Regulation of The Metaphase-Anaphase Transition in Mitosis in Mammalian Cells

by

Xu Naihan

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This is to certify that I have examined the above PhD thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

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<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>Anaphase-promoting complex</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BAPTA/AM</td>
<td>(Acetoxyethyl)-BAPTA</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Bub</td>
<td>Budding uninhibited by benzimidazole</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]</td>
<td>Intracellular free calcium concentration</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;/CaM-dependent protein kinase II</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependent protein kinase</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidine-2′-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECFP</td>
<td>Enhanced cyan fluorescent protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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</tbody>
</table>
EYFP  Enhanced yellow fluorescent protein
FACS  Fluorescence-activated cell sorter
FBS   Fetal bovine serum
FITC  Fluorescein isothiocyanate
FRET  Fluorescence resonance energy transfer
GFP   Green fluorescent protein
HRP   Horseradish peroxidase
IgG   Immunoglobulin G
IP$_3$ Inositol 1,4,5-trisphosphate
IP$_3$R Inositol 1,4,5-trisphosphate receptor
kD    Kilodalton
KN-92 $2-\left[\text{N-}(\text{methoxybenzenesulfonyl})\right] \text{amino-N-}(\text{4-}
\text{chlorocinnamyl})-\text{N-methylbenzylamine}$
KN-93 $2-\left[\text{N-2-hydroxyethyl}\right] \text{N-}(\text{methoxybenzenesulfonyl}) \text{amino-}
\text{N-(4-chlorocinnamyl})-\text{N-methylbenzylamine}$
Mad   Mitotic arrest deficient
MEM   Minimum essential medium
min   Minutes
ml    Milli-liter
MLC   Myosin light chain
MLCK  Myosin light chain kinase
mm    Milli-meter
mM    Milli-molar
MPF   Maturation/Mitosis promoting factor
MT    Microtubule

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEB</td>
<td>Nuclear envelope breakdown</td>
</tr>
<tr>
<td>μM</td>
<td>Micro-molar</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodine</td>
</tr>
<tr>
<td>Plk</td>
<td>Polo-like kinase</td>
</tr>
<tr>
<td>PM</td>
<td>Poration medium</td>
</tr>
<tr>
<td>Rhod</td>
<td>Rhodamine</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RM</td>
<td>Recovery medium</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Regulation of The Metaphase-Anaphase Transition in Mitosis in Mammalian Cells

by

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Abstract

A key step in mitosis is the separation of sister chromatids at the metaphase-anaphase transition, this step is controlled by the anaphase-promoting complex (APC) and it’s regulatory proteins. On the other hand, several earlier studies showed that Ca$^{2+}$ signaling is involved in regulating the metaphase-anaphase transition in meiosis. The present study is aimed at investigating the signaling transduction pathway in regulating the metaphase-anaphase transition in mitosis in mammalian cells. We tried to answer three questions: (1) Is Cdk1 inactivation required for the metaphase-anaphase transition in mammalian cells? (2) Is calcium signaling required for the activation of APC in mitosis in mammalian somatic cells? (3) How does calcium signaling play its role in mitosis? Using a GFP gene fusion technique and living cell imaging methods, we have conducted a series of experiments to answer those key questions. First, we measured the temporal and spatial dependent proteolysis of both securin and cyclin B and found that both of them are degraded before the onset of anaphase. By quantify the protein level of nondegradable cyclin B (Δ85) compared with endogenous cyclin B, we found that a low level of nondegradable cyclin B can cause cells arrested before M/A transition. These results indicate that Cdk1 inactivation is required for sister chromatids separation. Second, by examining the
differential effects of Ca\textsuperscript{2+} signaling blockers (BAPTA/AM, BAPTA, KN-93, heparin) on the metaphase-anaphase transition in HeLa cells, we found that the activation of APC depends on Ca\textsuperscript{2+} signaling via Ca\textsuperscript{2+}/CaM-dependent protein kinase II (CaMKII). The inhibition of APC by Ca\textsuperscript{2+} signal blockers may have two parallel pathways, one is through the spindle checkpoint via the destruction of mitotic spindle, while the another is suppression of CaMKII activity. Finally, we examined other important signaling molecules involved in the metaphase-anaphase transition, Mad2, Mad1 and Polo-like kinase 1. By measuring the dynamics of Mad2/Mad1 and cyclin B proteolysis in intact living cells, we demonstrated the degradation of cyclin B is related to the translocation of Mad2/Mad1. The detailed molecular mechanisms of the signaling transduction pathway in the metaphase-anaphase transition in mitosis mammalian cells were discussed.
CHAPTER 1

INTRODUCTION

1.1 Major objective and long-term significance

The long-term objectives of this research is to use a combination of molecular biological tools and imaging techniques to study the signal transduction pathways that regulate key control points of cell division, particularly, the separation of sister-chromatids at the metaphase-anaphase transition. Furthermore, we will investigate the functional roles of calcium signaling in regulating the metaphase-anaphase transition in mitosis in mammalian cells.

Cell division is one of the most important cellular processes. We hope that findings from this research will not only be important to the fundamental understanding of the basic regulating mechanisms of cells, but will also provide valuable insight into mechanisms of abnormal cell proliferation. Thus, this research showed benefit the development of anti-cancer drugs or anti-cancer treatments. Furthermore, Ca$^{2+}$ ion is a major second messenger that has many important physiological effects. Drugs based on intervening Ca$^{2+}$ signals have been used in treating cardiac and neurological diseases. A further understanding of the Ca$^{2+}$ signal transduction pathways in regulating cell division will provide new opportunities for developing innovative medical treatments for many important diseases or physiological disorders.

1.2 Rationale and background knowledge
1.2.1 Biology of the cell cycle

The defining feature of living organisms is the ability to multiply by replication of genetic materials. This process is subject to strict controls. In the case of single-cell organisms such as bacteria, the multiplication of the organism is closely linked to nutrient availability. For multicellular organisms (such as a human being), the proliferation of individual cells must be coordinated with the overall needs of the organism and therefore must be subjected to control by the entire system. This is achieved by subjecting the behavior of individual cells to control by signals emanating from other cells.

The multiplication of a single cell involves two essential processes: (i) the replication of deoxyribonucleic acid (DNA); and (ii) packaging the replicated DNA into two daughter cells by cell division. These two processes must be coordinated in time such that DNA replication is both initiated and completed before cell division occurs. The central problem of the cell cycle control is to explain how this coordination is achieved.

The duration of the cell cycle varies greatly from one cell type to another. Drosophila embryos have the shortest known cell cycle, each lasting as short as 8 minutes, while the cell cycle of a mammalian liver cell can last longer than a year. The cell cycle is traditionally divided into several distinct phases, of which the most dramatic is M-phase, during which the cells undergo mitosis; the process of nuclear division, leading up to the moment of cell division itself. In mitosis, the nuclear envelope breaks down, the contents of the nucleus condense into visible chromosomes, and the cell’s microtubules reorganize to form the mitotic spindle that will facilitate the separation of chromosomes. As mitosis proceeds, the cell seems to
pause briefly in a state called metaphase, in which the chromosomes, already duplicated, are aligned on the mitotic spindle, poised for segregation. The separation of the duplicated chromosomes marks the beginning of anaphase, during which the chromosomes move to the opposite poles of the spindle, where they decondense and re-form intact nuclei. The cell is then pinched into two by a process called cytokinesis, which is traditionally viewed as the end of the mitotic phase, or M phase, of the cell cycle. The other, much longer period of the cell cycle is known as interphase. This period of continuous cell growth includes S phase, when DNA replication occurs, and two gaps, the G₁ and G₂ phases, between S phase and M phase.

The sequences of cell-cycle events is governed by a cell-cycle control system, which cyclically triggers the essential processes of cell reproduction, such as DNA replication and chromosome segregation. In recent years, there has been tremendous progress in understanding the molecular mechanisms that govern cell cycle progression. The cell-cycle control system is based on two key families of proteins. The first one is the family of cyclin-dependent protein kinases (Cdk for short), which induce downstream processes by phosphorylating selected proteins on serines and threonines. The second is a family of specialized activating proteins, called cyclins, that bind to Cdk molecules and control their ability to phosphorylate appropriate target proteins. The cyclic assembly, activation, and disassembly of cyclin-Cdk complexes are the pivotal events driving the cell cycle. There are two main classes of cyclins: mitotic cyclins, which bind to Cdk molecules during G₂ and are required for entry into mitosis, and G₁ cyclins, which bind to Cdk molecules during G₁ and are required for entry into S phase. Each step of Cdk activation or inactivation marks a cell-cycle transition and presumably has an effect on the cell-cycle control system.
itself, initiating reactions that will eventually lead it to trigger the next downstream process.

1.2.2 Regulation of the metaphase-anaphase transition

One of the more demanding processes during the course of a cell cycle is that of the accurate transmission of genetic material from mother to daughter cells. The genetic material, which appears in the form of one or more chromosomes, must be duplicated with high fidelity prior to division to form two identical sister chromatids. The sister chromatids remain attached to each other until they separate at mitosis. This attachment allows the cell to identify these two DNA segments as identical chromosomes, and eventually to segregate them away from each other to the two daughter cells. Finally, there must be a mechanism to segregate one, and only one, copy of every chromosome to each of the daughter cells. Thus, one of the crucial steps in accurate chromosome segregation is the precise execution of the metaphase to anaphase transition [1].

The metaphase to anaphase transition is controlled by a ubiquitin-mediated degradation process. Many cellular proteins are degraded by this pathway, a major step in this destruction is catalyzed by a multimeric ubiquitin ligase known as the anaphase-promoting complex (APC) or cyclosome. APC activity rises abruptly at metaphase, resulting in the destruction of proteins that inhibits sister-chromatids separation. APC-dependent destruction of additional regulators initiates spindle disassembly, cytokinesis and the resetting of replication origins for the next cell-division cycle [2-5]. The vertebrate APC is a 20S complex which contains at least ten conserved subunits [6]. APC-dependent proteolysis requires conserved Trp-Asp
repeat (WD) proteins, Cdc20 (or Fizzy/Slp1/p55Cdc) and Cdh1 (or Hct1/Fizzy-related/Srw1/ste9) [7-11], which function as rate-limiting and cell-cycle stage-specific activators of the APC. In yeast, the timing of late mitotic events results from the sequential activation of the APC by Cdc20 and then Hct1, such that Cdc20 stimulates Pds1/Cut2 destruction whereas Hct1 targets the major mitotic cyclin Clb2 for destruction. In early embryos and probably in somatic cells, Cdc20 initiates APC-dependent degradation at the metaphase-anaphase transition [7, 12, 13]. By mediating the degradation of anaphase inhibitors (securin) and mitotic cyclins, Cdc20 has a central role in controlling entry into and exit from anaphase. Once mitotic cyclins are destroyed, Cdc20’s activation of the APC declines and is replaced by Cdh1 until cells enter S phase [7-9].

