shown that kinetochore recruitment of RZZ is not dependent on kinetochore proteins Bub3, BubR1, Mps1, Cenp-I, Cenp-F, or Cenp-E (Basu et al 1999, Liu et al 2003a, Liu et al 2003b, Wang et al 2004, Williams et al 2003). Although Zwint-1 is proposed to be a RZZ docking site in vertebrates because it interacts Zw10 and is recruited to kinetochores before RZZ, a Zwint-1 homolog has not been identified in *Drosophila* (Starr et al 2000).

POLAR BODIES

Female meiosis in multicellular organisms produces an oocyte and cellular remnants called polar bodies. Although the oocyte typically develops into the zygote upon fertilization, the generation and fate of polar bodies varies in many organisms. Polar bodies contain chromosomes that result from the first and second meiotic divisions and may or may not contribute to offspring development. Although polar bodies do not participate in egg fertilization in sexually reproducing animals, many animal and plant species undergo a form of asexual reproduction, called parthenogenesis, in which polar body nuclei fuse with each other or the oocyte nucleus to activate offspring development (Stenberg & Saura 2013).

Polar body generation is important for reducing the ploidy of gametes in sexually producing animals. However, polar bodies are largely dispensable for development and are not well-studied (Schmerler & Wessel 2011). In most animals, including humans and the model organisms *D. rerio*, *X. laevis*, and *C. elegans*, half of the segregated chromosomes from meiosis I and II are expelled from the oocyte after each division into small, separate polar body cells that shortly undergo apoptosis (Maddox et al 2012). Female meiosis and early embryonic development in *D. melanogaster*, however, consists of chromosome segregation without

cytokinesis, which results in retention of all nuclear products in the egg during meiosis, fertilization, and early embryonic development (Foe et al 1993). One of the female meiotic nuclear products fuses with the sperm pronucleus to form the zygote, while the remaining female meiotic nuclear products fuse to form an arrangement of condensed chromosomes called a polar body. Although polar bodies are formed differently in this organism, they are eventually degenerated and do not further contribute to development.

Studies of polar bodies in different systems can provide insight into asymmetric cell division, cell-cycle control, and cell-fate decisions during development. Studies of polar body extrusion have identified novel mechanisms that control the plane of cell division, spindle anchoring, and cytoskeletal rearrangements that are distinct from asymmetric divisions during mitosis and male meiosis (Maddox et al 2012). Additionally, studies of *D. melanogaster* early embryonic development have also identified regulatory mechanisms that are uniquely required to maintain local cell-cycle arrest in polar bodies prior to degeneration in this organism (Fischer et al 2004, Lee et al 2001, Perez-Mongiovi et al 2005). Furthermore, because polar bodies contribute to off-spring development in some organisms and not in others, further study of polar body fate may uncover unique molecular mechanisms that lead to these cell-fates (Schmerler & Wessel 2011).

INTRODUCTORY REMARKS

My dissertation research, presented in the next two chapters, is based upon the results of two independent screens to identify proteins involved in cell-cycle regulatory processes. In a biochemical screen for substrates of the APC, we identified *Drosophila* MCPH1 as a novel candidate APC substrate. Although previous studies have identified roles for MCPH1 in

regulating cellular processes such as DNA damage repair, cell-cycle control, and gene expression (Lin et al 2005, Shi et al 2012, Tibelius et al 2009), regulation of the activity of this protein by post-translational modifications is not well understood. In an effort to fill this gap in understanding, our studies further examine regulation of *Drosophila* MCPH1 and its human homolog by APC-dependent ubiquitination and proteasomal degradation and how regulation of their protein levels effects cell-cycle progression.

Additionally, we identified a novel allele of *rod* in a screen for genes required for cell-cycle regulation during *Drosophila* early embryogenesis. This allele, known as rod^{Z3}, is maternal-effect lethal and contains a point mutation that substitutes glutamatic acid for glycine 1973 in the C-terminal region of *Drosophila* Rod. Roles for this protein in regulating chromosome segregation and SAC activation have been previously identified in studies of *Drosophila* male meiosis and larval neural development in *rod*-null mutants (Karess & Glover 1989, Scaerou et al 1999). Although the Rod N-terminus is required for interaction with other RZZ subunits (Zw10 and Zwilch) (Civril et al 2010, Scaerou et al 2001), it is also unknown how the C-terminus contributes to its function. To identify the role of Rod in regulating *Drosophila* embryonic development and to better understand how the C-terminus contributes to Rod function, we characterized developmental defects and RZZ function in rod^{Z3}-derived embryos. Furthermore, it has been proposed that stabilization of Cyclin B via SAC activation is required to arrest polar bodies in a starburst configuration of condensed chromosomes during Drosophila embryogenesis (Fischer et al 2004, Perez-Mongiovi et al 2005). We test this hypothesis by examining polar body condensation and how modulation of Cyclin B levels affects this phenotype in rod^{Z3}-derived embryos.

CHAPTER II

THE *DROSOPHILA* MCPH1-B ISOFORM IS A SUBSTRATE OF THE APC^{CDH1} E3 UBIQUITIN LIGASE COMPLEX

The contents of this chapter have been published (Hainline et al 2014)

INTRODUCTION

The Anaphase-Promoting Complex (APC) is a multi-subunit E3 ubiquitin ligase that catalyzes ubiquitin-mediated proteasomal degradation of target proteins. A major function of the APC is to promote degradation of key cell-cycle proteins so as to coordinate orderly progression through the cell cycle (Peters 2006). Human and yeast APC are each composed of 14-15 identified subunits and two primary co-activators, Cdc20 and Cdh1 (Kulkarni et al 2013). Destruction of APC substrates is required in eukaryotes for the initiation of anaphase and exit from mitosis. Cdc20 associates with the APC in early mitosis, leading to the destruction of proteins that control the onset of anaphase, whereas Cdh1 promotes degradation of APC substrates that control late mitosis and the following G1 phase. These co-activators provide APC substrate specificity by facilitating the recognition of specific destruction motifs (e.g. degrons) such as the D-box (RxxLxxxxN) or KEN box (Lys-Glu-Asn) (King et al 1996, Min & Lindon 2012, Pfleger & Kirschner 2000). Mutations of these motifs block the recognition of the protein by the APC, preventing their APC-mediated destruction.

Xenopus egg extract contains many of the components necessary for ubiquitin-mediated degradation such as E1, E2, and E3 enzymes, ubiquitin, and the proteasome. Moreover, biochemical regulation of APC^{Cdc20} -and APC^{Cdh1}-mediated degradation has been well studied

and characterized in this system. *Xenopus* egg extract lacks Cdh1, and Cdc20 is the primary activator of APC (Lorca et al 1998). Addition of exogenous human Cyclin B lacking its N-terminal D-box (CycBΔ90) to interphase *Xenopus* egg extract drives the extract into mitosis and promotes the degradation of APC^{Cdc20} substrates (Glotzer et al 1991). Addition of exogenous Cdh1 to interphase *Xenopus* egg extract similarly promotes the degradation of APC^{Cdh1} substrates (Pfleger & Kirschner 2000).

The in vitro expression cloning (IVEC) strategy involves generating [^{35}S]methionine-labeled proteins by in vitro-coupled transcription and translation of small, random pools of cDNAs; these radiolabeled proteins can then be used for biochemical screening in a powerful approach that allows for rapid isolation of relevant cDNAs corresponding to "hits" in the screen (King et al 1997). IVEC has been successfully used in *Xenopus* egg extract to identify important APC substrates such as Geminin, Securin, Xkid, Tome-1, and Sororin (Ayad et al 2003, Funabiki & Murray 2000, McGarry & Kirschner 1998, Rankin et al 2005, Zou et al 1999). A weakness of the original IVEC strategy, however, is that, depending on the cDNA library being used, certain genes are over-represented whereas other genes are under-represented in the library. Thus, the same substrate is often identified over and over again, and substantial screening is necessary to identify relevant rare clones. Furthermore, the pools of cDNAs used for IVEC screening must be deconvoluted in order to isolate single hits as the identities of the clones in the pools are unknown.

To overcome these limitations, we previously modified the IVEC methodology to generate radiolabeled protein pools from Release 1 of the *Drosophila* Gene Collection (DGC), an annotated unigene set of 5,849 full-length cDNA clones representing 43% of the fly genome (Lee et al 2005, Stapleton et al 2002). Clones were individually arrayed in 17 X 384-well plates,

and in vitro transcription and translation was performed on small pools containing equivalent amounts of cDNA (or mRNA) for each gene. This *Drosophila* IVEC (DIVEC) approach has allowed for efficient genome-scale screening to identify substrates of the Pan Gu kinase and binding partners of p53 (Lee et al 2005, Lunardi et al 2010).

Given the conservation across phyla between cell cycle proteins, we herein applied the DIVEC approach to perform a biochemical screen for APC substrates in *Xenopus* interphase egg extract and identified *Drosophila* Microcephalin (dMCPH1) as a candidate. Human MCPH1 (hMCPH1) is a causative gene of autosomal recessive primary microcephaly (MCPH), a neurodevelopment disorder characterized by reduced brain size (Jackson et al 2002, Woods et al 2005). In humans, MCPH1 has been shown to prevent premature mitotic entry by regulating centrosomal recruitment of Chk1 at the G2/M transition as well as premature chromosome condensation by negatively regulating the activity of condensin II (Gruber et al 2011, Tibelius et al 2009, Trimborn et al 2006, Yamashita et al 2011). hMCPH1 has also been reported to have several functions in the DNA damage response (Gavvovidis et al 2012, Lin et al 2005, Peng et al 2009, Rai et al 2006, Tibelius et al 2009, Trimborn et al 2006, Yamashita et al 2011, Yang et al 2008). We previously reported that *Drosophila* syncytial embryos derived from *mcph1*-null females exhibit Chk2-mediated mitotic arrest in response to damaged or incompletely replicated DNA (Rickmyre et al 2007). Because *mcph1* mutants contain an intact DNA checkpoint, and MCPH1 has been shown to regulate premature chromosome condensation in other systems, we previously proposed that dMCPH1 prevents accumulation of DNA damage by delaying chromosome condensation until DNA replication is completed. Although MCPH1 is reported to function in multiple cellular processes, its regulation is not well understood. In this report, we demonstrate that dMCPH1 is a substrate of the critical cell cycle regulator, APC^{Cdh1}.

MATERIALS AND METHODS

cDNA clones and mutagenesis

cDNA clones encoding dMCPH1-B (clone LD42241), dMCPH1-C (clone LP15451), or p78 (GH13229) were obtained from the *Drosophila* Gene Collection Release 1 or the *Drosophila* Genomics Resource Center (Indiana University, Bloomington, IN), respectively. cDNA clones encoding hMCPH1, Cyclin B, NT-Cyclin B, Mos, Luciferase, and GFP were gifts from Marc Kirschner's lab (Harvard Medical School, Boston, MA). dMCPH1-B and dMCPH1-C were subcloned into vector pCS2 for in vitro transcription and translation reactions. dMCPH1-$B^{\Delta N}$, dMCPH1-$B^{DboxMut}$, and dMCPH1-B^{1-64} were generated from CS2-dMCPH1-B by mutagenesis to remove the first 40 amino acids, replace amino acids 36-40 with alanines, or remove the last 762 amino acids, respectively. dMCPH1-B, dMCPH1-$B^{DboxMut}$, and hMCPH1 were also subcloned into pCS2 derivatives encoding six N- or C-terminal Myc tags.

DIVEC screen and APC degradation assay

Xenopus interphase egg extract was prepared as previously described (Pfleger & Kirschner 2000). Baculoviruses encoding human His6-tagged CDH1 and His6-tagged Cyclin BΔ90 (gifts from Marc Kirschner's lab) were expressed in *Sf9* cells by baculovirus infection and purified over nickel beads. For the DIVEC screen, radiolabeled protein pools were generated from pools of cDNAs from the *Drosophila* Gene Collection Release 1 by transcription and translation in reticulocyte lysates using a Gold TNT T7 kit according to the manufacturer's protocol (Promega, Madison, WI) as previously described (Lee et al 2005). The identity of positive clones was confirmed by DNA sequencing.

For testing individual proteins in the APC degradation assay, 1 µl of radiolabeled protein was added to 10 µl of *Xenopus* egg extract supplemented with energy mix (1 mM HEPES, pH 7.7, 1 mM ATP, 10 mM creatine phosphate, and 1 mM $MgCl_2$) and 10 µg/ml ubiquitin. Egg extract was incubated with *Xenopus* Buffer control (100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, 50 mM sucrose, 5 mM EGTA), His6-Cyclin BΔ90 (60 µg/ml), or His6-CDH1 (0.4 nM) prior to starting the reaction with addition of radiolabeled proteins, and reactions were allowed to proceed at room temperature as previously described (Ayad et al 2003). All radiolabeled, in vitro-translated protein migrated at the expected size as assessed by SDS-PAGE/autoradiography. For radiolabeled degradation assays, loading controls were not necessary as equivalent volumes (0.5 µl) were removed at the indicated times for processing by SDS-PAGE/autoradiography. NT-Cyclin B peptide 100 µM was prepared as previously described in (Pfleger & Kirschner 2000). Pixel intensity measurements of autoradiograms were performed using ImageJ and statistical analysis was performed using the paired equal variance two-tailed t-test.

Drosophila stocks, embryo lysates, and immunoblotting

Flies were maintained at 25°C using standard techniques (Greenspan 2004). *morula* stocks (mr^1 and mr^2) were gifts from T. Orr-Weaver (Whitehead Institute, Cambridge, MA) (Reed & Orr-Weaver 1997). y^1 w^{1118} flies were used as the "wild-type" stock. Embryo lysates were made by homogenizing embryos (0-1 hour) in urea sample buffer (100 mM Tris, pH 7.6, 8 M urea, 2% SDS, 5% β-mercaptoethanol, and 5% Ficoll). Lysates were analyzed by SDS-PAGE and immunoblotting using standard techniques. Primary antibodies used included guinea pig anti-MCPH1 (1:200) (Rickmyre et al 2007); mouse anti-Cyclin B (1:200, F2F4, Developmental

Studies Hybridoma Bank, Iowa City, IA); and mouse anti-α-tubulin (1:5000, DM1α, Sigma-Aldrich, St. Louis, MO). HRP-conjugated secondary antibodies were used to detect primary antibodies by chemiluminescence.

In vitro ubiquitination assay

APC was purified by immunoprecipitation of Cdc27 from *Xenopus* interphase egg extract using Protein G Sepharose beads (GE Healthcare Life Sciences, Pittsburgh, PA) and anti-Cdc27 antibodies (AF3.1; Santa Cruz Biotechnology, Dallas, TX) as previously described (Wei et al 2004). For each ubiquitination reaction, 5 µl of APC-bound beads was incubated with 0.75 µM purified E1 (Boston Biochem, Cambridge, MA), 2 µM His-UbcH10 (Boston Biochem), 7.5 mg/ml ubiquitin (Boston Biochem), 0.5 µl 20X Energy Regeneration Mix (2 mg/ml creatine phosphokinase, 20 mM ATP, 200 mM Creatine Phosphate, 20 mM HEPES, 20 mM $MgCl_2$, 0.1% BSA), 5 µM ubiquitin aldehyde (Boston Biochem), and 10 mM DTT. 1 µl of in vitro transcription/translation reaction product and 0.4 nM His-Cdh1 or equal volume of Cdh1 dialysis buffer was incubated in each reaction for 90 minutes. Reaction products were separated by SDS-PAGE and visualized by autoradiography.