Insight into the control of anaphase initiation has come from recent studies of the spindle-assembly checkpoint, the signaling system that blocks the metaphase-anaphase transition in the presence of spindle damage or unattached kinetochores [14-16]. At the heart of the spindle assembly checkpoint is the kinetochores, a multilayered proteinaceous complex that assembles on the centromeric DNA of each chromosome [17]. During mitosis, the kinetochore mediates the interaction between the chromosome and spindle microtubules. The spindle assembly checkpoint ensures that, only when all the chromosomes are properly attached and aligned at the equatorial plane, anaphase onset is triggered, allowing the splitting of sister chromatids and their delivery to each spindle pole [18]. The checkpoint components include Mad1, Mad2, Mad3 (mitotic arrest deficient), Bub1, Bub3 (budding uninhibited by benzimidazole) and BubR1 (a Bub1-related kinase) [19-25]. The mitotic checkpoint is highly conserved in vertebrates. Mad1, Bub1, BubR1 and Bub3
are required for cell-cycle arrest upon spindle damage [19, 20, 26, 27] and for restraining anaphase entry in a normal cell cycle [20].

Recent work in yeast, frogs and mammals has produced an outline of spindle assembly checkpoint signaling. In brief, a signal is generated by the presence of unattached or improperly attached kinetochores that ultimately inhibits the activity of the APC. In both yeast and animal cells, the checkpoint component Mad2 inhibits activation of the APC by interacting with Cdc20 [28-31]. Mad2-Cdc20 interaction is required for blocking APC-Cdc20’s ubiquitin ligase activity, which then prevents proteolysis of securin and cyclin B. The unattached kinetochores on chromosomes provides sites for the activation of Mad2, activated Mad2 is then released into the cytoplasm and prevents the onset of anaphase by inhibiting the Cdc20-bound APC. After microtubules have attached to all the kinetochores, sites for Mad2 activation are no longer available, which eventually leads to APC activation by Cdc20 and triggering of anaphase onset [18]. In vertebrate cells, Mad1, Mad2, Bub1, BubR1 and Bub3 selectively accumulate at unattached kinetochores and disappear from them upon microtubule attachment [19, 20, 22, 23, 25, 26].

The final irreversible step in the duplication and distribution of genomes to daughter cells takes place at the metaphase to anaphase transition. At this point, aligned sister chromatid pairs split and separate. The first breakthrough in understanding how sister-chromatids separation is controlled came from the identification of cohesin, a protein complex that is required to hold sister chromatids together until the onset of chromosome segregation [32-34]. The 14S cohesin complex consists of the four subunits, Smc1, Smc3, Scc1 and Scc3, which are required both for establishing cohesion during S phase and for maintaining it until the onset of anaphase. Two other proteins, Scc2 and Scc4 form a separate complex
that is required for the association of cohesin with chromosomes [35-39]. At the time of chromosome segregation, cohesion between sister chromatids must be dissolved, securin and separin play very important roles in regulating this critical event. Separin, also called separase, is the protease responsible for cleaving cohesin at anaphase onset [40]. Securin is a cellular separin inhibitor. It binds to and inhibits separin for most of the cell cycle; but is only degraded at the onset of anaphase, thus releasing separin [41, 42].

Several reports show that the mechanism responsible for controlling sister-chromatid separation, as discovered in budding yeast, is conserved among eukaryotes. In yeast, sister chromatid separation is initiated by cleavage of cohesin's subunit Scc1/Mcd1 by the protease separase [40, 42]. This reaction removes cohesin from chromosomes and may directly dissolve cohesion between sister chromatids. In metaphase, separase is activated by APC, which mediates the ubiquitin-dependent proteolysis of the separase inhibitor securin [43-47]. In vertebrates, cohesin is removed from chromosomes in two steps. During prophase and prometaphase, the bulk of cohesin dissociates from the arms of condensing chromosomes by a mechanism that depends neither on the APC-separase pathway nor on cleavage of the Scc1/Mcd1. A small amount of cohesin remains in centromeric regions until metaphase and is removed from chromosomes only at the onset of anaphase. The disappearance of residual amounts of Scc1 from centromeres coincides with the APC- and separase-dependent cleavage of a small amount of Scc1 [39, 47, 48].

1.2.3 Calcium signal and cell division

Calcium is a universal signaling molecule. Its importance in regulating muscle
contraction, synaptic transmission, neurosecretion, the release of hormones and fluid secretion is well known [49, 50]. Given that calcium signals govern so many varied and diverse cellular functions, it is not surprising that it's important in regulating the cell division cycle. Findings over the last 15 years or so indicate that the resumption and progression of the cell cycle, both mitotic and meiotic, are accompanied by transient increase in cytosolic Ca^{2+}. They have been observed (a), in late G1 prior to the initiation of the S phase; (b), in G2 before entry into the M phase; (c), during mitosis between the metaphase and anaphase, and (d), during cytokinesis [51-60].

Calcium signals are generated by increasing the intracellular free calcium concentration ([Ca^{2+}]), through the modulation of channels that regulate calcium fluxes, both through the plasma membrane and through the intracellular membranes of calcium-storing organelles, in particular the endoplasmic reticulum (ER) [61, 62]. Release of calcium from intracellular organelles can be modulated by three known endogenous messengers: InsP_{3}, cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP). The existence of multitude Ca^{2+} messenger systems and Ca^{2+} stores can, in principle, provide the cells with versatile means to respond to and differentiate between diverse stimuli that signal through Ca^{2+}. Calcium signal regulates most cellular events in mitosis by binding protein instead of acting in its free ionic form [63]. There are a number of Ca^{2+} binding proteins have been proposed to mediate the effect of Ca^{2+} on the cell cycle. Calmodulin is the most important Ca^{2+} sensor in the cell cycle. It has been suggested that the calcium signal plays regulatory roles by binding to calmodulin, followed by activation of other enzymes such as Ca^{2+}/CaM-dependent protein kinase (CaMKII) and myosin light chain kinase (MLCK) [58, 64].
Calcium signal is involved in entry into mitosis. For example, $[\text{Ca}^{2+}]_i$ elevation has been found to be associated with the NEB (nuclear envelope breakdown). A highly localized brief release of $\text{Ca}^{2+}$ from the nuclear envelope stores or the perinuclear stores was detected during embryonic cell division [65-68]. Calcium transients trigger NEB and entry into mitosis in the sea urchin and mouse embryos and in mammalian somatic cells in culture. Calcium signal is also required for the exit from mitosis. There are a variety of earlier reports, using pharmacological agents such as calcium blockers, and $\text{Ca}^{2+}$ antagonists such as lanthanum, or inhibitors of inositol turnover such as lithium, that support the idea of a $\text{Ca}^{2+}$ transient preceding the onset of anaphase [69-71]. The clearest example of calcium’s involvement in anaphase onset comes from fertilization in *Xenopus* oocytes. Fertilization accompanied by a large calcium transient that leads first to cyclin B destruction [72, 73] through activation of the APC/proteasome pathway [74]. These experiments indicate that at least in the special case of fertilization, a calcium signal can lead to stimulation of APC activity and exit from meiosis. There is also evidence that calcium may trigger anaphase onset during mitosis. For example, a transient increase in intracellular calcium $[\text{Ca}^{2+}]$, occurs throughout the cell as sea urchin embryos enter anaphase of the first cell cycle. The transient just precedes chromatids separation and spindle elongation. Microinjection of calcium chelators or InsP$_3$ receptor antagonist heparin block chromosome separation [75]. At present, it is believed that the most likely downstream component of calcium signaling at metaphase-anaphase transition is the multifunctional $\text{Ca}^{2+}$/CaM-dependent protein kinase II (CaMKII). Lorca et al. (1993) showed that in *Xenopus* extracts, addition of CaMKII inhibitory peptide could block the degradation of cyclin B, while constitutively active form of CaMKII could rescue the blocking of metaphase-anaphase transition caused by the CaMKII
inhibitor. It was speculated that CaMKII phosphorylation could be a way to activate the proteasome [57, 74].

There has been study suggesting that calcium signals may be involved in the metaphase-anaphase transition in mammalian somatic cells, but so far the results were highly controversial. For example, some reported that a \([\text{Ca}^{2+}]_i\) transient could trigger the onset of anaphase in PtK1 cells and Swiss 3T3 fibroblasts [54, 76], while others suggested that calcium changes were absent and may not be directly related to the triggering of the anaphase onset [53]. At present, the question of calcium's involvement at anaphase in mammalian cells is still unresolved. One of the important objectives of this study is to investigate whether calcium signaling is involved in regulating the metaphase-anaphase transition in mitosis in mammalian cells.

Another important mitotic event, cytokinesis, is also regulated by calcium signal. During cytokinesis, localized \([\text{Ca}^{2+}]_i\) elevation was found to correlate with the cleavage furrow formation [59, 77, 78]. It is believed that an elevated \([\text{Ca}^{2+}]_i\) could play an important role in regulating cytokinesis by activating the target enzyme myosin light chain kinase (MLCK) which in turns phosphorylates the Ser-19 site of myosin light chain (MLC) and triggers the actomyosin contraction in the contractile band.

1.2.4 Functional roles of \(\text{Ca}^{2+}\) in the signal transduction pathway controlling the metaphase-anaphase transition

As discussed above, the signal transduction pathways in the metaphase-anaphase transition is very complicated. Based on discussions above and our recent studies, we
Figure 1-1  The hypothetical model of the signal transduction pathway in the metaphase-anaphase transition in mammalian cells.

This figure summarizes our proposed signaling pathways in the metaphase-anaphase transition in mammalian cells. Briefly, both cyclin B and securin are degraded by Cdc20-activated APC during the metaphase-anaphase transition, and their degradation are required for the sister-chromatids separation. The activation of APC can be inhibited by spindle-assembly checkpoint, Mad2 is the major component of spindle checkpoint. Mad2 localizes to unattached kinetochores, it releases from kinetochores when all the chromosomes aligned at the metaphase plate, resulting in the activation of Cdc20-APC. Calcium signal is involved in the regulation of the metaphase-anaphase transition. Ca\textsuperscript{2+} ions bind to calmodulin (CaM), the Ca\textsuperscript{2+}/CaM complex then binds to Ca\textsuperscript{2+}/CaM-dependent protein kinase II (CaMKII) and activates it. It is believed that Ca\textsuperscript{2+} signal activates APC, which results in a ubiquitin-dependent proteolysis of cyclin B and securin. The inhibition of APC by Ca\textsuperscript{2+} may have two parallel pathways, one is through the spindle checkpoint via the destruction of mitotic spindle, while the another is suppression of CaMKII activity. The broken arrow represents a somewhat hypothetical connection. The points where the signalling pathway can be blocked by applying specific inhibitors are marked by the symbol "—↓".
proposed a model to elucidate the possible roles of Ca\(^{2+}\) in regulating the metaphase-anaphase transition in mitosis in mammalian cells. The proposed pathways were summarized in Figure 1-1. In this hypothesis, we believed that both securin and cyclin B are degraded by APC-Cdc20 before the onset of anaphase; Cdk1 inactivation is required for the separation of sister chromatids. Calcium signal is involved in regulating the metaphase-anaphase transition in mitosis in mammalian cells, the calcium signaling pathway is through the activation of Ca\(^{2+}/CaM\)-dependent protein kinase II (CaMKII).