***Xenopus* embryo injection, immunostaining, and immunoblotting**

Capped mRNA encoding Mos, GFP, hMCPH1, dMCPH1-B, or dMCPH1-B^{DboxMut} was generated by in vitro transcription reactions using the mMessage mMachine kit per manufacturer's instructions (Life Technologies, Carlsbad, CA). Embryos were injected at the 2- or 4-cell stage with 2 ng of RNA and fixed in MEMFA (100 µM MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) after 4 hours. After fixation, embryos were washed 2X in

PBS and dehydrated stepwise (1 hour/step) in 75% PBS/25% methanol, 50% PBS/50% methanol, and 100% methanol and stored at 4°C. The percentage of injected embryos exhibiting cell-cycle defects was quantified and statistical analysis was performed using the Fisher exact test.

For tubulin staining, MEMFA-fixed embryos (in 100% methanol) were bleached in 10% H_2O_2/67% methanol for 8 hours at room temperature. Bleached embryos were rehydrated (1 hour/step) in 50% methanol/50% TBS (155 mM NaCl, 10mM Tris-Cl pH 7.5), 25% methanol/75% TBS, and finally 100% TBST (TBS plus 0.1% Triton-X-100). Embryos were then blocked in WMBS (TBS plus 10% fetal bovine serum and 5% DMSO) for 1 hour. Mouse anti-α-tubulin (DM1α, 1:500, Sigma), RNAse A (1 mg/ml), and propidium iodide (2 µg/ml) were then added and embryos were incubated overnight at 4°C. Embryos were washed 5X (1 hour each) with TBST and incubated in WMBS with RNAse A, propidium iodide, and Cy2-conjugated secondary antibodies (1:500, Sigma). Embryos were washed 5X (1 hour each) with TBST, placed in MatTek dishes (Ashland, MA), and imaged using a Leica TCS SP5 inverted confocal microscope (Buffalo Grove, IL).

For immunoblotting, capped mRNA encoding C-terminally Myc-tagged hMCPH1, dMCPH1-B, or dMCPH1-$B^{DboxMut}$ was generated, and 1 ng of RNA was injected into each cell of a two-cell staged *Xenopus* embryo. At 4 hours post-injection, the embryos were lysed in 6X Sample Buffer (300 mM Tris pH 6.8, 12% w/v SDS, 30% w/v glycerol, 600 mM DTT, and 0.01% w/v bromophenol blue). ¼ of each lysate was analyzed by SDS-PAGE and immunoblotting using standard techniques. Primary antibodies used included mouse anti-Myc-tag (1:500, 9E10) and mouse anti-α-tubulin (1:2000, DM1α, Sigma-Aldrich, St. Louis, MO).

HRP-conjugated secondary antibodies were used to detect primary antibodies by chemiluminescence.

Cell synchronization

24 hours after plating HeLa cells on 150 mm dishes at 20% confluency, cells were treated with nocodazole (25 ng/ml) for 13 hours. Plates were firmly tapped to loosen the rounded, mitotic cells from the dish. Cells were then collected by centrifugation for 5 minutes, and washed 3 times in fresh serum-free media. After the final wash, cells were resuspended in media containing 10% FBS and plated at 50% confluency in 6-well dishes. Cells were collected every 2 hours by removing media, washing in PBS, treating with 100 µl 0.25% trypsin-EDTA, and collecting in 1 ml media. Collected cells were washed once in PBS and lysed in non-denaturing lysis buffer (50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100). Lysates were analyzed by SDS-PAGE and immunoblotting using standard techniques. Antibodies used were rabbit anti- hMCPH1 (D38G5, 1:100, Cell Signaling Technology, Danvers, MA), rabbit anti-Cdk1 (1:4,000, Millipore, Billerica, MA), rabbit anti-Cyclin A (H-432, 1:500, Santa Cruz), rabbit anti-Cyclin B1 (H-20, 1:500, Santa Cruz), and rabbit anti-p27 (C-19, 1:100, Santa Cruz). HRP-conjugated secondary antibodies and chemiluminescence were used to detect primary antibodies.

RESULTS

DIVEC screen for APC substrates

In order to identify APC^{Cdc20} or APC^{Cdh1} substrates using DIVEC, bacterial stocks containing cDNA clones from the *Drosophila* Gene Collection Release 1 were individually

grown and their plasmids purified and pooled (Fig. 2.1A). Pooled clones (24 clones/pool) were used to generate radiolabeled proteins in rabbit reticulocyte lysate as previously described (Lee et al 2005). To test proteins for their capacity to undergo APC^{Cdc20}-or APC^{Cdh1}-mediated degradation, protein pools were incubated in *Xenopus* interphase egg extract supplemented with *Xenopus* buffer (XB), human CycBΔ90, or Cdh1. Candidate APC substrates were identified by their decreased band intensity after incubation in CycBΔ90 or Cdh1-supplemented extract relative to the buffer control as revealed by SDS-PAGE and autoradiography.

We identified two candidate substrates of APC in *Xenopus* egg extract using the DIVEC approach (Fig. 2.1B). We initially named these candidates "p78" and "p91" based on their apparent SDS-PAGE mobility. In the primary screen that involved the use of radiolabeled protein pools, both candidates were stable in the presence of XB and CycBΔ90 (mitotic extract containing activated APC^{Cdc20}), but they degraded in *Xenopus* egg extract supplemented with Cdh1, suggesting that they are substrates of APC^{Cdh1} and not APC^{Cdc20}. In addition, both candidates exhibited decreased mobility on SDS-PAGE when incubated in Cyclin BΔ90-supplemented (mitotic) extract, suggesting that they may be phosphorylated during mitosis.

The corresponding cDNA clones for the two candidate substrates were identified based on the predicted molecular weights of their encoded proteins and retesting in the degradation assay. We confirmed that the protein products generated by in vitro transcription and translation of these individual cDNA clones were degraded in Cdh1-supplemented *Xenopus* egg extract (Fig. 2.1C,D). p91 is encoded by clone LD43341 and corresponds to the *Drosophila mcph1* gene (Brunk et al 2007, Rickmyre et al 2007). p78 is encoded by clone GH13229 and corresponds to *CG32982,* an uncharacterized *Drosophila* gene. Cyclin B, a well-characterized APC substrate, was not identified in our screen because it is not present in the *Drosophila* Gene Collection

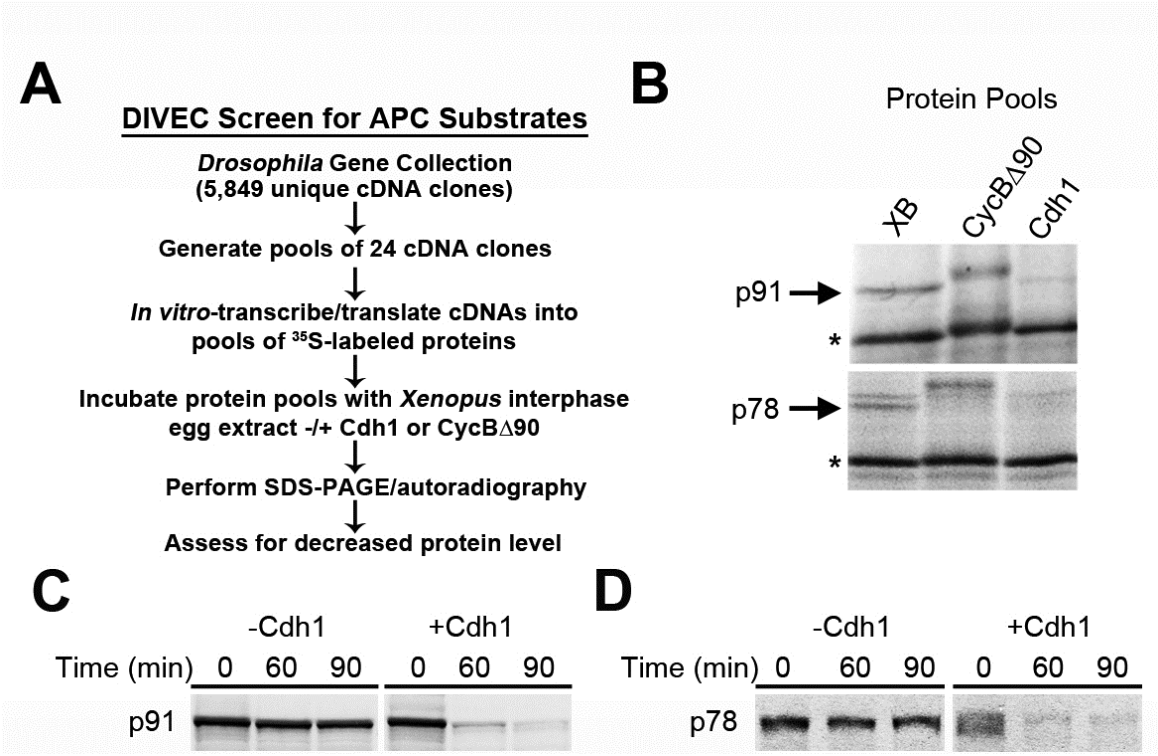

Figure 2.1 A *Drosophila* In Vitro Expression Cloning (DIVEC) screen identifies two novel APC substrates. (A) Schematic of the DIVEC screen strategy to identify APC substrates. ^{35}S-radiolabeled proteins were produced from pools of cDNA clones from the *Drosophila* Gene Collection Release 1 as previously described (Lee et al 2005). Radiolabeled protein pools were incubated in *Xenopus* interphase egg extract supplemented with *Xenopus* buffer (XB), non-degradable Cyclin B (CycBΔ90), or Cdh1. Reaction products were analyzed by SDS-PAGE and autoradiography to identify proteins degraded via APC-Cdc20 or APC-Cdh1. (B) p91 and p78 are candidate APC substrates. Autoradiogram of two protein pools containing p91 and p78. Both p91 and p78 exhibited an upward electrophoretic mobility shift in CycBΔ90-supplemented (mitotic) extract and decreased band intensity in Cdh1-supplemented extract. Asterisks mark proteins in the pools that did not exhibit decreased intensity in the supplemented extract and therefore served as negative controls. (C,D) Retesting of radiolabeled p91 and p78 (prepared from individual cDNA clones) by incubation in *Xenopus* interphase egg extract in the presence of Cdh1 confirmed that the clones encode putative APC substrates.

Release 1. Radiolabeled Cyclin B, however, was used as a positive control in our screen and was shown to degrade in both mitotic (activated APC^{Cdc20}) and Cdh1-supplemented interphase *Xenopus* egg extract (data not shown).

Drosophila MCPH1-B stability is regulated by APC

We previously identified a requirement for dMCPH1 during early embryogenesis in *Drosophila* (Rickmyre et al 2007). Two distinct isoforms of *Drosophila* MCPH1 (referred to as MCPH1-B and MCPH1-C) are produced by alternative splicing (Rickmyre et al 2007). Both isoforms are present in larval brains and imaginal discs. *Drosophila* MCPH1-B (dMCPH1-B) is predominantly expressed in the ovaries and syncytial embryos, whereas MCPH1-C (dMCPH1-C) is expressed primarily in the testes. The two isoforms differ primarily at their N- and C-termini. dMCPH1-B contains an additional 47 amino acids at its N-terminal end and lacks 200 amino acids at its C-terminal end when compared to the dMCPH1-C isoform (Fig. 2.2A). Both isoforms contain an N-terminal BRCT domain. Only dMCPH1-C, however, contains an additional pair of BRCT domains at its C-terminal end.

We identified the B isoform of dMCPH1 as a hit in our DIVEC screen for APC substrates. To demonstrate that the degradation of dMCPH1-B in *Xenopus* egg extract was specific to APC^{Cdh1} activity, we tested whether Cdh1-mediated degradation of dMCPH1-B in *Xenopus* interphase egg extract could be inhibited by addition of an N-terminal peptide of Cyclin B (NT-Cyclin B) containing a functional D-box (Fig. 2.3A). NT-Cyclin B is degraded in Cdh1-supplemented egg extract and competitively blocks APC^{Cdh1}-mediated degradation of Cdc20 (Pfleger & Kirschner 2000). Similarly, if dMCPH1-B degradation in Cdh1-supplemented *Xenopus* interphase egg extract were mediated by APC^{Cdh1}, addition of excess NT-Cyclin B

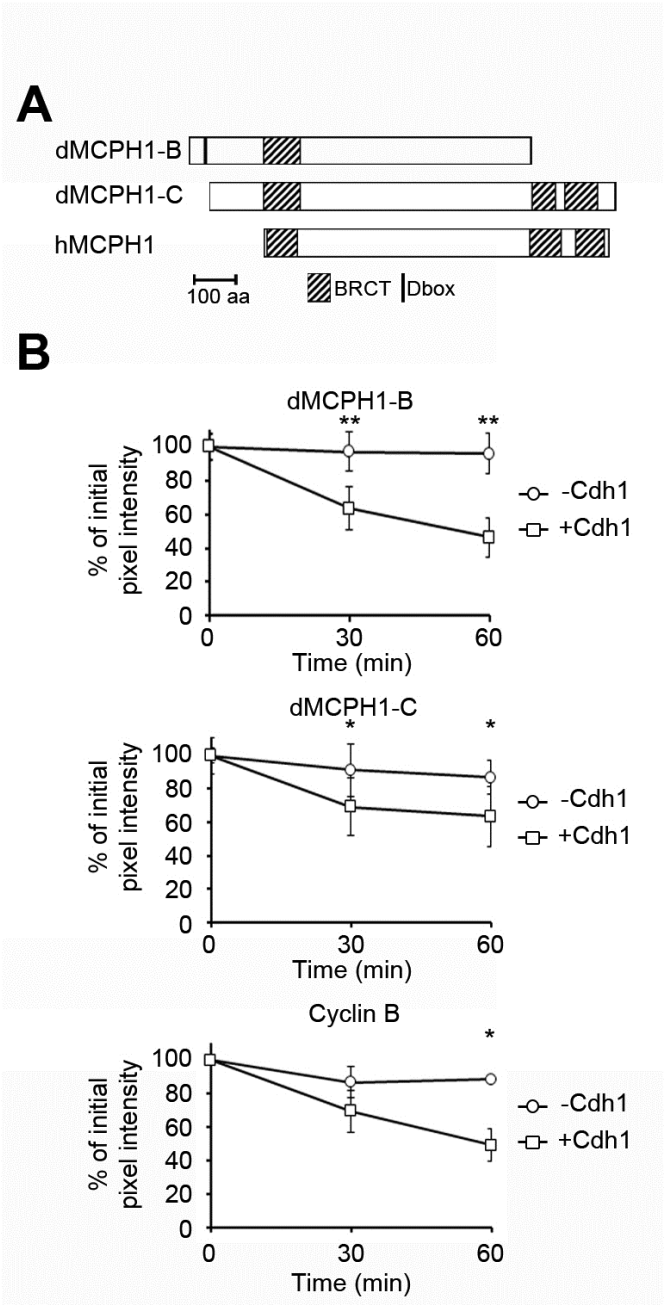

Figure 2.2 APC regulates stability of dMCPH1-B and dMCPH1-C. (A) Schematic representation of dMCPH1-B, dMCPH1-C, and hMCPH1. (B) Quantitation of pixel intensity of autoradiogram in Fig. 2B. Percent of initial pixel intensity was plotted over time for radiolabeled dMCPH1-B, dMCPH1-C, or Cyclin B incubated in *Xenopus* interphase egg extract in the absence or presence of Cdh1. $**p<0.005$, $*p<0.05$.

should inhibit its degradation. Consistent with this model, we found that addition of NT-Cyclin B potently blocked dMCPH1-B degradation in Cdh1-supplemented extract (Fig. 2.3A). We next asked if the MCPH1-C isoform is also a substrate of APC^{Cdh1}. We incubated radiolabeled dMCPH1-C in *Xenopus* interphase egg extract in the absence or presence of Cdh1 and assessed its levels after 30 and 60 minutes by performing SDS-PAGE/autoradiography (Fig. 2.1B, 2.2B). For dMCPH1-B and Cyclin B (positive control), we detected robust turnover in Cdh1-supplemented *Xenopus* interphase egg extract. Although we detected statistically significant Cdh1-mediated degradation for dMCPH1-C, it was not nearly as robust as that of dMCPH1-B or Cyclin B.