1.3 Specific aims

The specific aims of this project were mainly focused on resolving the following questions:

(1) *Is Cdk1 inactivation required for the metaphase-anaphase transition in mitosis in mammalian cells?*

Whether Cdk1 inactivation is required for the exit from mitosis or for the metaphase-anaphase transition is controversial. To clearly answer this question, we combined GFP-fusion technique and real-time living cell imaging technique to examine the temporal and spatial proteolysis of both securin and cyclin B in a single living cell. And we used both western blotting and immunofluorescence analysis to quantify the protein level in a single cell. (The results were discussed in Chapter 4)

(2) *Is calcium signaling involved in the metaphase-anaphase transition in mitosis in mammalian cells?*
As discussed above, whether calcium signal is involved in the regulation of the metaphase-anaphase transition in mammalian somatic cells is still controversial at present. To answer this question, we used both Western blotting analysis and living cell imaging to examine the effects of different Ca$^{2+}$ signaling blockers on the degradation of cyclin B and cell cycle progression. (The results were discussed in Chapter 5)

(3) **How does calcium signal affect the metaphase-anaphase transition?**

After having confirmed that calcium signaling is indeed involved in the regulation of the metaphase-anaphase transition in mammalian somatic cells, we then investigated how calcium signal affects this critical step in mitosis. We tried to clarify the linkage of calcium signal and the activation of APC during the metaphase-anaphase transition. (The results were discussed in Chapter 5 and Chapter 6)

(4) **How does spindle checkpoint regulate the activation of APC?**

Spindle assembly checkpoint and polo-like kinases are the very important regulatory signaling molecules in the metaphase-anaphase transition. Specifically, we would like to study the involvement of spindle checkpoint protein Mad1, Mad2 and Polo-like kinase 1 in regulating the metaphase-anaphase transition and their relationship with calcium signal. (The results were discussed in Chapter 6)

1.4 Experimental approaches

In this study, we combined advanced imaging techniques with newly developed molecular biology tools to study the signal transduction pathway in regulating the
metaphase-anaphase transition in a single living cell. Particularly, we labeled various signaling molecules with GFP (green fluorescent protein) by fusing their genes together and then monitor their translocation, activation, degradation and protein-protein interaction within the living cell using state-of-the-art optical techniques. The advantage of this approach is that we can provide the spatial and temporal specific information of both Ca$^{2+}$ signals and other regulatory components in intact cells. This approach is very useful in studying cell division process. Since the cell is a highly organized system in which the distributions of many important signaling molecules are spatially dependent, only with such an in vivo approach can we preserve the structural integrity of the living cells and obtain the four-dimensional information on the variation of a signaling molecule within the sub-cellular environment. We have utilized the GFP-labeling technique and real-time living cell imaging technique to study the dynamic redistribution of several important signaling molecules (including cyclin B, securin, Mad2, Mad1, Plk1, etc.) during cell division and obtained some very interesting findings. This approach, of course, also has certain limitations, it is much more difficult to do quantitative measurements in individual living cells, and it is also difficult to assay the activity of some molecules using optical method. The photo-damage of the cell caused by frequently being exposed to the fluorescent light can also limit the time for long-time optical measurement. Regardless of these limitations, this approach should be considered as a very powerful method that can well complement the biochemical studies in cell populations. We hope that by combining the results from our optical studies with the large amount of information obtained from the biochemical and molecular biology studies, one will be able to get a more comprehensive picture of the signal transduction pathway in regulating the metaphase-anaphase transition.
Another major technique we used in this study is the electroporation method, which was developed by our laboratory [79] and has been used to introduce plasmid DNA or large molecules into mammalian cells [80]. As many of the fluorescent indicators and the Ca\(^{2+}\) signaling inhibitors are not membrane-permeable, the application of the electroporation technique can help to solve the loading problem of these reagents into mammalian cells. Comparing with other transfection methods such as lipofectamine reagents, the electroporation method is not only highly efficient but also produces little toxicity to the living cells. In addition, by comparing with the microinjection method, the use of electroporation technique makes it possible to do quantitative studies in vivo dealing with a large number of cells.

As to the experimental model system, we mainly used cultured mammalian cells including HeLa (human cervix carcinoma cell line) and PtK2 (rat kangaroo kidney cell line) cells. The mammalian culture cell system has several advantages. First, the mitotic stages in mammalian cells can be easily identified by morphological markers or fluorescence DNA staining. Second, the cell cycle of the mammalian cells is relatively long (from 10 to 30 hours depending on cell type), so we have enough time to express an exogenous protein. Third, the mammalian cells are much thinner than embryonic cells, the distribution of fluorescent probes within the cell can be studied using our real-time living cell imaging system.

In order to dissect the Ca\(^{2+}\) signaling pathway involved in the regulation of the metaphase-anaphase transition, we applied various types of Ca\(^{2+}\) signal inhibitors and observe their effects on the mitotic process. Some of these inhibitors are cell permeant and thus can be applied in the medium. For those that are not membrane-permeant, we used in situ electroporation method to introduce those reagents into attached living cells [81].
1.5 Major contributions of this research

During cell division, each daughter cell inherits one copy of every chromosome. How sister chromatids are segregated with high fidelity to opposite poles of the cell is also a matter of great biomedical interest. Defects in genome segregation in somatic cells in all likelihood contribute to oncogenesis. Recent progress in characterizing proteins such as cohesin, condensin, separase, and the aurora B kinase, which control chromosome behavior during mitosis, is paving the way to molecular understanding of genome transmission (Nasmyth, 2002).

In this project, we focused on studying the signaling transduction pathway in regulating the sister chromatid separation during the metaphase-anaphase transition in mammalian somatic cells. Our research thus will provide some fundamental understanding on cell cycle research. The major contributions of this research are as follows:

(1) Using GFP (green fluorescence protein) gene fusion technique and our specifically built imaging system, we were able to examine the temporal and spatial proteolysis of cyclin B1 and securin (anaphase inhibitor) in the same living cells. We found that both of them are simultaneously degraded before the onset of anaphase. We combined both Western blotting and immunostaining method to quantify the protein level of nondegradable cyclin B compared with endogenous cyclin B, and we found that a low amount of nondegradable cyclin B can arrest cells before the metaphase-anaphase transition. Our findings suggest that different from embryonic cells and the yeast system, both Cdk1 inactivation and degradation of anaphase inhibitor are required for sister chromatids separation.
(2) Using both in vitro and in vivo assays, we examined the effects of various Ca$^{2+}$ signaling inhibitors on the metaphase-anaphase transition. Our findings provide clear evidence that the activation of APC depends on calcium signaling in mammalian somatic cells, the calcium signaling pathway is through the Ca$^{2+}$/CaM-dependent protein kinase II (CaMKII).

(3) We examined the mechanism of how Ca$^{2+}$ signals regulate the activation of APC in mammalian somatic cells. Results of our experiments using specific inhibitors and YFP-tubulin stable cell line suggest that Ca$^{2+}$ signal can affect the activation of APC through different pathways. By examining the redistribution of spindle checkpoint protein, Mad2, we demonstrated that calcium signal may affect APC activation through spindle checkpoint.

(4) We have examined the functional roles of some important regulatory components involved in the metaphase-anaphase transition. Using the confocal microscopy technology, we found evidence that proteolysis of cyclin B correlates with the translocation of Mad2/Mad1 from kinetochores to cytoplasm.
CHAPTER 2
LITERATURE REVIEW

2.1 An overview of the cell cycle

Cells reproduce by duplicating their contents and then dividing in two. This cell division cycle is the fundamental means by which all living things are propagated. In unicellular species, such as bacteria and yeasts, each cell division produces an additional organism. In multicellular species many rounds of cell division are required to make a new individual, and cell division is needed in the adult body, too, to replace cells that are lost by wear and tear or by programmed cell death. The details of the cell cycle may vary, but certain requirements are universal. First and foremost, to produce a pair of genetically identical daughter cells, the DNA must be faithfully replicated, and the replicated chromosomes must be segregated into two separate cells. The control mechanisms of cell cycle are very complicated. Biologists have spent over a century trying to answer the question of how cells grow and divide, but many aspects of the cell cycle control are not yet well understood. The regulation of cell cycle control is not only a fundamental biological question, but also related to human medical problems, such as cancer.

The cell cycle in eukaryotic cells is traditionally divided into several distinct phases. Of which, the most dramatic is mitotic phase, or M phase, the process of nuclear division, leading up to the moment of cell division itself. The much longer period that elapses between one M phase and the next is known as interphase. Replication of the nuclear DNA usually occupies only a portion of interphase, called the S phase of the cell cycle (S = synthesis). The interval between the completion of
mitosis and the beginning of DNA replication is called the G\textsubscript{1} phase (G = gap), and the interval between the end of DNA synthesis and the beginning of mitosis is called the G\textsubscript{2} phase. G\textsubscript{1}, S, G\textsubscript{2}, and M are the traditional subdivisions of the standard cell cycle. The duration of the cell cycle varies greatly from one cell type to another. Fly embryos have the shortest cell cycles, each lasting as little as 8 minutes, while cell cycle of a mammalian liver cell can last longer than a year. In a typical animal cell cycle, G\textsubscript{1} lasts 12 hours, S phase 6 hours, G\textsubscript{2} phase 6 hours and mitosis about 30 minutes.

The basic strategy of cell division is remarkably constant among eukaryotic organisms. There are five stages of the M phase that constitute mitosis, and the sixth stage, overlapping with the end of mitosis, is cytokinesis. These six stages form a dynamic sequences, the complexity and beauty of which are hard to appreciate from written descriptions or from a set of static pictures. Three features are unique to M phase: chromosome condensation, the mitotic spindle and the contractile ring. Chromosome condensation marks the initiation of the first stage, prophase. Then, the nuclear envelope breaks down and disassembles. This process, named the nuclear envelope breakdown (NEB), marks the start of prometaphase. A mitotic spindle forms and the duplicated microtubule organizing centers become the spindle poles. The condensed chromosomes gradually align at the equator of the mitotic spindle during the metaphase. This process is called congression. Each chromosome is held by in tension at this metaphase plate by the paired kinetochores and their associated microtubules, which are attached to opposite poles of the spindle. When all chromosomes are well aligned, sister chromatids start to separate. This is a critical event, which marks the transition from the metaphase to anaphase, also called onset of anaphase. Sister chromatids move along the microtubule fibers to opposite poles,
Figure 2-1  The course of mitosis in a typical animal cell.

In these micrographs of cultured newt lung cells, the microtubules have been visualized by indirect immunofluorescence, while chromatic is stained with a blue fluorescent dye. During interphase the centrosome forms the focus for the interphase microtubule array. In prophase, the centrosome divide and the resulting two asters move apart. The NEB occurs at prometaphase, allowing the spindle microtubules to interact with the chromosomes. All the chromosomes are aligned across the middle of the spindle at metaphase. Sister chromatids separate synchronously at early anaphase and the spindle poles move farther apart at late anaphase. At telophase the daughter nuclei re-form and cytokinesis is almost complete with the midbody persisting between the daughter cells. (Molecular Biology of the Cell, 1994)
the division of cytoplasm, cytokinesis, starts. A contractile ring pinches the cell into
two daughter cells, each containing a set of chromosomes and a spindle pole. As
cytokinesis proceeds, chromosomes decondense and the nucleus reassembles,
resulting in a new interphase nucleus. These events happen in the telophase. Then,
microtubule and stress fiber network return to their interphase pattern. These are the
"standard" M phase processes that usually occur in somatic cells (Figure 2-1).

2.2 The cell cycle control system

Progress from one phase of the cell cycle to the next is controlled by the activation or
inactivation of a member of a conserved family of serine/threonine protein kinases
known as the cyclin-dependent protein kinases (CDKs). The CDKs are inactive as
monomers and, therefore, need to bind a cyclin partner to be activated. Different
cyclin-CDK complexes are assembled and activated to control, for example, DNA
synthesis and mitosis [82](Figure 2-2). There are two main class of cyclins: mitotic
cyclins, which bind to Cdk molecules during G₂ and are required for entry into
mitosis, and G₁ cyclins, which bind to Cdk molecules during G₁ and are required for
entry into S phase. Each step of Cdk activation or inactivation marks a cell-cycle
transition and presumably has an effect on the cell-cycle control system itself,
initiating reactions that will eventually lead to trigger the next downstream process.

At least four major mechanisms appear to govern Cdk activity [83, 84]: (a)
The primary mechanism of Cdk activation is the binding of a cyclin subunit. Cyclins
were defined as proteins whose levels oscillate during the cell cycle. Homologue
among cyclins is often limited to a relatively conserved domain of about 100 amino
acids, the cyclin box, which is responsible for CDK binding and activation. Each
Cdk interact with specific subset of cyclins. (b) Complete activation of most Cdns also requires phosphorylation at a conserved threonine residue (Thr 161 in human CDC2, Thr 160 in CDK2) which lies in the T-loop. This phosphorylation is carried out by a CDK-activating kinase (CAK). CAK is a multi-subunit enzyme whose catalytic subunits are highly conserved. (c) The cyclin-CDK complex can also be inhibited by phosphorylation at two sites near the amino terminus (Thr14 and Tyr15 in human CDC2 and CDK2) in the ATP-binding site of the CDK. The major candidate for the Tyr15 kinase is Wee1/Mik1, an adjacent threonine residue is phosphorylated in animal cells by Myt1. Thr14 and Tyr15 are both dephosphorylated by a dual-specificity phosphatase termed CDC25, which remove the phosphates from tyrosine or/and threonine of the Cdns, thus activate the cyclin-CDK complex. (d) The forth major mechanism for CDK regulation involves a diverse family of proteins, termed the CKIs, that bind and inactivate CDK-cyclin complexes. Most CKIs bind tightly to the Thr160/161-phosphorylated CDK-cyclin complexes and indirectly inhibit kinase activity.

2.3 Checkpoints of cell division

"Cell cycle checkpoint" refers to the entire process that monitoring cell cycle events such as DNA replication, spindle assembly, generating signals in response to errors in the processes, and halting the cell cycle at a specific point [85]. These checkpoints, including G1/S boundary, S phase, G2/M transition, ensure that critical events in particular phase of the cell cycle are completed before a new phase initiated, thereby preventing the formation of genetically abnormal cells. There are several "checkpoints" or "transitions" during cell division. Generally, there are two major
Figure 2-2 Temporal control of the animal cell cycle.

The cyclin E, cyclin A and cyclin B-dependent kinases are active at different times in the cell cycle. On this basis, cyclin E-cdk2 appears to have a role in promoting S phase, cyclin A-cdk2 in S phase and at G2-to-M phase, and cyclin B-cdk1 during mitosis. Cyclin B1-cdk1 is activated at the end of G2 phase by the phosphatase Cdc25. [82]
checkpoints associated with cell division: “entry of M phase” and “exit of M phase”. However, defined by morphological markers, there are some specific milestones, including the chromosome condensation, the nuclear envelope breakdown (NEB), the metaphase-anaphase transition or the anaphase onset, and the cytokinesis. The chromosome condensation and NEB are the mitotic events associated with the entry of M phase, while the metaphase-anaphase transition and cytokinesis are closely related to the exit of M phase.

2.3.1 Entry of M phase

The initiation of mitosis in eukaryotic cells is governed by a spatially and temporally complex phosphorylation cascade which culminates in the activation of MPF. MPF consists of the cyclin-dependent protein kinase Cdc2 and a B-type cyclin regulatory subunit. The Cdc2/cyclin B was first identified in an embryonic system, and found to be able to promote egg maturation. Thus it was given the name maturation-promoting factor (MPF). Later, it was found that the Cdc2/cyclin B activity is required for the entry of M phase in mammalian cells. Therefore, the abbreviation of MPF also stands for mitosis-promoting factor [85-88].

During the G2 phase, cyclin B is gradually accumulated. Cyclin B binds to Cdc2 to form a heterodimer. MPF is kept inactive through Tyr15 phosphorylation of Cdc2 and, upon entry into mitosis, MPF is activated by dephosphorylation of this residue. The onset of mitosis is triggered by simultaneous activation of the Tyr15 phosphatase Cdc25 and inactivation of the Tyr15 kinase Wee1. As Cdc2-cyclin B is capable of phosphorylating and thereby activating and repressing the activity of Cdc25 and Wee1, respectively, it is thought that Cdc2 activation depends in part on a
positive feedback loop (Figure 2-3). Eukaryotic cells tightly couple MPF activation with a number of cell-cycle-dependent events. It triggers breakdown of the nuclear envelope, condensation of chromosomes, and the formation of mitotic spindle that can capture microtubules.

Evidence is now accumulating to suggest that the spatial organization of cell-cycle regulators is also important in the temporal control of the cell cycle [82]. The initiation of mitosis is accompanied not just by Cdc2 phosphorylation, but also by changes in the subcellular location of cyclin B1 [89-93]. Cdc2-cyclin B1 complex colocalize with cytoplasmic microtubules during interphase and abruptly translocate to the nucleus upon entry into mitosis [89, 91, 94, 95]. As many of the key substrates for Cdc2-cyclinB1 are in the nucleus, its exclusion from this organelle may provide a mechanism to limit its effects before mitosis. Nuclear translocation of cyclin B1 is therefore positioned to serve as an additional regulatory step in the control of mitotic initiation. Thus nuclear targeting of cyclin B1 and dephosphorylation of Cdc2 both contribute to the control of mitosis entry [82, 96-98].

More recently, Polo-like kinases have also been implicated in the regulation of Cdc2-cyclin B1 complex. It is capable of activating Cdc25 and being part of the positive feedback loop that amplified Cdc2-cyclin B1 activity both in Xenopus cell-free system and in vivo [99-104].

2.3.2 Exit of M phase

One of the most demanding processes during the course of a cell cycle is that of the accurate transmission of genetic materials from mother to daughter cells. The genetic materials, which appears in the form of one or more chromosomes, must be
Figure 2.3  Biochemical model of the embryonic cell cycle engine.

The different Cdc2/28-cyclin B complex formed during the embryonic cell cycle and the enzymes that act on them are shown. At the beginning of interphase, Cdc2/28 is unphosphorylated. Association of cyclin B with Cdc2/28 induces the phosphorylation tyrosine 15 and threonine161 to produce preMPF. At entry into mitosis, preMPF is converted into active MPF by Cdc25, which removes the phosphate from tyrosine15. Active MPF induces the destruction of cyclin, resulting in MPF inactivation.
duplicated with high fidelity prior to division to form two identical sister chromatids, which are associated with each other along their length by a putative cohesion factor. By metaphase, sister chromatids attach in a bipolar fashion to spindle microtubules that emanate from opposite poles of the cell and congress to the metaphase plate. At the onset of anaphase, inactivation of the cohesion factors leads to synchronous sister chromatid separation [1, 105].

The initiation and coordination of late mitotic events, from sister chromatids separation to cytokinesis, are governed by ubiquitin-dependent proteolysis of key regulatory proteins [2-5]. A major step in this destruction is catalyzed by a multimeric ubiquitin ligase known as the anaphase-promoting complex (APC) or cyclosome. APC activity rises abruptly at metaphase, resulting in the destruction of proteins that inhibit sister-chromatid separation. APC-dependent destruction of additional regulators initiates spindle disassembly, cytokinesis and the resetting of replication origins for the next cell division cycle [106].

2.3.2.1 Anaphase-promoting complex (APC)

The anaphase-promoting complex (APC/Cyclosome) is thought to be a ubiquitin ligase (or “E3” enzyme), which collaborates with a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2) to catalyse the transfer of ubiquitin molecules to lysine side chains on target proteins [4, 107-109]. The precise number of APC subunits is still not known, but multiple polypeptides have been found in all preparations, at least 10 in Xenopus and human cells, 9 in clams and 12 in budding yeast [106, 110-112]. Nine of the subunits identified in vertebrates show sequence homology with subunits of the budding yeast APC, providing one of many examples for the evolutionary conservation of the eukaryotic cell cycle machinery. The
primary structure of these subunits reveals little about their potential functions, except that one of the conserved subunits (APC2) shares sequence homology with the "cullins", a class of proteins that is found in other types of ubiquitin ligases and which may mediate the interaction with the E2 enzyme. The evolutionary conserved subunits CDC27, CDC16, and CDC23 and one additional subunit only found in vertebrates so far (APC7) contain multiple degenerate 34 amino acids repeats termed tetratricopeptide repeats (TPRs). TPR domains may mediate the interaction of different APC subunits.

Temporal control of APC activity is through post-translational mechanism, which specify overall enzymatic activity and substrate selection. In *Xenopus* early embryonic cell division, APC is active in mitosis, but has only a basal level of activity in interphase [110]. In mammalian tissue culture cells and in yeast, APC becomes active at the onset of anaphase and its activity persists through most of G1. The APC activity is low in S, G2 and in early mitosis, as assayed by cyclin ubiquitination [113-115]. During mitosis, the APC degrades multiple substrates, such as anaphase inhibitors securin and cyclin B at different stages of mitosis (Table 2-1).