During our characterization of dMCPH1-B degradation, we found that an N-terminally Myc-tagged, but not a C-terminally Myc-tagged, version of dMCPH1-B degraded in *Xenopus* interphase egg extract (Fig. 2.3C), suggesting that the N-terminal Myc-tag might mask a nearby degron. These findings were consistent with a model in which the first 47 amino acids of dMCPH1-B that is not shared with dMCPH1-C contains the relevant degron that mediates degradation by APC^{Cdh1}. To test this possibility, we generated an N-terminal truncation mutant of dMCPH1-B (dMCPH1-$B^{\Delta N}$) in which the first 40 amino acids was deleted. We found that this mutant was stable in Cdh1-supplemented extract, indicating that the N-terminal end of dMCPH1-B contains a degron necessary for APC^{Cdh1}-mediated degradation (Fig. 2.3C).

We identified a putative D-box motif (RRPLHDSN) within the first 40 amino acids of dMCPH1-B and generated a mutant in which the first four amino acids of this sequence were replaced with alanines (dMCPH1-$B^{DboxMut}$). We found that, in contrast to the wild-type protein, dMCPH1-$B^{DboxMut}$ was stable in Cdh1-supplemented extract (Fig. 2.3C). These data indicate the

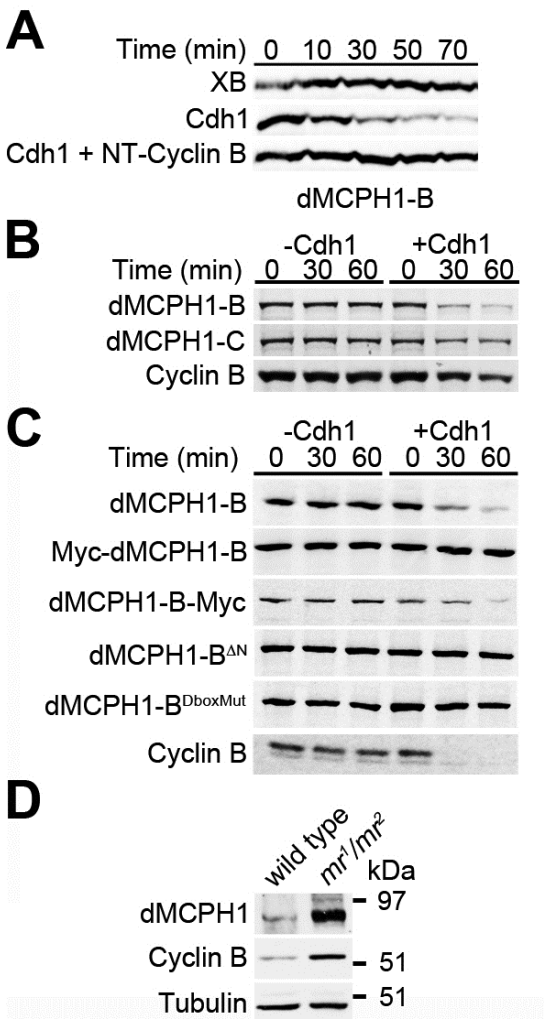

Figure 2.3 dMCPH1-B stability is regulated by APC. (A) dMCPH1 degradation in *Xenopus* egg extract is stimulated by Cdh1. Radiolabeled dMCPH1-B was incubated in *Xenopus* interphase egg extract supplemented with XB (buffer control), Cdh1, or Cdh1 plus an N-terminal Cyclin B peptide (NT-Cyclin B). (B) Degradation of dMCPH1-C in *Xenopus* egg extract. Radiolabeled dMCPH1-B, dMCPH1-C, or Cyclin B was incubated in *Xenopus* interphase egg extract in the absence or presence of Cdh1. See Figure S1B for quantification of gel band intensities. (C) A free N-terminal end of dMCPH1-B, which contains a putative D-box, is required for its Cdh1-stimulated degradation in *Xenopus* egg extract. Wild-type dMCPH1-B and a C-terminally Myc-tagged version (dMCPH1-B-Myc) degraded in *Xenopus* egg extract in the presence of Cdh1. In contrast, an N-terminally Myc-tagged version (Myc-dMCPH1-B), an N-terminal deletion mutant (dMCPH1-$B^{\Delta N}$), or a N-terminal D-box mutant (dMCPH1-$B^{DboxMut}$) failed to degrade in Cdh1-stimulated *Xenopus* egg extract. (D) Immunoblot analysis of dMCPH1, Cyclin B, and alpha-Tubulin levels in lysates derived from embryos (0-1 hour) of APC2 mutant (*mr^1/mr^2*) females indicate that dMCPH1 levels are increased in the mutant embryos compared to wild-type embryos.

D-box sequence found within the N-terminal 40 amino acids of dMCPH1-B mediates its APC^{Cdh1}-dependent degradation.

Mutants of the *Drosophila morula (mr)* gene, which encodes the homolog of the vertebrate APC2 subunit of APC, have increased levels of Cyclin B due to reduced APC activity (Reed & Orr-Weaver 1997). Syncytial embryos laid by females transhetersozygous for mr^1 and mr^2 alleles (mr^1/mr^2) arrest in mitosis shortly after a few cell cycles. dMCPH1-B is primarily expressed in syncytial embryos (Brunk et al 2007, Rickmyre et al 2007). If dMCPH1-B were an APC substrate, we reasoned that its levels should be increased in *morula* mutant flies. To test this possibility, we prepared lysates from 0-1 hour syncytial embryos derived from wild-type or mr^1/mr^2 females and assessed endogenous dMCPH1, Cyclin B (positive control), and alpha-tubulin (loading control) levels by immunoblotting (Fig. 2.3D). Embryos derived from mr^1/mr^2 females had increased levels of both dMCPH1-B and Cyclin B compared to wild-type, suggesting that dMCPH1-B is an APC substrate in vivo. mr^1/mr^2-derived embryos also contain dMCPH1, which exhibits slower mobility on SDS-PAGE (Fig. 2.3D). Because mr^1/mr^2-derived embryos are reported to arrest in mitosis, it is possible that this form of dMCPH1 is the result of mitotic phosphorylation.

dMCPH1-B is ubiquitinated by APC

We next sought to determine whether dMCPH1-B is a direct substrate of APC^{Cdh1} using a purified system as previously described (King et al 1995, Pfleger & Kirschner 2000). The APC was purified from *Xenopus* interphase egg extract by immunoprecipitation using an antibody against the Cdc27 subunit. Purified APC was then used for in vitro ubiquitination reactions containing recombinant human E1, E2 (UbcH10), Cdh1, and ubiquitin. The radiolabeled NT-

Cyclin B peptide (positive control) was polyubiquinated as evidenced by the presence of higher molecular weight laddering on SDS-PAGE (Fig. 2.4A). In contrast, no laddering was detected for firefly luciferase (negative control).

We next tested whether dMCPH1-B was ubiquitinated in our purified system and whether addition of Cdh1 would enhance ubiquitination. We found that ubiquitination of dMCPH1-B and NT-Cyclin B (positive control) was dramatically enhanced in the presence of Cdh1 in our reconstituted ubiquitination system, consistent with dMCPH1-B being an APC^{Cdh1} substrate (Fig. 2.4B). Ubiquitination of the D-box mutant, dMCPH1-$B^{DboxMut}$, was observed in the presence of Cdh1, albeit at a much reduced level (Fig. 2.4B). This phenomenon has been observed with other APC substrates in the purified system (Araki et al 2005, Fang et al 1998b, Pfleger & Kirschner 2000), and the low level of ubiquitination observed likely reflects the fact that the purified system lacks many regulatory proteins present in an extract or cell. To further confirm that the N-terminal end of dMCPH1-B contains a functional D-box, we showed that the first 64 amino acids of dMCPH1-B (dMCPH1-B^{1-64}) was ubiquitinated in the purified system and that ubiquitination was enhanced in the presence of Cdh1 (Fig. 2.4B). These results indicate that dMCPH1-B is a direct substrate of APC^{Cdh1} in vitro and that the N-terminal D-box of dMCPH1-B plays a major role in mediating its ubiquitination by APC.

Steady state-levels of hMCPH1 do not change in a cell cycle-dependent manner in cultured human cells

Human MCPH1 (hMCPH1) contains one N-terminal and two C-terminal BRCT domains and is more similar to dMCPH1-C in organization than dMCPH1-B (Fig. 2.2A).

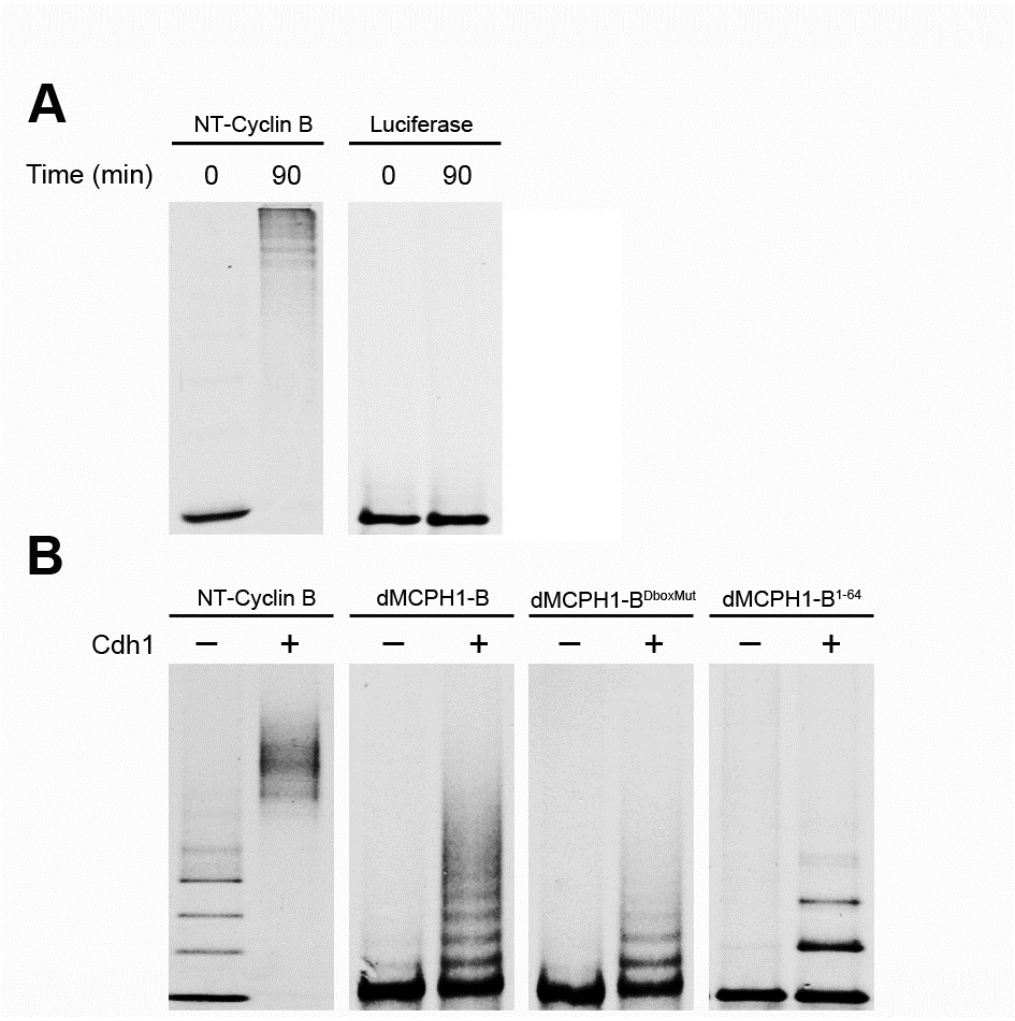

Figure 2.4 dMCPH1-B is ubiquitinated by APC in vitro. (A) Establishment of an in vitro APC ubiquitination assay. Radiolabeled N-terminal peptide of Cyclin B (NT-Cyclin B) and Luciferase protein were incubated in a reaction containing APC purified from *Xenopus* interphase egg extract, purified human E1, His-UbcH10, His-Cdh1, ubiquitin, and an energy regeneration system. Reactions were terminated by addition of sample buffer followed by SDS-PAGE/autoradiography. (B) dMCPH1-B is an in vitro substrate of APC^{Cdh1}, and its ubiquitination is mediated in large part by its N-terminal D-box. Radiolabeled NT-Cyclin B, dMCPH1-B, dMCPH1-$B^{Dboxmut}$, and the N-terminal 64 amino acid fragment of dMCPH1-B (dMCPH1-B^{1-64}) were incubated in the APC ubiquitination assay and reaction products assessed by SDS-PAGE/autoradiography.

Although hMCPH1 lacks an N-terminal degron similar to dMCPH1-B, it contains several putative D-boxes and a candidate KEN box. To determine if hMCPH1 is also degraded via APC^{Cdh1}, radiolabeled hMCPH1 was incubated in *Xenopus* interphase egg extract in the absence or presence of Cdh1. In contrast to dMCPH1-B, hMCPH1 did not degrade in Cdh1-supplemented extract (Fig. 2.5A). The observed doublet is consistent with an alternative translation initiation downstream (35 amino acids) of the canonical start site using the rabbit reticulocyte translation system. It is possible that the incapacity of *Xenopus* interphase egg extract to support hMCPH1 degradation by APC^{Cdh1} is due to differences between the amphibian and human systems.

We next assessed the steady-state levels of hMCPH1 throughout the cell cycle in cultured human cells. HeLa cells were synchronized by nocodazole block and release, and aliquots were taken at two-hour time points in order to assess endogenous levels of hMCPH1, Cyclin B, Cyclin A, p27, and Cdk1 by immunoblotting (Fig. 2.5B). From 2-10 hours after nocodazole release, p27 levels were elevated, and Cyclin A and Cyclin B levels were decreased, consistent with cell-cycle progression into G1. By 10 hours after nocodazole release, p27 levels were decreased, whereas Cyclin A and Cyclin B levels were increased, indicating cell-cycle progression through S, G2, and M-phase. Throughout the time course, hMCPH1 levels remained constant. Taken together, these data suggest that the overall cellular levels of hMCPH1 do not fluctuate in an APC-dependent manner.

Overexpression of hMCPH1 or dMCPH1-B results in cell-cycle defects

Because APC-mediated degradation of substrates is required for cell-cycle progression, we sought to determine if increasing MCPH1 levels would lead to disruption of cell division.

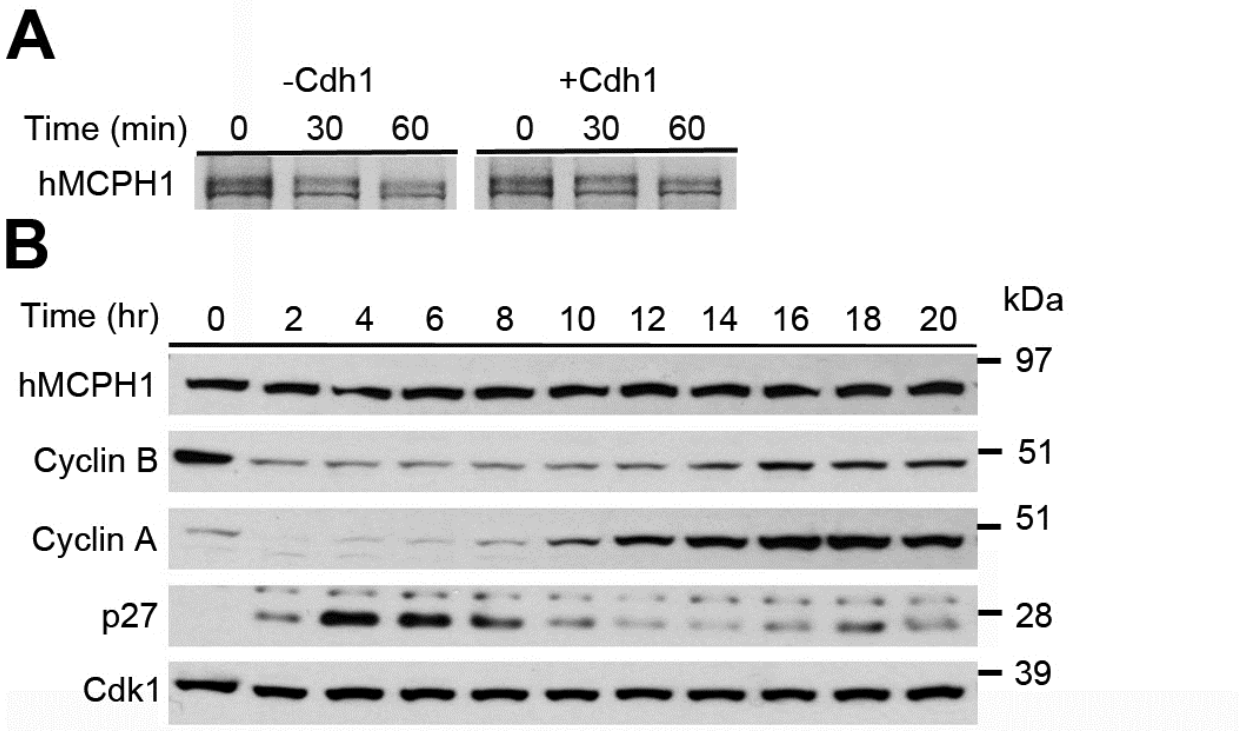

Figure 2.5 APC does not regulate the stability of hMCPH1. (A) Autoradiogram of radiolabeled hMCPH1 incubated in *Xenopus* interphase egg extract in the absence or presence of Cdh1. (C) Levels of hMCPH1 do not notably fluctuate in a cell cycle-dependent manner. Immunoblot analysis of hMCPH1, Cyclin B, Cyclin A, p27, and Cdk1 in lysates derived from synchronized HeLa cells 0-20 hours after nocodazole release. Degradation of Cyclin A and B occurs in prophase and metaphase, respectively, whereas degradation of p27 marks late G1/S.

The *Xenopus* embryo system has been previously used as an in vivo readout of cell cycle progression (Fang et al 1998a, Ivanovska et al 2004, McGarry & Kirschner 1998, Pfleger et al 2001, Rankin et al 2005). An advantage of the *Xenopus* embryo system is that the non-injected cells act as a negative control within the same embryo.

We tested whether injecting mRNAs encoding hMCPH1 or dMCPH1-B into developing *Xenopus* embryos at the 2-4 cell stage would lead to disruption of cell division. Because Cdh1 is absent in the early embryo, levels of injected MCPH1 should not be regulated by APC, leading to inappropriate activity during these early embryonic cell cycles (Lorca et al 1998). Injected embryos were allowed to develop, fixed, and assessed for cell division defects (Fig. 2.6A,B). Mos (a component of cytostatic factor; positive control) is required to maintain metaphase arrest during meiosis II by inhibiting APC activity (Tunquist & Maller 2003). Injection of Mos mRNA resulted in a block in cell division in the injected half of the embryo. Injection of GFP (negative control) had no observable cell cycle effect on the injected cells. In contrast, 92% of embryos injected with hMCPH1 and 67% of those injected with dMCPH1-B exhibited reduced cell number and increased cell size, likely due to cell-cycle arrest. The levels of the human and *Drosophila* MCPH1 proteins expressed in embryos are nearly equivalent as assessed by immunoblotting (Fig. 2.7). Thus, we attribute the difference in potency between hMCPH1 and dMCPH1-B to be due to differences in sequence identity between the insect and vertebrate proteins. Finally, injections of the D-box mutant of dMCPH1-B also result in embryos with cell cycle arrest (Fig. 2.6A,B). As expected, levels of the mutant are comparable to that of the wild-type dMCPH1-B protein (not degraded due to the absence of Cdh1 in the early embryo) (Fig. 2.7).

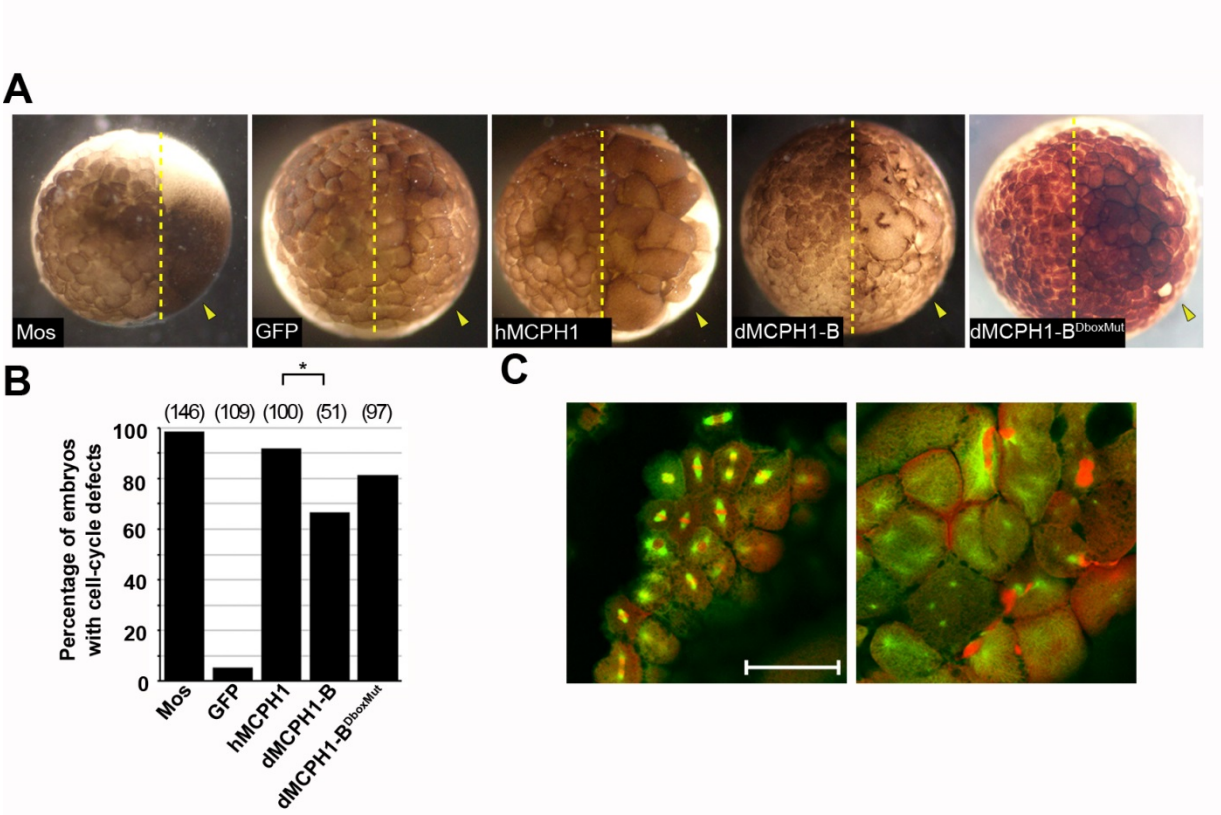

Figure 2.6 Overexpression of dMCPH1-B or hMCPH1 results in cell-cycle defects. (A) Representative images of whole *Xenopus* embryos fixed four hours after injection of Mos, GFP, full-length human MCPH1 (hMCPH1), dMCPH1-B , or dMCPH1-B^{DboxMut} RNA at the 2-4-cell stage. Arrows indicate injected halves of embryos. (B) Quantification of *Xenopus* embryos displaying cell division defects 4 hours post-injection. Total number of embryos injected is indicated in parentheses. *p<0.005 (C) Confocal sections of the uninjected (left) and injected (right) areas of a representative whole embryo following injection with hMCPH1 mRNA. Microtubules, green; DNA, red. Scale bar,100 μm

Embryos injected with hMCPH1 were fixed and stained for tubulin and DNA to further examine the cell-cycle defects associated with hMCPH1 overexpression (Fig. 2.6C). In contrast to the uninjected cells, hMCPH1-injected cells contained abnormal spindle arrangements, free centrosomes, lack of DNA, and/or DNA trapped between daughter blastomeres. These findings are consistent with a previous study in which Sororin, another substrate of APC^{Cdh1}, was overexpressed in *Xenopus* embryos (Rankin et al 2005).

DISCUSSION

In our DIVEC screen for APC substrates in *Xenopus* egg extract, we identified two candidates: the protein encoded by *CG32982*, a previously uncharacterized *Drosophila* gene, and dMCPH1-B, a splice variant of *Drosophila mcph1*, the homologue of a human microcephaly gene. We show that dMCPH1-B undergoes Cdh1-dependent degradation in *Xenopus* egg extract and not Cdc20-dependent degradation. We show that APC-mediated degradation of dMCPH1 is restricted primarily to the splice variant dMCPH1-B, which contains an N-terminal D-box sequence required for Cdh1-mediated degradation. This restriction may allow for tissue- or developmental-specific regulation of dMCPH1 levels during the cell cycle. Consistent with this idea, we show that dMCPH1 levels are up-regulated in syncytial embryos with reduced APC activity (mr^1/mr^2), a developmental stage in which dMCPH1-B is preferentially expressed. The low level of dMCPH1-C degradation may reflect cryptic APC^{Cdh1} site(s) that is recognized in our optimized system. Alternatively, our system may be missing a co-factor required for efficient turnover of dMCPH1-C by APC^{Cdh1} in *Drosophila* embryos that allows for differential regulation of dMCPH1-B and dMCPH1-C by the APC.

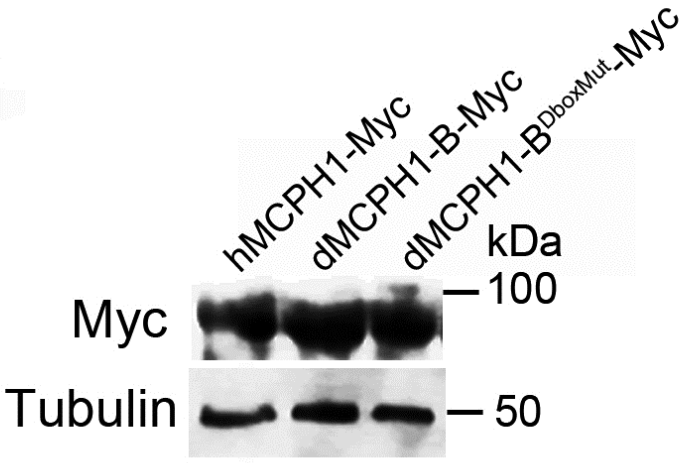

Figure 2.7 *Xenopus* embryos express hMCPH1-Myc, dMCPH1-B-Myc, and dMCPH1-B^{DboxMut}-Myc at similar levels. (A) Immunoblot for Myc and tubulin (loading control) of lysates derived from *Xenopus* embryos after injection with mRNA encoding hMCPH1-Myc, dMCPH1-B-Myc, or dMCPH1-B^{DboxMut}-Myc.

Because dMCPH1-B is preferentially expressed during *Drosophila* syncytial embryogenesis and is down-regulated by the APC, one would predict that dMCPH1-B levels would oscillate throughout the cell cycle during this developmental stage. However, oscillations in total levels of APC substrates, such as mitotic cyclins, are not observed until the later cycles of syncytial embryogenesis (Raff et al 2002). In fact, localized degradation of Cyclin B by the APC is proposed to control cell-cycle progression during these syncytial cycles (Raff et al 2002). Thus, it is not surprising that Brunk et. al (2007) observed no change in total levels of dMCPH1 during the cell cycles of syncytial embryogenesis. It is possible that dMCPH1-B, like Cyclin B, is targeted for degradation in a localized manner.

In vitro ubiquitination assays also revealed that the N-terminal D-box of dMCPH1-B is sufficient for APC^{Cdh1}-mediated ubiquitination. The finding that the N-terminal D-box is also not required for APC^{Cdh1}-mediated ubiquitination suggests that dMCPH1-B contains additional degrons. This finding is not surprising because many APC substrates have been shown to contain multiple APC-targeting motifs (Min & Lindon 2012). Although dMCPH1-B contains multiple predicted D-box motifs, we show that deletion of the N-terminal D-box is sufficient to significantly block its Cdh1-dependent degradation in *Xenopus* interphase egg extract. dMCPH1-C also contains many of these putative D-boxes motifs, as well two motifs in the C-terminal region that are not shared with dMCPH1-B. These motifs potentially mediate the low level of degradation in APC^{Cdh1}-activated *Xenopus* egg extract.

Two isoforms of human MCPH1 produced by alternative splicing have been previously described and are structurally similar to *Drosophila* dMCPH1-B and C (Gavvovidis et al 2012). The full-length form of hMCPH1 (used in the current study) contains an N-terminal and two C-terminal BRCT domains, whereas the short form lacks the C-terminal paired BRCT domain

region. A previous report has shown that the C-terminal paired BRCT domains of full-length hMCPH1 interact with Cdc27, a subunit of the APC, and the authors hypothesized that hMCPH1 is a substrate of the APC or may regulate APC activity (Singh et al 2012). In our current study, however, we were not able to observed changes in bulk steady-state hMCPH1 levels in cultured human cells during the cell cycle.

MCPH1 has been shown to be a rapidly evolving gene that exhibits low sequence similarity between homologs (Ponting & Jackson 2005). Therefore, it is perhaps not surprising that several functions of MCPH1 appear to be species-specific. For example, only hMCPH1 has been shown to regulate condensin II-dependent chromosome condensation (Yamashita et al 2011). Thus, it is possible that APC-dependent regulation of *Drosophila* MCPH1 is not a conserved feature in humans. Alternatively, similar to the situation with Cyclin B in early embryos of *Drosophila*, levels of hMCPH1 may be regulated locally. Alternatively, the activity of hMCPH1 could be regulated via its binding partners/effectors. Indeed, binding partners, SET/Phosphatase Inhibitor 2 and E2F1, are potential or known APC substrates, respectively (Brautigan et al 1990, Budhavarapu et al 2012, Leung et al 2011, Peart et al 2010, Yang et al 2008). Thus, the regulation of these two MCPH1 binding partners by the APC could serve as a mechanism to regulate MCPH1 activity in a cell cycle-dependent manner in vertebrates.