### 2.3.2.2 Regulation of APC activity

Recent studies have led to the surprising revelation that the core APC, despite its large size and complexity, does not actually contain everything it needs to promote ubiquitination of target proteins. One more subunit is required to complete the enzyme. Two related versions of this activating subunit have been identified in numerous organisms, there are conserved Trp-Asp repeat (WD) proteins, Cdc20 (or
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<th>Function of Substrate</th>
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<th>Species</th>
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<tr>
<td>Anaphase inhibitors</td>
<td>Securin/PTTG</td>
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<td>Pipples</td>
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<td>Cut2</td>
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<td>Regulatory subunits of</td>
<td>B-type cyclins</td>
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<td>UbcH10/E2-C</td>
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<td>Regulators of DNA replication</td>
<td>Geminin</td>
<td><em>X. laevis, D. melanogaster</em></td>
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Fizzy/Slp1/p55<sup>Cde</sup>) and Cdh1 (or Hct1/Fizzy-related/Srw1/ste9) [7-11], which function as rate-limiting and cell-cycle stage-specific activators of the APC. In yeast, the timing of late mitotic events result from the sequential activation of the APC by Cdc20 and then Hct1. Such that Cdc20 stimulates Pds1 destruction whereas Hct1 targets the major mitotic cyclin, Clb2, for destruction. The roles of Cdc20 and Cdh1 in substrates targeting are different in animal cells. In early embryos and probably in somatic cells, Cdc20 alone seems capable of promoting the destruction of all APC substrates, including cyclins. Cdc20 initiate APC-dependent degradation at the metaphase-anaphase transition [7, 9, 13, 116]. By mediating degradation of anaphase inhibitors Pds1/Cut2 and mitotic cyclins, Cdc20 has a central role in controlling entry into and exit from anaphase [117]. Once cyclins are destroyed, Cdc20's activation of the APC declines and is replace by Cdh1 until cells enter S phase (Figure 2-4).

In yeast and human cells, Cdc20 and Cdh1 both bind to the APC but their association is regulated differently. Transcripts of both genes accumulate in G2, peak in mitosis and disappear in G1 phase. The Cdc20 protein level follows a similar pattern, accumulating from late G2 through mitosis and dropped dramatically in early G1. Changes in Cdc20 levels are generally paralleled by changes in the amount of Cdc20 associated with the APC. In contrast, Cdh1 is a stable protein whose level does not vary significantly through the cell cycle, it only binds to APC during late anaphase and G1 [13, 114, 118].

What triggers the sudden activation of APC-Cdc20 at the metaphase-anaphase transition? Recent experiments suggest that the activation of APC-Cdc20 depends on the prior phosphorylation of APC core subunits by Cdk1/cyclin B. Cdc20 only promote the activity of the phosphorylated, mitotic form of the APC. On the other
In embryonic cells, the Cdc20-APC is stimulated by mitotic Cdk1 activity and is responsible for the destruction of anaphase inhibitors and cyclins; the dashed arrows indicate that the ability of Cdk1 and Polo-like kinases to stimulate the Cdc20-APC is poorly inhibited Hct1 subsystem (enclosed in dashed box) is bolted on to allow stable APC activation in G1. In budding yeast, Cdc20-APC may target some cyclins for destruction, but at least on cyclin type (Clb2) is targeted primarily by the Cdh1-APC. [106]
hand, phosphorylation of Cdc20 during mitosis has been also observed in animal system but its role remains controversial [10, 39, 119]. Some studies suggested that the activation of APC requires phosphorylation of Cdc20 by Cdk1/cyclin B [118], whereas another study shows that efficient activation of mitotic APC by a non-phosphorylatable version of Cdc20. Phosphorylation might augment Cdc20's activity but is probably not essential for the activity of APC-Cdc20 in vitro. Throughout mitosis, Cdh1 is unable to associate with APC due to its phosphorylation by the Cdc2 kinase. Later in mitosis, an unidentified mechanism causes dephosphorylation of Cdh1. Dephosphorylated Cdh1 in turn binds to and activates APC. Cdh1 maintains APC activity in G1, certain Cdh1-specific substrates identified so far, such as vertebrate Cdc20, Plk1 and ARK2 [115].

Polo-like kinases in many species are also required for mitotic exit. It is a member of highly conserved family of Polo-related protein kinases. The vertebrate Plk1 phosphorylate core APC subunit (Cdc27, Cdc16 and Tsg24) \textit{in vitro} and causes APC activation [118, 120], although effective phosphorylation by Plk1 may require the presence of Cdc2 kinase. Protein kinase A can also phosphorylate the APC, but its phosphorylation appears to be inhibitory [117, 118, 120].

### 2.3.2.3 Spindle assembly checkpoint

Once the mitotic spindle is formed, progression through mitosis is controlled largely by the proteolysis of specific proteins [2], which may be spatially regulated by the mitotic apparatus. More specifically, structures such as kinetochores and spindle poles appear to integrate signals between the chromosomes and the anaphase-promoting complex (APC), which controls the degradation of mitotic regulators.
This is essential for the proper control of chromosomes segregation by the "spindle-assembly checkpoint" [121]. The spindle-assembly checkpoint mechanism ensures equal separation of sister chromatids into two daughter cells by delaying anaphase initiation until all chromosomes are aligned properly at the metaphase plate [82, 106, 115, 117]. Genetic studies in yeast have identified a group of genes, Mad1, Mad2, Mad3, Bub1, Bub2 and Bub3 involved in checkpoint control [21, 26, 122-124]. The mitotic checkpoint is highly conserved. In vertebrates, the protein Mad2 is an essential component of the spindle-assembly checkpoint system. Mad2 associates with Cdc20 and APC to form a ternary complex and thereby inhibit activation of APC by Cdc20 [30, 31, 114, 125, 126]. This Mad2-Cdc20-APC ternary complex must be readily reversible. When all chromosomes are aligned at the metaphase plate, the checkpoint signal disappears and Mad2 dissociate from the ternary complex. The resulting Cdc20-APC complex becomes active in degrading the anaphase inhibitors, leading to separation of sister chromatids. Interestingly, the purified recombinant Mad2 exists in either a monomeric or a tetrameric state, only the tetramer inhibits Cdc20-APC activation, suggesting that the spindle checkpoint may activate Mad2 by altering its structure [114].

Numerous studies suggested that an activated form of Mad2 checkpoint protein generated specifically at unattached kinetochores binds and inhibits the actions of the APC [127]. However, two recent studies indicate that Mad2 is not the whole story, BubR1 kinase is a more potent inhibitor than Mad2 alone [128, 129]. The role of other checkpoint components would be upstream of Mad2, acting at the unattached kinetochore to convert Mad2 to its APC inhibitory form. Both new studies reported the purification of a complex from HeLa cells containing BubR1 and other checkpoint proteins. The complex characterized by Sudakin et al. (2001) and
Figure 2-5  The spindle-assembly checkpoint.

Unaligned kinetochores are associated with an active Mad/Bub complex that sequesters Cdc20 and prevents the APC from the kinetochores have captured microtubules (black lines). Mad2 dissociates from the kinetochore, releasing Cdc20 to bind to the APC (center), which is now able to ubiquitinate Pds1/Cut2 and the B-type cyclins (right). As a consequence of Pds1/Cut2 degradation by the proteasome, the cohesins located between sister chromatids dissociate from chromatin, leading to sister-chromatid separation. [82]
named the mitotic checkpoint complex (MCC) contains BubR1, Bub3, Cdc20 and Mad2 in near equal stoichiometry. The MCC was found to inhibit APC ubiquitination activity in vitro ~3,000-fold more effectively than purified recombinant Mad2 alone [130] (Figure 2-5).

2.3.2.4 Sister-chromatid separation

One of the key processes in cell division is the precise segregation of chromosomes. The first breakthrough in understanding how sister chromatids separation is controlled came from the identification of cohesin, a protein complex that is required to hold sister chromatids together until the onset of chromosome segregation [32, 33]. The 14S cohesin complex consists of the four subunits (Figure 2-6a), Smc1, Smc3, Scc1 and Scc3, which are required both for establishing cohesion during S phase and for maintaining it until the onset of anaphase. Two other proteins, Scc2 and Scc4 form a separate complex that is required for the association of cohesin with chromosomes [35-39].

At the time of chromosome segregation, cohesion between sister chromatids must be dissolved. Securin and separin play very important roles in regulating this critical event. Destruction of Pds1/Cut2 at the onset of anaphase depends on APC activity and is essential for sister chromatids separation. These proteins are members of a class of anaphase-inhibitory proteins existing in all eukaryotes, including Pds1 in budding yeast, Cut2 in fission yeast, Pim1 in Drosophila and hPTTG in vertebrate, they are now called securin because their role in controlling the onset of sister separation [43-46, 131]. Securin bind to Esp1/Cut1-like proteins, they are usually large proteins with molecular weight from 180-200 Kda, containing a conserved C-
Figure 2-6  Cohesin and sister chromatids separation.

(A) Cohesin and its friends. A model illustrating how cohesin subunits connect sister chromatids during DNA replication and maintain its association until the onset of anaphase. (B) The APC-separin pathway. A model illustrating how APC-Cdc20 initiate anaphase through the activation of separin and subsequent cleavage of cohesin subunit. [33]
terminal "separin" domain, and now called "separin" or "separase". They belong to a family of cystenine CD-clan proteases that also includes the caspases. Several lines of evidence strongly suggest that separin is the Scc1 protease [40-42, 47, 132] (Figure 2-6b). Separase is inhibited independently by affinity binding to securin and by specific inhibitory phosphorylation by Cdk1-cyclin B [133]. Like caspases, separases in vertebrate cells undergo autolysis upon their activation by the APC [134].

Several reports now show that the mechanism that controls sister-chromatid separation, as discovered in budding yeast, is conserved among eukaryotes. In yeast, sister chromatid separation is initiated by cleavage of cohesin's subunit Scc1/Mcd1 by the protease separase [40, 42]. This reaction removes cohesin from chromosomes and may directly dissolve cohesion between sister chromatids. In metaphase, separase is activated by APC, which mediates the ubiquitin-dependent proteolysis of the separase inhibitor securin [43-47]. In vertebrates, cohesin is removed from chromosomes in two steps. During prophase and prometaphase, the bulk of cohesin dissociates from the arms of condensing chromosomes by a mechanism that depends neither on the APC-separase pathway nor on cleavage of the Scc1/Mcd1. A small amount of cohesin remains in centromeric regions until metaphase and is removed from chromosomes only at the onset of anaphase.

During the early stage of mitosis — between prophase, when chromosomes start to condense, and prometaphase when they bi-orient on the mitotic spindle. This dissociation is thought to be independent of separase and is accompanied by the splitting of chromosomes into two morphologically defined chromatids, which takes place along chromosome arms but not in the neighborhood of centromeres [39, 48]. At about the same time, a related complex, called condensing [135], binds to the axis
of both chromatids and organizes chromosomal DNA in a manner that is essential for sister chromatid disentanglement [136-140]. Like cohesin, condensin is composed of a pair of SMC proteins, Smc2 and Smc4, which form a heterodimer whose heads bind three non-SMC (structural maintenance of chromosomes) proteins [135, 141].