We show herein that overexpression of either hMCPH1 or dMCPH1-B in *Xenopus* embryos, an assay that has been previously used to characterize important cell-cycle regulators, leads to cell-cycle defects (Fang et al 1998a, Ivanovska et al 2004, McGarry & Kirschner 1998, Pfleger et al 2001, Rankin et al 2005). This finding suggests that tight regulation of the levels of MCPH1 may be required for proper cell-cycle progression. Because hMCPH1 is known to negatively regulate mitotic entry and chromosome condensation, the cell-cycle defects we

observe in *Xenopus* embryos overexpressing MCPH1 may be due to misregulation of these processes (Alderton et al 2006, Tibelius et al 2009, Trimborn et al 2006, Yamashita et al 2011). Although MCPH1 has been implicated in many cellular processes, regulation of its activity is not well understood. Future studies to elucidate how the activities and/or levels of MCPH1 are controlled will be important to fully understand how this evolutionarily conserved, highly evolving protein functions in regulating critical processes within the developing organism.

CHAPTER III

rough deal (*rod*) IS REQUIRED FOR POLAR BODY ARREST AND SYNCYTIAL MITOSIS DURING *DROSOPHILA* EMBRYOGENESIS

INTRODUCTION

Cell division is driven by oscillations in the activity of Cdks and their associations with regulatory cyclin subunits. While key events in early mitosis, such as chromosome condensation, nuclear-envelope break down, and spindle assembly, are driven by Cyclin B-Cdk activity, mitotic exit is dependent on mechanisms that inactivate this complex (Sullivan & Morgan 2007). The spindle assembly checkpoint (SAC) regulates the metaphase-anaphase transition by inhibiting the proteolytic degradation of Cyclin B and Securin until all sister chromatids are bi-oriented on the mitotic spindle. When checkpoint signaling is silenced, proteolytic degradation of securin activates the protease separase that promotes sister-chromatid separation and degradation of Cyclin B inhibits Cdk1 activity. In the absence of Cyclin B-Cdk1 activity, mitotic exit is directed by dephosphorylation of its targets.

The SAC consists of a regulatory protein network that is recruited to unattached kinetochores (Jia et al 2013). Kinetochore recruitment of SAC mediators, such as the Mad1-Mad2 complex and BubR1, promotes the assembly of diffusible inhibitors of Cdc20, a coactivator of the Anaphase-promoting complex (APC). When kinetochore-microtubule (MT) attachments are formed, the minus-end directed MT motor dynein-dynactin transports Mad1-Mad2 as well as several additional SAC mediators towards the spindle poles. This “shedding” of SAC mediators allows Cdc20 to activate the APC, which then facilitates the ubiquitin-mediated proteolysis of securin and Cyclin B.

Drosophila melanogaster is an ideal model organism for studying the cell cycle during development (Foe et al 1993, Lee & Orr-Weaver 2003). *Drosophila* achieves rapid embryogenesis by using a streamlined cell cycle that is not dependent on transcription or growth. In the early cell cycles of *Drosophila* embryogenesis, nuclei divide synchronously in a common cytoplasm (syncytium). These divisions are modified cell cycles in which S-phase and M-phase oscillate without gap-phases or cytokinesis. They are also transcriptionally silent and are driven by stockpiles of maternal mRNAs and proteins. During the syncytial cycles, the unfertilized nuclear products of female meiosis, referred to as polar bodies, coalesce to form a "starburst" of condensed DNA at the periphery of the embryo. Polar bodies are arrested in this condensed state until they are culled from the embryo during cellularization.

Defective polar body DNA condensation has been reported in mutants with either reduced Cdk1 activity or misregulated levels of Cyclin B. Embryos derived from females with a temperature-sensitive, inactivating mutation in Cdk1 have decondensed, interphase-like polar bodies when females are exposed to high temperatures (T.T. Su and P.H. O'Farrell, unpublished). Mutant females of the *png* kinase and its regulatory subunits *plutonium* (*plu*) and *giant nuclei* (*gnu*) also produce syncytial embryos with decondensed polar bodies (Fenger et al 2000, Lee et al 2001). The PAN GU (PNG) kinase complex promotes the translation of Cyclin B during female meiosis and syncytial embryogenesis (Vardy & Orr-Weaver 2007). Due to low levels of Cyclin B in *png*, *plu*, and *gnu*-derived embryos, DNA replication occurs in the absence of mitosis, resulting in polyploid interphase-like polar bodies. Additionally, *mps1* and *bubR1* mutant females produce embryos with similar polar body defects (Fischer et al 2004, Perez-Mongiovi et al 2005). *mps1* and *bubR1* are SAC mediators of SAC activation. Because the SAC

indirectly regulates the proteolytic degradation of Cyclin B, it is another proposed mechanism by which polar body condensation is maintained.

In a screen for regulators of *Drosophila* syncytial embryogenesis, we identified a novel allele of *rough deal* (*rod*). Rod is a 240 kDa subunit of the Rod-Zw10-Zwilch (RZZ) complex, which is required for SAC activation in metazoans. RZZ is required for the recruitment of Mad1-Mad2 and dynein-dynactin to unattached kinetochores. Previous studies of *Drosophila rod*-null alleles identified its requirement for accurate chromosome segregation during the mitotic divisions of larval neuroblasts and the meiotic divisions of spermatogenesis (Karess & Glover 1989, Scaerou et al 1999). We report the identification of rod^{Z3}, the first non-null mutant allele of an RZZ subunit. rod^{Z3} is maternal-effect lethal and encodes a G-E substitution in the Rod C-terminus that does not perturb the levels of RZZ subunits or RZZ complex formation. rod^{Z3}-derived embryos do not maintain condensed polar bodies and have severe mitotic defects. Additionally, the RZZ complex is not recruited to polar body or metaphase kinetochores in these embryos, which results in SAC inactivation. This allele is a valuable tool in establishing a required role for the RZZ complex in *Drosophila* syncytial embryogenesis and the role of the Rod C-terminus in mediating kinetochore recruitment.

MATERIALS AND METHODS

Drosophila Stocks

Flies were maintained at 25°C using standard techniques. *y w* was used as wild type for embryo experiments. The Zuker stock designation has been shortened and superscripted to indicate that Z3-0733 is an allele of *rod* (e.g. Z3-0733 becomes rod^{Z3}). *bw*; rod^{Z3} *st* / *TM6B* and 2XCyclin B stocks were gifts from Charles Zuker (Columbia University) and Christian Lehner

(University of Zurich) respectively. *rod*$^{+tC3L9}$ was generated as previously described (Scaerou et al 1999) and has a transgene insertion of 34 kb of *Drosophila* genome containing the full *rod* gene. *mad2*^{P} contains a P-element insertion near the beginning of exon 3 and does not produce detectable Mad2 protein (Buffin et al 2007). [GFP-Zw10, RFP-Spc25] and [GFP-Zw10, RFP-Spc25]; *rod*^{Z3} stocks were gifts from Roger Karess (University of Paris).The *shtd*^{3} stock was obtained from the Bloomington stock center.

Embryo Fixation and Colchicine Treatment

0-2 hour embryos (unless otherwise noted) were collected from females as previously described (Rothwell & Sullivan 2000). For Tubulin, Centrosomin, and/or DNA staining embryos were dechorionated in 50% bleach, and then fixed and devitellinized by shaking in a 1:1 mixture of methanol and heptane. For visualizing RFP/GFP fluorescence during cortical divisions, 0-30 minute embryos were collected, aged for 1 hour and 15 minutes, dechorionated in 50% bleach, and fixed/devitellinized in methanol/heptane. For pH3 immunostaining, embryos were dechorionated in 50% bleach, fixed in a 1:1 mixture of 4% formaldehyde (in PBS) and heptane, and devitellinized as described above. For colchicine treatment, 0-1 hour embryos were collected, dechorionated, and incubated in 1:1 mixture of 250 μM colchicine (in PBS) and heptane for 30 minutes. Treated embryos were then formaldehyde fixed and devitellinized as described above.

Embryo Immunofluorescence and Microscopy

Fixed embryos were rehydrated in PBS and incubated in 1 mg/ml RNAse A for 1 hour. Embryos were then incubated in primary antibody overnight at 4°C, washed, and then incubated

in Cy2 and/or Cy5 conjugated secondary antibodies (Jackson ImmunoResearch) for 3 hours at room temperature. Primary antibodies used were anti-alpha-Tubulin (YL1/2, AbD Serotec), anti-Centrosomin (gift from W.Theurkauf, University of Massachusetts Medical School), and anti-pH3 (06-570, Millipore). Embryos were either stained with propidium iodide and mounted in clearing solution (Fenger et al 2000) or mounted in Prolong-Gold with DAPI (Life Technologies). Embryos were visualized with a Nikon Eclipse 80i microscope equipped with a CoolSNAP ES camera (Photometrics). Statistical analysis of all imaging quantifications was performed using the Fisher Exact test.

Embryo Immunoblotting

0-1 hour embryos were homogenized in non-denaturing lysis buffer (50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100). Lysates were analyzed by SDS-PAGE and immunoblotting using standard techniques. Primary antibodies used were anti-Tubulin (DM1α), anti-Rod (Scaerou et al 1999), anti-Zw10, and anti-Zwilch (gifts from M.Goldberg, Cornell University). HRP-conjugated secondary antibodies and chemiluminescence were used to detect primary antibodies.

For immunoprecipitations, 500 μg of embryo lysate was incubated with 5 μl anti-Rod serum (Scaerou et al 1999) or normal rabbit serum overnight at 4°C. Lysates were then incubated with 25 μl of Protein G Sepharose beads (GE Healthcare) for 3 hours at 4°C. Beads were pelleted, washed three times in non-denaturing lysis buffer, and boiled in 30 μl 6X Sample Buffer (300 mM Tris pH 6.8, 12% w/v SDS, 30% w/v glycerol, 600 mM DTT, and 0.01% w/v bromophenol blue). The resulting supernatant and 20 μg of input lysate were analyzed by SDS-PAGE and by immunoblotting.

For sucrose density gradient analysis, 2 mg of embryo lysate was layered on top of a sucrose gradient column of 5%, 10%, 20%, and 30% sucrose. Columns were then centrifuged in a L8-70M ultracentrifuge (Beckman) with a SW55Ti rotor for 4 hours at 46,000 rpm and 4°C. 19 fractions were collected and 8% of each fraction was analyzed by SDS-PAGE and immunoblotting.

Quantification of Egg Hatch Rates

Eggs were collected from females daily over five days, aged ~40 hours post-collection at 25°C, and then scored for hatching. The number of hatched embryos was determined by subtracting the number of unhatched (intact) embryos from the total number collected. Hatch rate is the ratio of hatched to total embryos expressed as a percentage.

RESULTS

***rod*^{Z3}-derived embryos do not maintain polar body condensation**

In an effort to identify new genes required for the S-M cycles in syncytial embryogenesis, we previously screened a large maternal-effect lethal collection generated in the laboratory of Charles Zuker (Koundakjian et al 2004). From this collection, we previously identified two novel cell-cycle regulators, *mcph1* and *nopo*, as well as novel alleles of *gnu*, *wee1*, *grapes*, *telomere fusion*, and *aurora* (Lee et al 2003, Merkle et al 2009, Rickmyre et al 2007). Of these mutants, only embryos derived from *gnu* females exhibited the large, polyploid interphase nuclei phenotype. We now report the identification of a novel allele of *rod* from this collection, and embryos derived from these mutant females contain polar bodies exhibiting a similar phenotype.

Syncytial embryos derived from wild type (WT) females contain a starburst configuration of condensed polar body DNA at the anterior dorsal surface. Those derived from

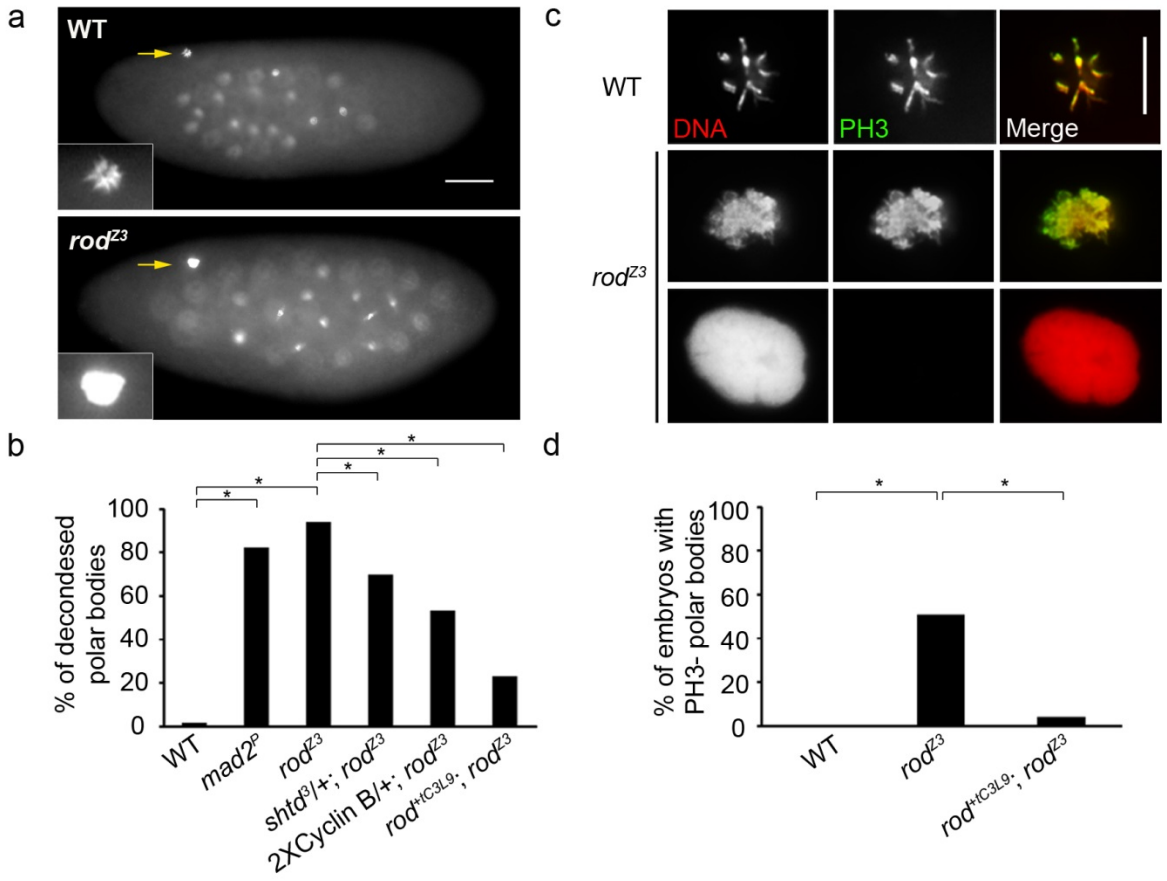

Figure 3.1 *rough deal* (*rod*) is required for maintenance of polar body DNA condensation (a) Representative images of DNA-stained syncytial embryos from wild-type (WT) and rod^{Z3} females. Scale bar, 50 µm. Insets, polar bodies marked by arrows (b) Quantification of percent of decondensed polar bodies in syncytial embryos derived from females of the stated genotypes. *p<0.0001. N=140 polar bodies (except 2XCyclin B/+; rod^{Z3}, N=60). (c) Representative images of polar bodies from WT, rod^{Z3}, and rescue-derived syncytial embryos stained for phosphorylated Histone 3 (pH3) and DNA. Exposure time for the DNA channel was optimized for each polar body. Scale bar, 10 µm. (d) Quantification of the percentage of syncytial embryos with PH3 negative polar bodies. *p<0.0001. N=60 embryos.

rod^{Z3} females, however, contain large, interphase-like polar bodies with decondensed DNA (Fig 3.1a, c). The increased size of these polar bodies and intensity of their DNA stain compared to WT suggest an increase in DNA content, possibly due to aberrant DNA replication. While decondensed polar bodies a rarely found in WT-derived embryos, the majority (94%) of polar bodies in *rod*^{Z3}-derived embryos are decondensed (Fig. 3.1b). Additionally, expression of the *rod*$^{+tC3L9}$ transgene, which contains the full *rod* genomic region, in the *rod*^{Z3} background rescues these polar body defects (Fig 3.1b).

We also determined if polar bodies in *rod*^{Z3}-derived embryos contained phosphorylated Histone H3 (pH3), a marker of chromosome condensation. Although all
WT-derived embryos have pH3 positive polar bodies, confirming results from previous studies (Fischer et al 2004, Perez-Mongiovi et al 2005), roughly half of *rod*^{Z3}-derived embryos do not (Fig 3.1c,d). *rod*^{Z3}-derived embryos have two populations of decondensed polar bodies: those that are pH3 positive with irregularly-shaped and partially decondensed DNA and those that are pH3 negative with rounded, interphase-like DNA. These results suggest DNA decondensation is not always complete in *rod*^{Z3} polar bodies.

So far, decondensed polar bodies have been reported in mutants of two other genes required for SAC activation, *mps1* and *bubR1* (Fischer et al 2004, Perez-Mongiovi et al 2005). *rod* is the third SAC gene identified that is associated with this phenotype. Because Mps1, BubR1, and the RZZ complex have SAC-independent functions as well (Althoff et al 2012, Ditchfield et al 2003, Hewitt et al 2010, Jelluma et al 2008, Jones et al 2005, Lampson & Kapoor 2005, Maure et al 2007, Santaguida et al 2010, Sliedrecht et al 2010, Starr et al 1998, Wainman et al 2012), it is not clear if polar body decondensation is regulated by SAC activation specifically.

Although *Drosophila mad2*-null mutants have an inactive SAC, they do not exhibit the mitotic defects previously reported for mutants of other SAC genes (Buffin et al 2007). Because of this finding, it has been proposed that the SAC is not required for mitosis in *Drosophila* and that Mad2 solely functions to promote SAC activation. To better understand the role of SAC activation in polar body condensation, we also examined embryos derived from *mad2*-null ($mad2^{P}$) females for polar body defects. Similar to those of rod^{Z3}, 82% of polar bodies in $mad2^{P}$-derived embryos were large and interphase-like with semi- or fully decondensed DNA (Fig 3.1b). This result supports the hypothesis that SAC activation is required to maintain polar body condensation.

It has been speculated that the SAC maintains polar body M-phase through inhibition of the APC and consequent stabilization of Cyclin B (Fischer et al 2004, Perez-Mongiovi et al 2005); however, direct evidence is lacking. To determine if increasing Cyclin B levels via reduction of APC activity suppresses the polar body defect of rod^{Z3} mutants, we generated rod^{Z3} mutants carrying one copy of a lethal allele of the APC subunit *APC1* (*shattered* or *shtd* in *Drosophila*). Additionally, to determine if directly increasing Cyclin B levels suppresses the polar body defect, we introduced two additional copies of the *Cyclin B* gene into the rod^{Z3} mutant background. 68% and 53% of $shtd^{3}$*/+;* rod^{Z3} and *2xCyclin B/+*; rod^{Z3}-derived embryos, respectively, have decondensed polar bodies (Fig 3.1b). This suppression of polar body decondensation in rod^{Z3}-derived embryos further supports the hypothesis that *rod* is required to maintain polar body condensation by promoting the stabilization of Cyclin B.

rod^{Z3} is a novel maternal-effect lethal allele of *rod*

We confirmed that rod^{Z3} females are completely sterile. While 94% of embryos derived from WT females hatch into larvae, none hatch from rod^{Z3} females (Fig. 3.2a). Expression of the rod^{+tC3L9} transgene in rod^{Z3}-derived embryos rescues this reduced hatch rate (Fig. 3.2a), indicating that rod^{Z3} sterility is due to a lack of Rod function.

Sequencing analysis of rod^{Z3} genomic DNA revealed a point mutation in *rod* that is predicted to substitute a glutamic acid for glycine near the Rod C-terminus (amino acid 1973.) *Drosophila* Rod is a large 240 kDa protein (2089 amino acids) with a predicted N-terminal β-propeller region and a series of α-helices extending from the β-propeller to the C-terminus, similar to the reported structure of human Rod (Civril et al 2010). Though glycine 1973 is not conserved in mice and humans, it is conserved among *Drosophila* species. Previous studies of the human RZZ complex have shown that the central domain of Rod (amino acids 715-1485) is required for interaction with Zw10 and the N-terminal domain (amino acids 1-350) is required for interaction with Zwilch (Civril et al 2010, Scaerou et al 2001). However, no functional or interaction domains have been identified in the Rod C-terminus. Thus, characterization of rod^{Z3} provides a unique opportunity to examine how the Rod C-terminus contributes to RZZ function.

Immunoblotting revealed that levels of Rod, Zw10, and Zwilch in rod^{Z3}-derived embryos are similar to those in WT embryos (Fig. 3.2c,d). Lysates from WT and rod^{Z3}-derived syncytial embryos were also fractionated by sucrose density gradient. Immunoblotting of these fractions for Rod revealed that the rod^{Z3} mutation does not affect Rod's capacity to form high molecular weight complexes (Fig. 3.3a). Furthermore, Zw10 and Zwilch co-immunoprecipitate with Rod similarly in WT and rod^{Z3}-derived syncytial embryos (Fig. 3.3b,c), which indicates that the mutation does not affect the capacity of Rod to associate with these RZZ subunits. Taken

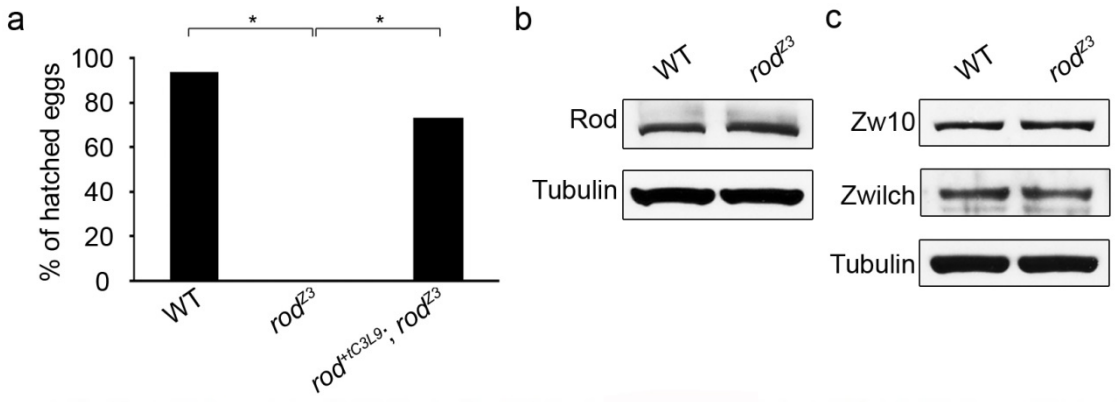

Figure 3.2 *rod*^{Z3} is a maternal-effect lethal allele of *rod* (a) Quantification of the percentage of hatched eggs from WT, *rod*^{Z3}, and rescue females. **p*<0.0001. N=900 eggs. (b) Immunoblot for Rod and Tubulin (loading control) of lysates from WT and *rod*^{Z3}–derived syncytial embryos. (c) Immunoblot for Zw10, Zwilch and Tubulin (loading control) of lysates from WT and *rod*^{Z3}–derived syncytial embryos.

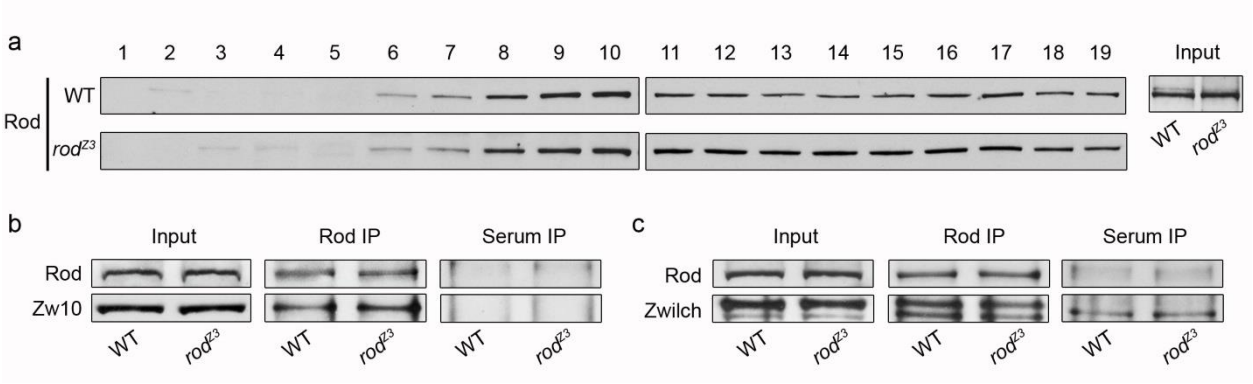

Figure 3.3 The RZZ complex is intact in *rod*^{Z3}–derived embryos. (a) Rod from WT and *rod*^{Z3}–derived syncytial embryos fractionates similarly through a sucrose density gradient. Immunoblot for Rod of sucrose density gradient fractionations and Input lysate. (b, c) Zw10 (b) and Zwilch (c) immunoprecipitate with Rod from lysates of WT and *rod*^{Z3}–derived syncytial embryos. Immunoblots for Rod (b, c), Zw10 (b), and Zwilch (c) of immunoprecipitations using normal rabbit serum (control) and anti-Rod rabbit serum and Input lysates. The higher molecular weight band in the Zwilch blots corresponds to Zwilch, while the lower band is unrelated.

together, these data suggest that the *rod*^{Z3} mutation does not affect RZZ steady state levels or complex formation.

rod^{Z3}–derived embryos undergo aberrant syncytial mitosis

To determine when developmental arrest occurs, we examined the capacity of *rod*^{Z3}-derived embryos to reach gastrulation. The morphology of 3-5 hour WT and *rod*^{Z3}-derived embryos was assessed by DNA staining and microscopic analysis. The majority (73%) of WT-derived embryos undergo gastrulation, while the remaining embryos undergo cellularization (22%) and syncytial cycling (4%) (Fig. 3.4a). However, *rod*^{Z3}-derived embryos arrest during cellularization (43%) and the syncytial cycles (57%) with fragmented and pyknotic DNA (Fig. 3a).

The syncytial divisions were further assessed in 0-2 hour embryos. Roughly half of DNA-stained *rod*^{Z3}-derived syncytial embryos contained unevenly spaced, asynchronously dividing nuclei (Fig 3.4b,c). Similarly, roughly half of *rod*^{Z3}-derived syncytial embryos contain 50% or more prophase and metaphase figures with abnormal centrosome attachments (Fig. 3.4d,e,f). Aberrant metaphase spindles in *rod*^{Z3}-derived embryos are typically bipolar with unfocused poles or multipolar (Fig. 3.4e). Additionally, the percentage of *rod*^{Z3}-derived embryos with asynchronous or aberrant mitoses is rescued in the presence of the *rod*$^{+tC3L9}$ transgene (Fig. 3.4b,f). These results suggest that *rod* is required for proper progression of the syncytial divisions.

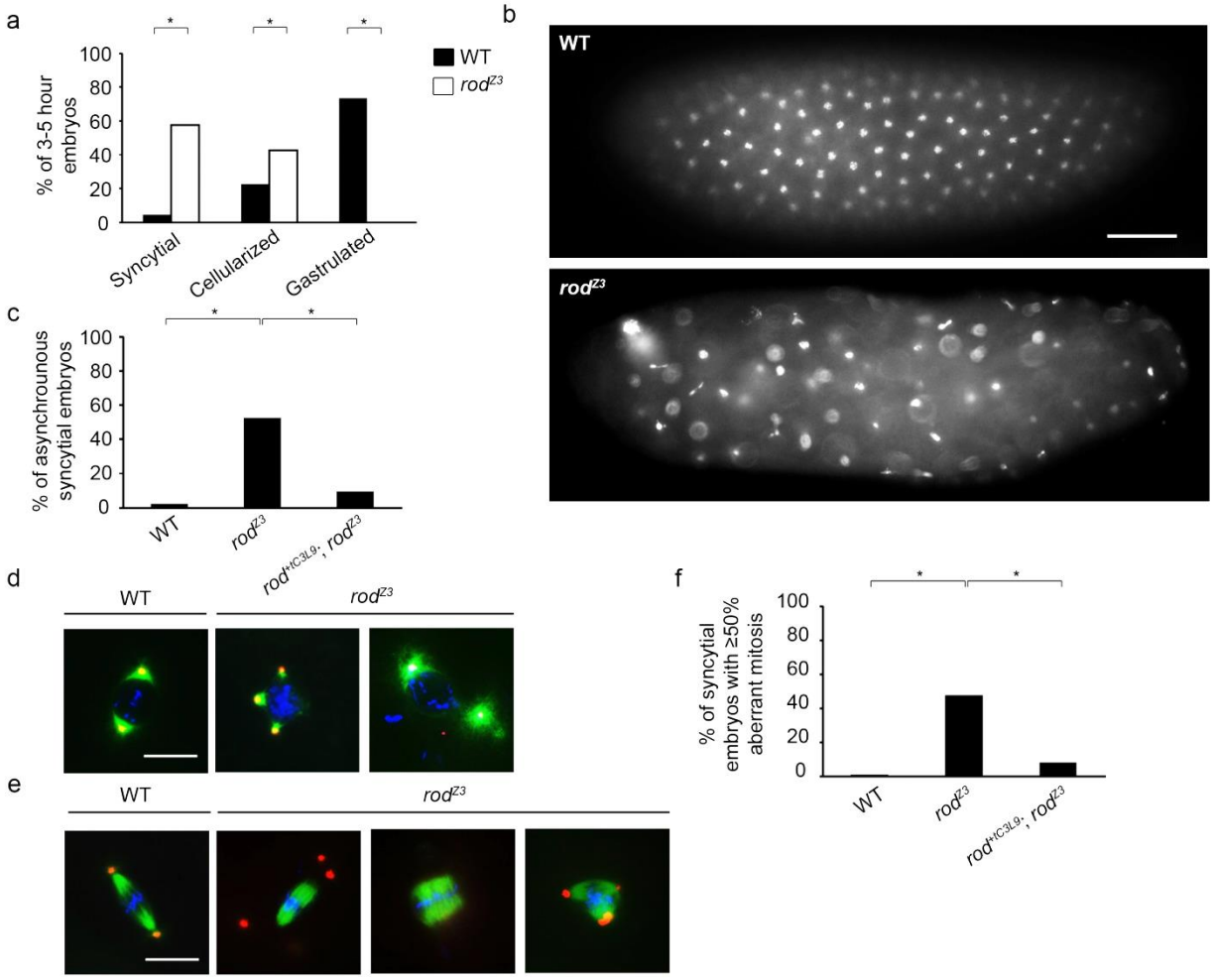

Figure 3.4 *rod*^{Z3}–derived embryos are defective in syncytial mitosis. (a) 3-5 hour *rod*^{Z3}–derived embryos are arrested during the syncytial divisions and cellularization and do not gastrulate. Quantification of the percentage of 3-5 hour WT and *rod*^{Z3}–derived embryos that are syncytial, cellularized, or gastrulated. **p*<0.0001. N=200 embryos. (b) Representative images of DNA-stained (synchronous) WT and (asynchronous) *rod*^{Z3}–derived syncytial embryos. Scale bar, 50 μm. (c) Quantification of the percentage of asynchronous syncytial embryos from WT, *rod*^{Z3}, and rescue females. **p*<0.0001. N=90 embryos. (d,e) Representative images of (d) prophase and (e) metaphase from WT and *rod*^{Z3}–derived syncytial embryos stained for alpha-tubulin (green), centrosomin (red), and DNA (blue). Scale bar, 10 μm. (f) Quantification of the percentage of syncytial embryos with greater than or equal to 50% aberrant mitoses from WT, *rod*^{Z3}, and rescue females. **p*<0.0001. N=100 embryos.