What drives chromatid individualization during prophase? Recently, in vitro study found that cohesin is phosphorylated by Polo-like kinase as cells enter mitosis [142-144], phosphorylation of cohesin by Plks severely reduced its ability to bind to chromatin, indicating that Polo-like kinase regulates the dissociation of cohesin from chromosomes in early mitosis.

The disappearance of residual amounts of Scc1 from centromeres coincides with the APC- and separase-dependent cleavage of a small amount of Scc1 [39, 47, 48] (Figure 2-7). It is still unknown, however, if this cleavage reaction, which affects maximally 10% of the total cellular cohesin, is required for anaphase. Recently, J-M Peters (2002) identified two separase cleavage sites in human cohesin subunit Scc1, comparison of these sites with known separase cleavage sites in budding yeast and fission yeast yields glutamate-X-X-arginine as the consensus for the sequence preceding the scissile peptide bond [42, 145, 146].

2.3.3 Cytokinesis

Sister chromatids are pulled into the opposite spindle poles during anaphase. When chromosomes are near the spindle poles, the division of cytoplasm starts. This is called cytokinesis. The cleavage furrow forms in the equatorial region of the cell. The classic experiments of the early twentieth century suggested that the astral microtubules have been implicated in determine the site of cleavage [147]. However,
A

1. Pds1
2. APC/C\(_{Cdc20}\)
3. Esp1

- Sister chromatids
- Cohesin
- Centromere

Metaphase → Anaphase

B

1. Securin
2. APC/C\(_{Cdc20}\)

- Chromosome condensation
- Bulk of cohesin dissociates

Prophase → Metaphase → Anaphase
Figure 2-7  Control of sister-chromatid cohesion in yeast and vertebrates.

(A) In budding yeast, sister chromatids are held together by cohesin complexes at metaphase. Degradation of Pds1 by APC/C\textsuperscript{cdc20}-dependent proteolysis release Esp1, allowing it to cleave Scc1/Mcd1 and leading to the initiation of anaphase. (B) In vertebrates, the bulk of cohesin dissociates from chromosome during prophase, perhaps as a result of chromosome condensation. A small amount of cohesin remains on chromosomes, predominantly around centromeres. This pool of cohesin is cleaved by separin at the metaphase-anaphase transition. Activation of separin at the cell-cycle transition is brought about by destruction of securin by APC/C\textsuperscript{cdc20}. [34]
recent experiments indicated that the central spindle is required for the formation of the contractile ring [148-152]. An actomyosin contractile ring forms at anaphase and starts to contract by the actin-myosin interaction, resulting in the ingestion of plasma membranes [153].

Cell cycle kinases, such as Cdc2-cyclin B complex and Polo-like kinase are known to play major regulatory roles in cytokinesis. Cdk1 has shown to affect microtubule dynamics and phosphorylate myosin II regulatory light chain on its inhibitory sites, thereby blocking cell cleavage until the end of anaphase [154-157]. Plks may also be important regulators of cytokinesis. Plks colocalize with a kinesin-related motor protein and could phosphorylate this protein in vitro [158], and this motor in turn required for the organization of the central spindle, the formation of a contractile ring and cytokinesis [99, 100, 159]. During cytokinesis, Rho-mediated signaling is required for the regulation of cortical activities during cytokinesis [160, 161] in addition to the recruitment of myosin II and actin filaments to cleavage furrow. There is evidence that Rho-activated citron kinase regulates cleavage furrow contractility [162] and the Rho-kinase regulates separation of the intermediate filaments that extend through the midbody in mammalian cells [163]. Rho-kinase might also regulate contractility by inactivating the myosin II light chain phosphatase, an inhibitor of myosin II activity, in the cleavage furrow [164].

2.4 Calcium signals associated with regulation of cell division

Calcium is a universal signaling molecule. Its importance in regulating muscle contraction, synaptic transmission, neurosecretion, the release of hormones and fluid secretion is well known [49, 50]. Calcium signals are generated by increasing the
intracellular free calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}), through the modulation of channels that regulate calcium fluxes, both through the plasma membrane and through the intracellular membranes of calcium-storing organelles, in particular the endoplasmic reticulum (ER) [61, 62]. Release of calcium from intracellular organelles can be modulated by three known endogenous messengers: InsP\textsubscript{3}, cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP). The existence of multitude Ca\textsuperscript{2+} messenger systems and Ca\textsuperscript{2+} stores can, in principle, provide the cells with versatile means to respond to and differentiate between diverse stimuli that signal through Ca\textsuperscript{2+}. The outcome of increase in [Ca\textsuperscript{2+}], is the activation of secondary messenger proteins, one such protein is the calcium binding protein calmodulin (CaM), which alters cell function by modulating the activity of a variety of kinases, phosphatases and proteases, for example, the calcium-calmodulin kinase family (CaM kinase). Given that calcium signals govern so many varied and diverse cellular functions, it is not surprising that it’s important in regulating the cell division cycle [165].

Findings over the last 15 years or so indicate that the resumption and progression of the cell cycle, both mitotic and meiotic, are accompanied by transient increase in cytosolic Ca\textsuperscript{2+}. They have been observed (a), in late G\textsubscript{1} prior to the initiation of the S phase; (b), in G\textsubscript{2} before entry into the M phase; (c), during mitosis between the metaphase and anaphase, and (d), during cytokinesis [51-60].

2.4.1 Calcium signals and mitosis entry

[Ca\textsuperscript{2+}], elevation has been found to be associated with the NEB (nuclear envelope breakdown). A highly localized brief release of Ca\textsuperscript{2+} from the nuclear envelope
stores or the perinuclear stores was detected during embryonic cell division [65-68]. Calcium transients trigger NEB and entry into mitosis in the sea urchin and mouse embryos and in mammalian somatic cells in culture. Using different Ca^{2+} buffers to blunt Ca^{2+} spikes, the entry into NEB can be inhibited, whereas the photoactivation of a Ca^{2+} spike from a nitr-5 loaded cell or the injection of InsP₃ can stimulate the precocious entry into NEB [54, 65, 66, 166]. For example, injection of the Ca^{2+} chelators EGTA and BAPTA into sea urchin embryos delayed NEB, whereas injection of Ca^{2+} with high free Ca^{2+} concentrations induced precocious NEB. In Swiss 3T3 fibroblasts, artificially generated global Ca^{2+} pulses could precipitate precocious NEB in prophase cells, while increased levels of intracellular Ca^{2+} buffering achieved through microinjection of BAPTA could delay or block NEB in prophase cells [54].

Strong evidence suggests that an InsP₃ triggered calcium signal stimulates mitotic progression via a calmodulin/CaM kinase pathway. There is a peak of phosphoinostide lipid turnover and of InsP₃ concentration just before NEB occurs [167]. Lithium treatment blocks NEB; inhibition can be overcome by microinjection of InsP₃ [168]. Injection of InsP₃ itself induces a precocious NEB [66]. NEB is also prevented by microinjection of the InsP₃ antagonist heparin [167]. These data support a mechanism in which an episode of InsP₃ production just before NEB triggers a calcium signal that induces mitosis. The autoinhibitory domain peptide from CaMKII prevents NEB when injected into sea urchin embryos [169]. A calmodulin inhibitory peptide generated from the calmodulin binding site of myosin light chain kinase also prevents NEB [170], these observations suggest that the calcium signal triggers NEB by activating calmodulin and CaMKII.
2.4.2 Calcium signals and mitosis exit

At the exit of mitosis, when the congression is fully established, cells reach the metaphase-anaphase transition checkpoint. During the metaphase-anaphase transition, the release from the spindle assembly checkpoint mechanism leads to activation of the anaphase-promoting complex (APC) which causes proteolysis of mitotic cyclins and is also essential for disjunction of sister chromatids. There are a variety of earlier reports, using pharmacological agents such as calcium blockers, and Ca\(^{2+}\) antagonists such as lanthanum, or inhibitors of inositol turnover such as lithium, that support the idea of a Ca\(^{2+}\) transient preceding the onset of anaphase [69-71]. The clearest example of calcium’s involvement in anaphase onset comes from fertilization in Xenopus oocytes. Fertilization accompanied by a large calcium transient that leads first to cyclin B destruction [72, 73], presumably through activation of the APC/proteasome pathway [74]. These experiments indicates that at least in the special case of fertilization, a calcium signal can lead to stimulation of APC activity and exit from meiosis.

Is fertilization a special case, or does calcium also control mitosis exit during normal cell division? There is longstanding evidence that calcium may also trigger anaphase onset during mitosis. Calcium signals at mitosis are less dramatic than the large calcium wave of fertilization. For example, a transient increase in intracellular calcium [Ca\(^{2+}\)], occurs throughout the cell as sea urchin embryos enter anaphase of the first cell cycle. The transient just precedes chromatids separation and spindle elongation. Microinjection of calcium chelators or InsP\(_3\) receptor antagonist heparin block chromosome separation [75].
The importance of calcium signals to mitosis in mammalian somatic cells is questionable. Some reports have demonstrated mitosis-associated calcium spikes. In 1983, Izant showed that anaphase in PtK1 cells could be advanced by microinjecting calcium and delayed by injecting calcium-EGTA buffers [171]. The development of fluorescence indicator fura 2 [51] allowed the detection of brief calcium increases at anaphase onset in mammalian cells [52, 76]. However, these calcium transients were shown to occur several minutes before anaphase onset [172]. Inhibition of [Ca$^{2+}$], by BAPTA or EGTA injection in Swiss 3T3 cells can eliminate the [Ca$^{2+}$], transient as well as block the cell division at metaphase. Some reports suggests that calcium changes are small or absent. In Swiss 3T3 fibroblasts and REF52 cells, Ca$^{2+}$ is actively involved in NEB, but Ca$^{2+}$ signals are not likely to be necessary for the metaphase-anaphase transition. Serum removal and permeant BAPTA abolished the calcium spike without preventing anaphase onset [54]. Present evidence in mammalian cell lines leaves the question of calcium's involvement at anaphase unresolved, though it is evident that a calcium signal is essential for entry into mitosis [56, 75, 173].

2.4.3 Calcium signals and cytokinesis

Cytokinesis is another important mitotic checkpoint. It has been suspected that calcium signal may play an important role in regulating cytokinesis [56]. Some early studies reported the elevation of [Ca$^{2+}$], associated with cytokinesis [51, 174]. Using fluorescent and bioluminescent calcium indicators, calcium wave associated with furrow extension were found in medaka fish embryos [77] and zebrafish embryos [59]. In *Xenopus* embryos, a calcium wave was also appeared at the cell cleavage
region after furrow formation [55]. In zebrafish embryos, there are calcium signals associated with furrow positioning, propagation and deepening [78]. Microinjection of BAPTA and heparin blocked the cell cleavage in various embryonic cells [78].

It is well known that cell cleavage depends on the contraction of a submembrane contractile band composed mainly of actin and myosin II [175]. An elevated \([\text{Ca}^{2+}]_i\) could trigger the actomyosin contraction by a mechanism analogous to that found in smooth muscle [156]. When free \([\text{Ca}^{2+}]_i\) is increased in a local region, \(\text{Ca}^{2+}\) can bind to calmodulin and activate a calcium/calmodulin-dependent protein kinase called “myosin light chain kinase” (MLCK). Once activated, MLCK can promote the growth of the contractile band by phosphorylating the Ser-19 site of the regulatory light chain of myosin (MLC) [157]. Such phosphorylation causes a conformational change in the myosin head and exposes its actin-binding site. This change allows the myosin filaments to interact with the actin filaments and triggers the contraction of the actomyosin band. As a result, a cleavage furrow starts to form. This mechanism can thus explain why a localized elevation of \([\text{Ca}^{2+}]_i\) could trigger the formation of the contractile band, it can also explain why a \(\text{Ca}^{2+}\) wave travelling along the cell equator was able to guide the extension of the cleavage furrow. Furthermore, it explains why the post-cleavage \(\text{Ca}^{2+}\) transients were needed to maintain the cleavage furrow structure, since the MLC must be continuously phosphorylated at the controlled site by MLCK in order to maintain the contraction between actin and myosin.

2.5 Calcium signaling pathway in cell division
It is interesting to know how the calcium signal regulates various cellular events in mitosis. The possibility that Ca$^{2+}$ may require the presence of a binding protein instead of acting in its free ionic form was suggested by the data from muscle contraction studies [63]. There are a number of Ca$^{2+}$ binding proteins have been proposed to mediate the effect of Ca$^{2+}$ on the cell cycle. As could be expected, calmodulin has received most of the attention, and a large body of data points to it as the most important Ca$^{2+}$ sensor in the cell cycle. It has been suggested that the calcium signal plays regulatory roles by binding to calmodulin, followed by activation of Ca$^{2+}$/CaM-dependent protein kinase (CaMKII) and myosin light chain kinase (MLCK) [58, 64].

2.5.1 Calmodulin – intracellular calcium receptor

Calmodulin (CaM) is a 17 KD, ubiquitous Ca$^{2+}$ binding protein that is highly conserved throughout eukaryotic species. A typical animal cells contains more than $10^7$ molecules of CaM, which contains much as 1 % of the total protein mass of the cell. It has four EF-hand Ca$^{2+}$ binding sites with different affinities and specificities. Under physiological conditions, CaM contains two high-affinity (Kd < $10^{-5}$) binding sites in the carboxyl-terminus and two low-affinity (Kd $10^{-4}$ to $10^{-5}$ M) sites in the amino-terminus [176, 177]. Binding of calcium with these sites results in a conformational change [178]. Ca$^{2+}$/CaM does not have enzymatic activity. It acts by binding to various target proteins and thereby altering their activities. Binding of Ca$^{2+}$/CaM with its target proteins causes further structural changes of Ca$^{2+}$/CaM [179]. Since its discovery in the early 1970s, calmodulin has been found to regulate many fundamental cellular activities [63].
Early indications for its role in cell division came from experiments in which the mitotic cycle was arrested by anti-calmodulin drugs added to exponentially growing cells or cells re-entering the cycle under the influence of mitogens. Monoclonal antibody against calmodulin also inhibited the synthesis of DNA in permeabilized mammalian cells [180]. In *Xenopus* unfertilized eggs, exit from metaphase is prevented by a cytostatic factor (CSF), which is the product of a proto-oncogene c-mos. Ca²⁺ elevation at the micromolar level is required for the release of the CSF arrested metaphase. A synthetic peptide inhibitor, which is derived from the myosin light chain kinase (MLCK) sequence and binds CaM with high affinity, suppresses cyclin degradation and blocks the exit of metaphase [72, 73, 181]. A role of calmodulin in the cell cycle was also supported by its detection in the mitotic apparatus in rat kangaroo PtK2 cells and Chinese hamster ovary cells [182] and plant endosperm [183] during cell division.

Specific points of CaM intervention in the cell cycle are now considered to be the G1/S boundary, the transition from G2 to mitosis, and the metaphase-anaphase transition. This is supported by experiments in which CaM was constitutively elevated in mammalian cell lines by expressing a chicken CaM gene using a chicken promoter, or decreased by expressing a CaM anti-sense RNA. The inducible increase in the CaM level accelerated the rate of progression through G1 whereas the decreased CaM levels induced by the anti-sense RNA caused arrest in G1, G2 and metaphase [184, 185]. CaM activation during mitosis had been observed directly using a novel fluorescent calmodulin probe GFP-CaM. It was found that its activation occurs at the spindle poles just before anaphase. A calmodulin inhibitory peptide very markedly delays anaphase onset [170]. Recent study in our lab examined the dynamic re-distribution of calmodulin (CaM) in HeLa cells during cell
division by using GFP-CaM fusion gene [80]. We found evidence that CaM may play an active role in determining the position and timing of the cleavage furrow formation. Microinjection of CaM-specific inhibitor Trp-peptide into HeLa cells in early anaphase, cell cleavage was either blocked or greatly delayed. Injection of a point-mutated peptide that has a much lower affinity to CaM did not block cell cleavage and only caused a slight delay. Thus, the blockage of cytokinesis was specifically due to the inhibition of CaM. These findings strongly suggested CaM play an active role in regulating the initiation of cytokinesis.

The role of CaM in cell division is not well understood by now. One hypothesis is that Ca$^{2+}$/CaM regulates mitotic progression by triggering the activation of Ca$^{2+}$/CaM-dependent protein kinases [186, 187]. The mitotic specific targets include multifunctional Ca$^{2+}$/CaM-dependent protein kinase II (CaMKII) and non-muscle light chain kinase (MLCK).

2.5.2 Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII)

Most of the effects of Ca$^{2+}$/CaM are mediated by a family of Ca$^{2+}$/CaM-dependent protein kinases. The multifunctional Ca$^{2+}$/CaM-dependent protein kinase II (CaMKII) is a kinase that has been well characterized in various species. It is serine/threonine protein kinase. Its substrates include microtubule-associated protein II (MAP-2), tau, vimentin, synapson I, and tyrosine hydroxylase [188]. The CaMKII holoenzyme has 12 subunits, including $\alpha$ (54 KD), $\beta$ (58 KD), $\beta'$ (60 KD), $\gamma$ (59 KD), and $\delta$ (60 KD). Each of the subunits can undergo autophosphorylation upon with CaM. Rat CaMKII $\alpha$ subunit has a N-terminal catalytic domain containing the conserved blocks of homology found in serine/threonine kinase, a central regulatory domain containing
the CaM binding region, an autophosphorylated site (Thr-286) and a C-terminal connection domain. The three-dimensional structure model of CaM suggests that by interaction with CaM, CaMKII undergoes a conformational change, exposing Thr-286 for inter-subunit phosphorylation and allowing phosphorylation of other subunits. The autophosphorylation of Thr-286 prevents the reassociation of the autoinhibitory domain with the body of the kinase, thus, the enzyme becomes Ca\(^{2+}\)/CaM independent [189].

Using anti-CaM antibodies and indirect immunostaining methods, people found that CaMKII was co-localized with the mitotic apparatus [190] and was particularly present at the microtubule-organizing center in mitotic 3Y1 cells. During cell division, CaMKII abruptly appeared at the midbody region in the two new daughter cells.

### 2.5.2.1 CaMKII and G2/M transition

Several evidences in a variety of experimental systems suggests a role for CaMKII in G2/M progression. Baitinger have reported that this enzyme mediates the Ca\(^{2+}\)/CaM requirement for nuclear envelope breakdown that is required for resumption of meiosis following fertilization of sea urchin eggs [169]. Planas-Silva and Means revealed that inducible expression of a constitutively active form of CaMKII\(\alpha\) in a mouse cell line resulted in a G\(_2\) arrest [191]. Arrest in G2 by overexpression of a constitutively active form of CaMKII has also been reported in Saccharomyces pombe [192]. These observation imply that not only is a CaM kinase-dependent phosphorylation event(s) required for G\(_2\)/M progression but this substrate(s) must also be dephosphorylated for entry into mitosis to occur.
The prevailing model of cdc2 activation at mitosis involves cyclin-dependent translocation of the cdc2 subunit into the nucleus and autoactivation of cdc2 through a positive feedback loop involving an activating dephosphorylation of cdc2 by cdc25 phosphatase and further activation of cdc25 through phosphorylation by cdc2. This mechanism begs the question of what event triggers the autoactivation loop to begin with. One possibility is that CaMKII may phosphorylate cdc25, which will then dephosphorylate the inactive p34cdc2/cyclin B complex, leading to its activation. It has been found that CaMKII inhibitors KN-93 could prevent cdc25 activation in HeLa cells [193, 194].

2.5.2.2 CaMKII and the metaphase-anaphase transition

Another process which may involve CaMKII is the metaphase-anaphase transition. Experiments on this transition have been performed on frog oocytes which are naturally arrested in the prophase of the first meiotic division. The hormone progesterone eliminates the block, causing meiosis progression to a second spontaneous arrest in metaphase. The release of the second block is promoted by the entry of the sperm and by the associated Ca\(^{2+}\) wave produced by the emptying of the intracellular stores, both the cytostatic factor (CSF) and the mitotic promoting factor (MPF) are inactivated by the Ca\(^{2+}\) upsurge. The inactivation of MPF precedes that of CSF and is mediated by CaMKII: cyclin B degradation was found to occur when a constitutively active mutant of the kinase was added to extracts of metaphase-arrested frog eggs, even in the absence of Ca\(^{2+}\) [72]. Additional support for the role of CaMKII came from the prevention of cyclin B degradation by specific inhibitors, and by the finding that injection of a constitutively active form of the kinase into
unfertilized frog eggs inactivated p34cdc2 in the absence of Ca\textsuperscript{2+} spike. The attractive speculation has been put forward that CaMKII phosphorylation could be a way to activate the proteasome [57, 74].

2.5.3 Myosin light chain kinase (MLCK)

Another important target of the Ca\textsuperscript{2+}/CaM is the myosin light chain kinase (MLCK). Actin and filamentous myosin II undergo precisely regulated reorganization during mitosis, including a disassembly of the stress fibers during prophase to prometaphase, an increase in cortical association during metaphase-to-anaphase and a redistribution in the contractile ring during cytokinesis. The regulatory light chain of myosin II (MLC) could be phosphorylated by myosin light chain kinase (MLCK), along with other protein kinases, such as cdc2 protein kinase and protein kinase C in vitro [156].