GFP-Zw10 does not localize to kinetochores in *rod*^{Z3}–derived embryos

We next examined the localization of the RZZ complex in WT and *rod*^{Z3}-derived syncytial embryos. WT embryos expressing GFP-Zw10 and RFP-Spc25 (an outer kinetochore marker) contain GFP-Zw10 foci at polar body and metaphase kinetochores (Fig. 3.5a,b). GFP-Zw10 is also found on metaphase spindle fibers (Fig. 3.5b), consistent with previous reports of RZZ pole-ward shedding from metaphase kinetochores (Buffin et al 2007). Although GFP-Zw10 is expressed at similar levels in WT and *rod*^{Z3} females, GFP-Zw10 does not localize to polar body or metaphase kinetochores, or spindle fibers in *rod*^{Z3}-derived embryos (Fig. 3.5a,b). In contrast to the irregularly-shaped, semi-decondensed polar bodies of *rod*^{Z3}-derived embryos, the rounded and interphase-like polar bodies lack both RFP-Spc25 and GFP-Zw10 foci (Fig 3.5a), suggesting that these polar bodies do not have fully-assembled kinetochores. Taken together, these data suggest that the *rod*^{Z3} mutation affects either kinetochore recruitment or turnover of the RZZ complex, such that it does not accumulate at or shed from kinetochores.

The SAC is inactive in *rod*^{Z3}–derived embryos

To determine if *rod*^{Z3}–derived syncytial embryos have the capacity to activate the SAC in response to spindle damage, we quantified the percentage of untreated and colchicine-treated syncytial embryos with at least 50% pH3 positive, mitotic nuclei (hereafter referred to as mitotic embryos). The percentage of mitotic WT-derived embryos dramatically increases upon colchicine treatment (Fig 3.6). However, this dramatic increase in mitotic embryos after colchicine treatment is not seen in *mad2*^{P} (negative control) and *rod*^{Z3}-derived embryos (Fig. 3.6). Furthermore, the percentage of mitotic embryos after colchicine treatment of *rod*^{Z3}-derived

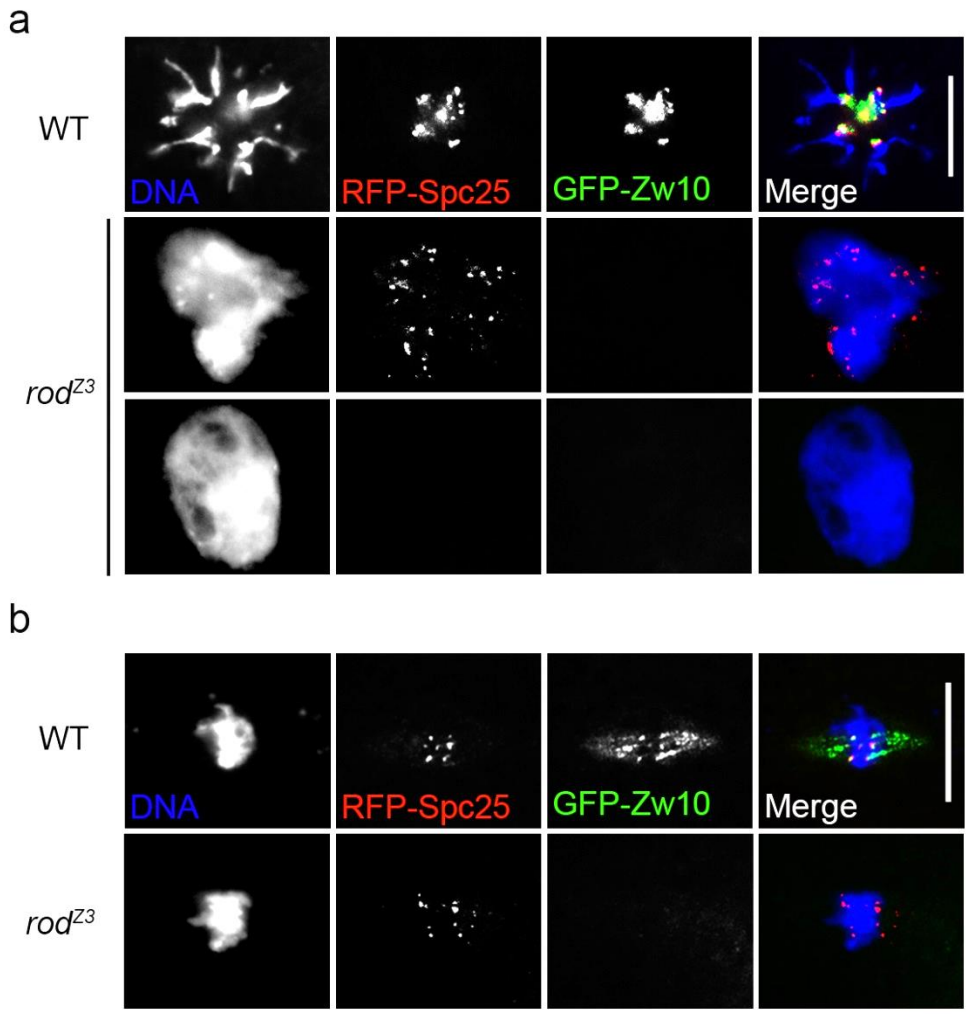

Figure 3.5 GFP-Zw10 does not localize to polar bodies and syncytial kinetochores in *rod*^{Z3}–derived embryos. (a) GFP-Zw10 does not localize to polar bodies in *rod*^{Z3}–derived embryos. Polar bodies from fixed WT and *rod*^{Z3}–derived syncytial embryos expressing RFP-Spc25 (Red), GFP-Zw10 (green) and stained for DNA (blue). Scale bar, 10 μm. $p<0.0001$. N=12 polar bodies. (b) GFP-Zw10 does not localize to metaphase kinetochores or spindle fibers in *rod*^{Z3}–derived embryos. Metaphase nuclei from fixed WT and *rod*^{Z3}–derived syncytial embryos expressing RFP-Spc25 (Red), GFP-Zw10 (green) and stained for DNA (blue). Scale bar, 10 μm. $p<0.0001$. N=30 metaphases, from 12 embryos.

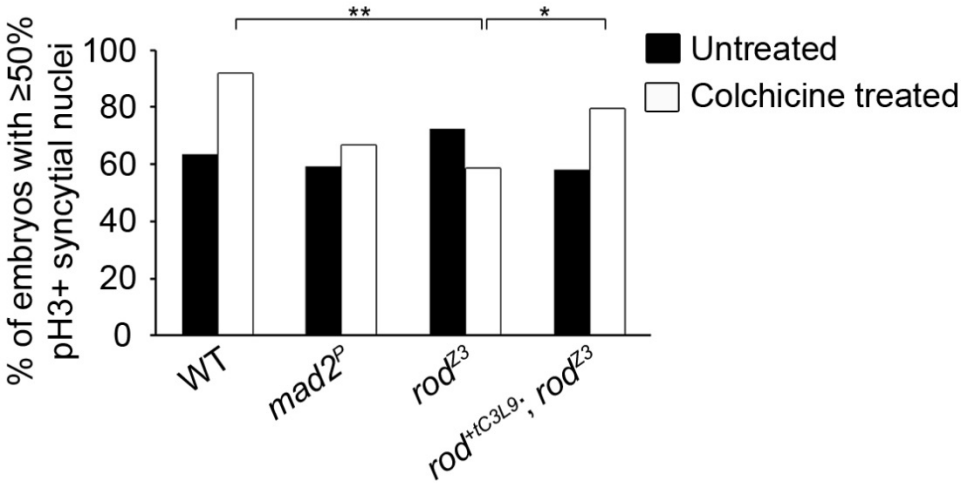

Figure 3.6 SAC is inactive in *rod*^{Z3}–derived embryos. Quantification of the percentage of syncytial embryos with greater than 50% PH3 positive (mitotic) syncytial nuclei. ***p*<0.0001, **p*<0.005. N=80 embryos, except rescue-derived embryos (N=140).

embryos significantly increases in the presence of the rod^{+tC3L9} transgene (Fig. 3.6). These results suggest that WT-derived embryos activate the SAC in response to spindle damage, leading to mitotic arrest, while $mad2^{P}$ and rod^{Z3}-derived embryos do not. Additionally, SAC activation in rod^{Z3}-derived embryos is rescued in the presence of the rod^{+tC3L9} transgene.

DISCUSSION

The SAC is required to maintain polar body condensation in *Drosophila*

Previous studies have shown that Mps1 and BubR1, proteins which promote SAC activation at mitotic kinetochores, localize to *Drosophila* polar body kinetochores as well and are required to maintain polar body condensation during *Drosophila* embryogenesis (Fischer et al 2004, Perez-Mongiovi et al 2005). We show that the RZZ complex has similar polar body localization and is also required to maintain polar body condensation. rod^{Z3}-derived embryos contain large interphase-like polar bodies, similar to those reported in *mps1* and *bubR1* mutants. Live imaging of $bubR1^{Rev1}$-derived embryos revealed that their polar bodies cycle between periods of condensation and decondensation (Perez-Mongiovi et al 2005). Because the polar body condensation state correlated with the cell-cycles of neighboring nuclei, it was suggested that these polar body cycles are under the control of the embryonic mitotic oscillator. Because polar bodies in rod^{Z3} embryos are not always fully-decondensed, it is possible that they undergo similar cycles in DNA condensation.

Furthermore, we show that directly or indirectly increasing Cyclin B levels in rod^{Z3}-derived embryos suppresses polar body condensation defects. This suggests that *rod* maintains polar body condensation by maintaining levels of Cyclin B. Overexpression of Cyclin B also suppresses polar body condensation defects in *png*-derived embryos (Lee et al 2001). However,

rod and *png* regulate Cyclin B levels through different mechanisms. During embryogenesis, the PNG kinase complex promotes the translation of Cyclin B by regulating the polyadenylation of its mRNA and antagonizing the translational repressor PUMILIO (PUM) (Vardy & Orr-Weaver 2007). While Rod, on the other hand, regulates the recruitment of factors that promote activation of the SAC, which when activated, inhibits the proteolytic degradation of Cyclin B. Although previous studies have shown that Cdk1 and Cyclin B activity are required for polar body condensation, we further establish that polar bodies maintain Cdk1-Cyclin B activity through activation of the SAC.

The Rod C-terminus mediates kinetochore recruitment of *Drosophila* RZZ

The KNL1-Mis12-Ndc80 (KMN) network is a conserved core component of the kinetochore which is required for MT-binding and SAC activation (Foley & Kapoor 2013). The KNL1 subunit is a large protein scaffold that is required to recruit SAC signaling proteins such as Bub1, Bub3, BubR1, and the RZZ complex to unattached kinetochores in multiple systems. In vertebrates, KNL1 recruitment of RZZ is mediated by the interaction of Zw10-interacting protein1 (Zwint1) with Zw10 and KNL1 (Kops et al 2005). Zwint1 and KNL1 are also both required for RZZ kinetochore recruitment. However, a Zwint1 homolog does not exist in *C. elegans* and *Drosophila* (Essex et al 2009, Karess 2005). Although KNL1 is required for RZZ recruitment in *C. elegans*, it is not known if this recruitment is mediated indirectly via another protein or by direct KNL1-RZZ interactions. In *Drosophila*, KNL1 is also required for SAC activation and recruitment of BubR1 and Mad2, suggesting that a role in recruiting RZZ may be conserved in this system as well (Feijao et al 2013). Although the mechanism is unknown, it is

likely that the *Drosophila* RZZ is recruited to kinetochores by an interaction with another kinetochore protein such as KNL1 or one of its binding partners.

Our studies indicate that RZZ is not recruited to kinetochores in rod^{Z3}-derived embryos. Because the levels of RZZ subunits and RZZ complex formation are unperturbed in these embryos, the G1973E mutation encoded by rod^{Z3} likely disrupts RZZ kinetochore recruitment specifically. It is possible that this mutation disrupts the conformation of the RZZ complex such that recruitment mediated by any of subunits or by the complex as a whole is disrupted. Alternatively, this mutation may inhibit a specific interaction between the Rod C-terminus and a binding partner at the kinetochore. rod^{Z3} will be a valuable tool in further elucidating the mechanism of RZZ recruitment to kinetochores in *Drosophila.*

Previous studies in *Drosophila* have shown that RZZ recruitment to unattached kinetochores is required for recruitment of Mad1-Mad2 and dynein-dynactin (Buffin et al 2005, Starr et al 1998). Because RZZ is not recruited to kinetochores in rod^{Z3}-derived embryos, we predict that kinetochore Mad1-Mad2 and dynein-dynactin are also not present. We also predict that SAC inactivation in rod^{Z3}-derived embryos is due to a lack of kinetochore Mad1-Mad2. Additional studies are required, however, to confirm these predictions.

rod^{Z3} sterility is due to mitotic defects that are independent of the SAC

rod^{Z3}-derived embryos undergo aberrant syncytial mitosis and a developmental arrest prior to gastrulation. These embryos contain asynchronously dividing nuclei with centrosome attachment defects during mitosis**.** Defects in mitotic spindle function during syncytial embryogenesis can uncouple the nuclear and centrosomes cycles (Archambault & Pinson 2010). The resulting replication of detached centrosomes and fusion of free centrosomes to neighboring

mitoses leads to aberrant mitotic figures and failure to segregate chromosomes. Furthermore, centrosome detachment during syncytial embryogenesis inhibits the nuclear positioning and migration required for morphogenesis. It is possible that *rod*^{Z3}-derived embryos undergo developmental arrest for this reason.

Because the SAC is not required for mitosis in *Drosophila*, mitotic defects which result from the loss of SAC signaling protein function is attributed to the loss of their SAC independent functions (Buffin et al 2007). The mitotic defects of *rod*^{Z3}-derived embryos are therefore likely due to SAC independent functions of the RZZ complex or the proteins it recruits (i.e. Mad1 or dynein-dynactin). In addition to an inactive SAC response, *Drosophila mad1*-null larval neuroblasts have increased lagging chromosomes at anaphase due to persistent merotelic kinetochore-MT (K-MT) attachments (Emre et al 2011). This suggests that Mad1 functions independently of the SAC to promote proper K-MT attachments. In addition to transporting SAC mediators toward the spindle poles once K-MT attachments are made, kinetochore dynein-dynactin also promotes chromosome congression and tension at kinetochores that stabilizes K-MT attachments (Yang et al 2007). If defective RZZ recruitment in *rod*^{Z3}-derived embryos results in loss of kinetochore Mad1 or dynein-dynactin as well, then it is possible their secondary mitotic defects may result from loss of Mad1 or dynein-dependent regulation of k-MT attachments.

Future studies will be important to further elucidate how the Rod C-terminus affects RZZ recruitment to kinetochores and what SAC independent function of Rod is required for proper cell-cycle progression in *Drosophila* syncytial embryogenesis.

CHAPTER IV

CONCLUDING REMARKS

SUMMARY

In a biochemical screen for substrates of the APC, we identified a novel candidate substrates of APC^{Cdh1}: *Drosophila* MCPH1-B. *Drosophila MCPH1* is the homolog of human *Microcephalin*, the first causative gene identified for autosomal recessive primary microcephaly (MCPH). Two isoforms of *Drosophila* and human MCPH1 are produced by alternative splicing. In both organisms, the long isoform contains two C-terminal BRCT domains in addition to one near the N-terminus, while the short isoform does not. We determined that only the short isoform of *Drosophila* MCPH1 (dMCPH1-B) is robustly targeted for Cdh1-dependent degradation in *Xenopus* interphase egg extract. This degradation is mediated by an N-terminal D-box that is not present in the long isoform (dMCPH1-C). Additionally, dMCPH1 accumulates in early embryos with reduced APC activity, suggesting that it is an APC substrate in vivo. An N-terminal peptide of dMCPH1-B containing the D-box is sufficient for in vitro ubiquitination by APC^{Cdh1} and the D-box is required for robust in vitro ubiquitination of full length dMCPH1-B.

Because full-length human MCPH1 (hMCPH1) was reported to interact with the APC, we also examined its stability in our *Xenopus* egg extract system. hMCPH1 does not undergo Cdh1-dependent degradation in *Xenopus* egg extract and its steady-state levels do not oscillate during the cell cycle in human cultured cells. These results suggest that, unlike its *Drosophila* homolog, stability of hMCPH1 is not regulated by APC^{Cdh1}. Furthermore, injection of mRNA

encoding either hMCPH1 or dMCPH1-B into *Xenopus* embryos results in cell-division defects, which suggests that tight regulation of their levels is required for cell-cycle progression.

In a genetic screen for regulators of *Drosophila* syncytial embryogenesis, we also identified rod^{Z3}, a novel maternal-effect lethal allele of *rough deal* (*rod*). rod^{Z3} is predicted to encode a G-E substitution in the Rod C-terminus at amino acid 1973. This mutation does not affect the levels of RZZ subunits or RZZ complex formation.

We first characterized defects in polar body condensation in rod^{Z3}-derived embryos. Polar bodies in these embryos are either pH3 positive with irregularly-shaped, partially decondensed DNA or pH3 negative with rounded, interphase-like DNA. *mad2*-null-derived embryos contain similar polar body defects, suggesting that SAC activation is specifically required to maintain polar body condensation. Furthermore, polar body defects in rod^{Z3}-derived embryos are suppressed by directly or indirectly increasing Cyclin B levels, confirming that polar body condensation is maintained by promoting the stabilization of Cyclin B.

Additionally, rod^{Z3}-derived 3-5 embryos undergo a developmental arrest prior to cellularization. Roughly half of rod^{Z3}-derived syncytial embryos contain asynchronously dividing nuclei and similarly, roughly half contain prophase and metaphase figures with abnormal centrosome attachments. In rod^{Z3}-derived embryos, GFP-Zw10 does not localize to polar body or mitotic kinetochores and mitotic arrest does not occur when the SAC is activated by colchicine treatment. These results suggest that the Rod C-terminus is required to mediate RZZ kinetochore recruitment and that this recruitment is required to maintain polar body condensation and cell-cycle progression during Drosophila syncytial embryogenesis.

DISCUSSION AND FUTURE DIRECTIONS

Regulation of dMCPH1 by the APC

Future studies that determine the effects of Cdh1-dependent degradation of dMCPH1-B on its function will provide valuable information about how dMCPH1 function is regulated during the cell cycle. Although studies in a variety of systems have reported several roles for MCPH1 in regulating cell-cycle processes and gene expression, regulation of this protein by post-translational modifications is not well understood.

Because dMCPH1-B is required for regulating the S-M cycles of syncytial embryogenesis and is preferentially expressed in this stage of development, this system is initially attractive for examining the effects overexpression of wild type or a stabilized form of dMCPH1-B (dMCPH1-B^{DboxMut}) on cell-cycle regulation. However, preliminary studies in our lab indicate that overexpression of wild type dMCPH1-B during embryogenesis results in mitotic arrest of syncytial nuclei with aberrant spindle organization and centrosome attachments (Jamie Rickmyre, unpublished). These phenotypes, which are similar to those reported for embryos derived from *mcph1*-null females, are secondary defects of disrupted cell-cycle regulation during *Drosophila* embryogenesis (Rickmyre et al 2007). Because these phenotypes result from disruptions in DNA replication, DNA damage repair, centrosome replication, or spindle function, it is difficult to identify primary cell-cycle defects in this system (Archambault & Pinson 2010).

Examining the effects of dMCPH1-B knockdown and stabilization on cell-cycle regulation in cultured *Drosophila* Schneider (S2) cells is a promising alternative approach. Large-scale RNA expression profiling of *Drosophila* cell lines has revealed that it is expressed in these cells (Cherbas et al 2011). Future studies of dMCPH1 knockdown in S2 cells by RNA interference (RNAi), may elucidate primary defects in cell-cycle regulation due to its loss of

function. Previous studies in vertebrate systems have identified that MCPH1 is required for activation of DNA checkpoints and negatively regulating chromosome condensation and mitotic entry. Although previous studies from our lab have established that dMCPH1 is not required for activation of the DNA damage checkpoint, dMCPH1 knockdown in S2 cells may reveal a conserved role in regulating chromosome condensation or mitotic entry (Rickmyre et al 2007). Furthermore, expression of dMCPH1-$B^{DboxMut}$ in MCPH1-knockdown cells may reveal how APC-dependent degradation of dMCPH1 affects its role in regulating cell-cycle progression.

Regulation of hMCPH1 by post-translational modifications

Centrosomal localization and regulation of centrosomal activities is a common feature of proteins encoded by MCPH genes, including MCPH1 (Mahmood et al 2011). Additionally, studies of human MCPH7 (SCL/TAL1 interrupting locus, or STIL) and the *Drosophila* homolog of MCPH4 (Abnormal Spindle Microcephaly-associated, or ASPM) have identified them as substrates of APC^{Cdh1} (Araki et al 2005, Arquint & Nigg 2014). The identification of dMCPH1 as an APC^{Cdh1} substrate may indicate that APC regulation is another shared or conserved feature of MCPH proteins.

Although our results suggest that hMCPH1 is not targeted for Cdh1-dependent degradation and its steady-state levels do not oscillate during the cell cycle, its regulation by the APC may involve additional factors that were not assessed in our studies. Use of alternative approaches in future studies may reveal that regulation of hMCPH1 by the APC^{Cdh1} is conserved.

It is possible that the C-terminal BRCT domains of full-length hMCPH1, which are known to mediate the interaction between hMCPH1 and the APC subunit Cdc27, may inhibit or block its ubiquitination by the APC (Singh et al 2012). In this case, APC regulation may be

restricted to the short isoform of hMCPH1 (hMCPH1-S), as it is in *Drosophila*. To test this possibility, we could examine the stability of radiolabeled, in vitro transcribed and translated hMCPH1-S in Cdh1-supplemented extract. Additionally, we could examine the steady-state levels of hMCPH1-S during the cell cycle by immunoblotting lysates derived from HeLa cells 0-20 hours after synchronization by nocodazole block. The antibody used in a similar experiment (Fig 2.5) to detect full length hMCPH1 recognizes a central domain of hMCPH1 that is found in both isoforms. Because previous studies have primarily examined exogenously expressed hMCPH1-S, this antibody may be a valuable tool to determine the expression and stability of endogenous hMCPH1-S.

Phosphorylation of APC^{Cdc20} substrates, such as securin, and APC^{Cdh1} substrates, such as Cdc6 and Skp2, inhibits their ubiquitination by the APC (Holt et al 2008, Mailand & Diffley 2005, Rodier et al 2008). It is possible that phosphorylation of hMCPH1 may also inhibit its APC-dependent ubiquitination, resulting in local degradation when the phosphorylation is removed. Although little is known about post-translational modifications of hMCPH1, a large-scale proteomics study reported that it is phosphorylated at a cluster of amino acids (S333, S337, and T339) in mitosis-arrested, but not G1-arrested HeLa cells (Dephoure et al 2008). To determine if phosphorylation affects APC-dependent degradation of hMCPH1, radiolabeled hMCPH1, generated by in vitro transcription and translation in reticulocyte lysate, could be treated with lambda-phosphatase and then examined for its stability in Cdh1-supplemented egg extracts. If dephosphorylation of hMCPH1 results in Cdh1-dependent degradation, the specific phosphorylated residue(s) required for inhibiting APC-dependent degradation could be identified in future studies.

Much work has been done to determine the roles of hMCPH1 in regulating cellular processes, its localization, and its interactions with other proteins. Elucidating how post-translational modifications, such as phosphorylation and ubiquitination, regulate these activities is important to fully understand the molecular pathway in which it functions. For example, it is known that centrosomal hMCPH1 inhibits activation of cyclin B-Cdk1 during G2/M and upon mitotic entry, centrosomal hMCPH1 is reduced (Tibelius et al 2009). It is unknown, however, how localization of hMCPH1 to centrosomes is regulated. Recent studies have shown that centrosomal MCPH7/STIL is also reduced upon mitotic entry and that this change in localization is dependent upon cyclin B-Cdk1 activity (Arquint & Nigg 2014). It is possible that centrosomal localization of hMCPH1 is regulated in a similar manner. To test this possibility, live imaging of GFP-tagged hMCPH1 could be used to examine the localization changes upon treatment with the Cdk inhibitor roscovitin. Analysis of hMCPH1's amino acid sequences also reveals the presence of three predicted sites of Cdk phosphorylation (S297, S365, and S487). GFP-hMCPH1 mutants could be generated that mimic or inhibit phosphorylation at these sites as well as the mitotic phosphorylation sites previously identified by mass spectrometry. Live imaging of these mutants could identify the specific phosphorylated residues required for centrosomal localization of hMCPH1.

RZZ in *Drosophila* syncytial embryogenesis

Our studies indicate that RZZ recruitment to kinetochores is defective in rod^{Z3}-derived embryos. Furthermore, our studies suggest that RZZ recruitment to syncytial kinetochores is required for SAC activation and regulation of syncytial mitosis. Because RZZ-dependent recruitment of Mad1-Mad2 to kinetochores is required for SAC activation in *Drosophila* and

rod^{Z3}-derived embryos have an inactive SAC response, we predict that *rod*^{Z3}-derived embryos have defective Mad1-Mad2 recruitment (Buffin et al 2005). Additionally, because RZZ is also required for the recruitment of dynein-dynactin to kinetochores, it is possible that dynein-dynactin recruitment is defective in these embryos as well (Starr et al 1998). Future experiments examining the localization of GFP-Mad1, GFP-Mad2, or GFP-p50 (a subunit of dynactin to kinetochores in *rod*^{Z3}-derived embryos could determine the effects of defective RZZ kinetochore recruitment on the recruitment of Mad1, Mad2 and dynein-dynactin.

Additionally, the mitotic defects of *rod*^{Z3}-derived embryos may be due to uncoupling of the nuclear and centrosomes cycles when spindle function is compromised during syncytial embryogenesis. Because kinetochore-localized Mad1 and dynein-dynactin mediate k-MT interactions during mitosis, defective Mad1 and dynein-dynactin recruitment to kinetochores in *rod*^{Z3}-derived embryos may cause defects in spindle function that ultimately derail the nuclear and centrosome cycles (Emre et al 2011, Yang et al 2007). To test this possibility, we could examine embryos derived from *mad1*-null females or derived from females that are transhetersozygous for hypomorphic *dynein* alleles for mitotic defects similar to those observed in *rod*^{Z3}-derived embryos. We could also examine if introduction of one copy of a *mad1*-null or a *dynein heavy chain* (*DHC*) null allele into the *rod*^{Z3} background enhances the mitotic defects.

Much work has been done to elucidate the mechanism or RZZ kinetochore recruitment in vertebrates. However, little is known about this mechanism in organisms that lack Zwint1, such as *Drosophila* and *C. elegans*. Future studies that determine how RZZ kinetochore recruitment is mediated in these organisms could reveal currently unknown mechanisms that may be conserved in vertebrates as well. Because KNL1 is conserved in *Drosophila* and *C. elegans*, is known to be required SAC activation in both systems, and is required for RZZ recruitment in *C. elegans*, it is

a promising candidate as binding partner/recruitment factor for *Drosophila* RZZ (Essex et al 2009, Feijao et al 2013). Co-immunoprecipitations of RZZ subunits and KNL1 from lysates of *Drosophila* embryos could reveal a direct or indirect interaction. Additionally, mass spectrometry analysis could reveal proteins that immunoprecipitate with Rod from wild type embryos that do not immunoprecipitate with Rod from rod^{Z3}-derived embryos. This method may reveal previously unknown proteins that are required for RZZ kinetochore recruitment.

Characterization of rod^{Z3} larval neuroblasts

We are currently collaborating with Dr. Roger Karess and members of his lab (Lenaig Defachelles and Alexandra Menant) at the University of Paris. A manuscript presenting their studies in rod^{Z3} neuroblasts and our studies in rod^{Z3}-derived embryos is currently being prepared for publication. Results from the Karess lab indicate the GFP-Zw10, GFP-Mad1, and GFP-Mad2 are recruited to kinetochores of rod^{Z3} neuroblasts, but at much reduced levels. Additionally, these proteins do not accumulate at kinetochores in response to SAC activation in rod^{Z3} neuroblasts as they normally do in wild type neuroblasts. Furthermore, recruitment of GFP-Dynein-Light-Intermediate Chain (DLIC) to kinetochores and dynein-dependent shedding of RZZ away from kinetochores is also reduced in rod^{Z3} neuroblasts. Reduced recruitment of RZZ in rod^{Z3} neuroblasts is consistent with the results from our studies in rod^{Z3}-derived embryos. However, detectable RZZ at neuroblast kinetochores, even at reduced levels, was not expected. Interestingly, their results indicate that Mad1, Mad2, and dynein recruitment to kinetochores is affected as well. Surprisingly, rod^{Z3} neuroblasts are capable of arresting in mitosis in response to colchicine treatment, suggesting they have an active SAC response. Although Mad1 and Mad2

levels at kinetochores are reduced to 30% of wild type in *rod*^{Z3} neuroblasts, their results suggest this is sufficient for SAC activation.

It is possible that low levels of RZZ recruitment in *rod*^{Z3} neuroblasts are due to residual maternal contributions of wild type Rod. In *Drosophila*, although some maternally deposited RNA and proteins persist through early stages of development, they are not normally present in adulthood. Maternal contributions of embryos derived from *rod*^{Z3} adult females only encode the mutant form of Rod. This scenario may explain why RZZ recruitment defects and SAC activation are more severe in *rod*^{Z3}-derived embryos. Future studies are directed at determining if maternally contributed Rod persists during larval neural development

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