Yamakita et al. (1994) demonstrated that in rat embryo REF-4A cells, the phosphorylated forms of MLC increased from \(~\text{16\%}\) in nonmitotic cells to \(~\text{31\%}\) in mitotically arrested cells, and could reach as high as \(~\text{49\%}\) in arrest-released cells when cells start cytokinesis. It was shown that during the prophase and metaphase, MLC is phosphorylated by cdc2 kinase on the negative regulatory sites (Ser-1/2). When in anaphase, MLC is phosphorylated by MLCK on the positive regulatory site (Ser-19). This suggests the involvement of MLCK in regulation of cytokinesis.

However, microinjection of catalytic fragment of MLCK into normal rat kidney (NRK) cells after nuclear envelope breakdown can delay the onset of anaphase with no effects on the time course from the onset of anaphase to mid cytokinesis (Fishkine et al., 1991). These results make the involvement of MLCK in mitotic progression more controversial.
2.6 Source of intracellular calcium elevation

Intracellular \( \text{Ca}^{2+} \) is important in regulating virtually all cellular processes from fertilization, the beginning of life, to apoptosis, the programmed cell death. A principle mechanism is that calcium signals are generated by increasing the intracellular free calcium concentrations \([\text{Ca}^{2+}]_i\), through the modulation of channels that regulate calcium influxes, both through the plasma membrane and through the intracellular membranes of calcium storing organelles, in particular the endoplasmic reticulum (ER). Most of the evidence supports that during cell division, \([\text{Ca}^{2+}]_i\) elevation is from the release of intracellular calcium stores, endoplasmic reticulum (ER). For example, Swiss 3T3 cells are found to be able to go through mitosis in an EGTA-containing medium [53, 54], suggesting that the \([\text{Ca}^{2+}]_i\) may not come from an extracellular source.

One prevalent route for calcium release from the ER is through InsP\(_3\) receptor channels. InsP\(_3\) is generated from phosphatidylinositol bisphosphate (PIP\(_2\)), a membrane lipid, by phospholipase C. Levels of InsP\(_3\) increase at anaphase [167] and peaks of \([\text{Ca}^{2+}]_i\), occur just before mitosis and around anaphase. Dominant negative inhibitory PDGF receptor SH2 domains block the activation of the phospholipase C\(_\gamma\) isoform [195, 196] and markedly delay both entry into mitosis and anaphase onset in sea urchin. InsP\(_3\) production is prevented by treatment of cells with lithium [197], embryos treated with lithium arrest at NEB and during mitosis [168]. Heparin, an inhibitor of InsP\(_3\) receptor channel, prevents NEB when microinjected into sea urchin embryos before mitosis [68] and sister chromatids disjunction when microinjected after NEB, during prophase [75]. Terasaki (1991) has shown that the ER of sea urchin embryos gathers around the mitotic spindle during mitosis. Recently,
Whitaker demonstrated that ER penetrates the spindle and is most abundant at the spindle poles and at the metaphase plate [165]. In the Xenopus system, first cleavage is blocked by microinjection of an anti-phosphoinostide antibody or by heparin [198]. It was also reported that the calcium elevation in the zebrafish embryos could also be blocked by microinjection of heparin [59, 78].

In addition to the InsP₃-sensitive Ca²⁺ stores, two new types have now been functionally defined. One type is sensitive to a novel nucleotide, cyclic ADP-ribose (cADPR), and the other is sensitive to nicotinic acid adenine dinucleotide phosphate (NAADP) [199-201]. These novel Ca²⁺ stores are found to be widely present in all eukaryotes from protist and plant to human cells [200, 202-204]. The mechanisms of Ca²⁺ release activated by these Ca²⁺ agonists are different. The action of cADPR, in conjunction with calmodulin, is to greatly increase the Ca²⁺ sensitivity of the Ca²⁺-induced Ca²⁺ release mechanism such that it can be activated by Ca²⁺ event when it is in nanomolar range. The NAADP mechanism, however, shows no dependence on Ca²⁺ [205-207]. This and self-inactivation property make NAADP ideally suited for functioning as a Ca²⁺ trigger. The localized Ca²⁺ release by NAADP, through interaction with the Ca²⁺-induced Ca²⁺-release mechanisms mediated by cADPR and InsP₃, is then amplified into Ca²⁺ spiking.

In human HeLa cells, cADPR is shown to play a role in regulating the cell doubling time [208]. HeLa cells transfected with CD38, a cADPR metabolising enzyme, show increased consumption of pyridine nucleotides (NAD + NADH), accumulation of cellular cADPR, depletion of Ca²⁺ stores and elevation of basal Ca²⁺ levels. These effects culminate in shortening of the S phase of the cell cycle and reducing the cell doubling time by as much as 75% as compared to untransfected cells [209]. It thus appears that elevation of cADPR can stimulate cellular DNA
synthesis and accelerate cell division. The Ca^{2+} signaling pathways mediated by cADPR and NAADP was widely distributed among species. Moreover, the general characteristics of the two pathways observed in cells from such divergent species as human and protist are remarkably similar, suggesting that these highly conserved signaling mechanisms are likely to confer important evolutionary advantages to organisms.
CHAPTER 3

MATERIALS AND METHODS

3.1 Cell biology techniques

3.1.1 Mammalian cell lines and cell culture

Cell lines used in this study, including HeLa and PtK2, were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell culture medium, fetal bovine serum and antibiotics were obtained from Gibco-BRL, Inc.

Both HeLa and PtK2 cells were cultured as a monolayer in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin (base) and 100 μg/ml streptomycin (base). The medium used was buffered with bicarbonate (30 mM). Cell cultures were maintained at 37°C, 5 % CO₂, in a humidified incubator. This allowed maintenance of the correct pH which was readily monitored by the color of the phenol red present in the medium. The cells were replated every 2 to 3 days when the monolayer reached confluence. For subculture of a cell monolayer, cells were treated with a mixture of trypsin (0.05 mg/ml) and EDTA (2 mg/ml). The trypsin and EDTA mixture should not be left for longer than necessary time. Culture medium was added then to prevent excessive trypsin action.

3.1.2 Cell synchronization

To synchronize cells at prometaphase, HeLa cells in culture plates were treated with nocodazole, a microtubule disrupting drug, at 200 ng/ml for 10-12 hours. Cells
entered mitosis were rounded-up and detached from the bottom. The detached cells were collected by shake-off and washed in PBS for three times to remove the drug. The detached cells released from nocodazole block were in early metaphase and would finish mitosis within one hour. For synchronization with a double thymidine block, cells were grown in the presence of 2 mM thymidine (Sigma) for 18 hours, washed with PBS, and grown in fresh medium without thymidine for 8 hours. Cells were then incubated with 2 mM thymidine for 18 hours to block cells at G1/S boundary.

3.1.3 Immunofluorescence

The cell monolayer growing on coverslip was rinsed three times with PBS and then fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde at room temperature for 15 minutes. The fixed cells were rinsed in PBS and permeabilized in 0.2% Triton X-100 (in PBS) for 15 minutes. Non-specific binding was blocked by incubating cells in PBS containing 3% BSA for 1 hour. Cells were then incubated with anti-cyclin B1 (1:200 dilution, mouse monoclonal antibody, PharMingen), anti-PTTG (1:200 dilution, goat polyclonal antibody, Santa Cruz), anti-p55CDC (1:200 dilution, rabbit polyclonal antibody, Santa Cruz), anti-β-tubulin (1:200 dilution, mouse monoclonal antibody, Sigma) or human autoantibody control ANA positive (1:50 dilution, Sigma) at room temperature for 2 hours. After washing in PBS for 5 times, followed by treatment of secondary antibody at a dilution of 1:200 (rhodamine-conjugated goat anti-mouse antibody, Calbiochem CA; rhodamine-conjugated rabbit anti-goat antibody, Calbiochem CA; FITC-conjugated goat anti-rabbit antibody, Calbiochem CA; TRITC-conjugating goat anti-human antibody, Calbiochem CA) for 1 hour after
thoroughly washing with PBS. Auto fluorescence of glutaraldehyde was quenched with a freshly prepared solution of 0.1% NaBH₄ (in PBS) for 10 minutes. Then the coverslip was washed and mounted onto a slide with moviwol 4-88 (Calbiochem, CA).

### 3.1.4 Western blotting

HeLa cells were synchronized at prometaphase by nocodazole treatment. Cells were washed with PBS three times, either harvested immediately or transferred into fresh medium for 0.5, 1, 1.5, 2, 3, 4, 6 or 8 hours with or without drug treatment and then harvested. Cells were scraped down with disposable cell scraper (COSTAR) and then lysed in lysis buffer (50 mM Tris, pH8.0, 150 mM NaCl, 1% NP-40) with protease and phosphatase inhibitors (50 mM NaF, 100 μg/ml PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 μM EDTA, 10 μg/ml pepstatin A, 1 mM benzamidine, 0.5 μM okadaic acid), the lysates were then centrifuged for 20 minute at 14,000 rpm at 4°C to make the high speed supernatant.

The protein concentrations of cell lysates were determined using Bradford reagent (Bio-Rad). The cell lysates were analyzed by 10% SDS-PAGE, the protein were then transferred to a nitrocellulose membrane, which was blocked with 5% non-fat milk and then incubated with primary antibody for 2 hours at room temperature or overnight at 4°C. The membrane was washed 4 times for 10 minutes each in TBS with 0.2% Tween-20 and then incubated with the horseradish peroxidase-conjugated secondary antibody (Bio-Rad) at 1:5000 dilution for 1 hour. Finally, the blotting patterns were detected using the ECL™ western-blotting analysis system (Amersham Life Science Ltd.).
3.1.5 Chromosome spread

HeLa cells (with or without expressing the GFP-cyclin B1-Δ85 gene) were arrested in M-phase by nocodazole treatment. At 0.5 hr and 3.5 hr after releasing from nocodazole arrest, cells were collected and treated with a hypotonic buffer (10 mM Tris, 10 mM NaCl, 5 mM MgCl₂) for 15 min before collapsing them on a coverslip by cytopsin. The cells were fixed using cold methanol for 20 min, and then incubated with primary (ANA-C) and secondary (TRITC-conjugated anti-human IgG) antibody. DNA was stained with Hoechst 33342. The slides were viewed using a Zeiss Axiovert 35 microscope.

3.1.6 Determining the protein level of cyclin B1-Δ85 in a single cell

The relative amount of GFP-cyclin B1-Δ85 protein was determined by comparing the intensity of GFP fluorescence of each Δ85-positive cell. To convert the GFP fluorescence into protein level, we conducted a calibration experiment based on an immunostaining analysis. We first synchronized the HeLa cells (with or without expressing the GFP-cyclin B1-Δ85 gene) at early M-phase by treating them with nocodazole. Cells were fixed at 0.5 or 3.5 hr after release from the nocodazole treatment. They were then immunostained with anti-cyclin B antibody that recognized both the full-length and truncated cyclin B1 (GNS-11, from PharMingen). At 3.5 hr after nocodazole release, the endogenous cyclin B1 was destroyed. Under this condition, the immunofluorescence intensity of the Δ85-positive cell reflected mainly the protein level of the nondegradable cyclin B1-Δ85, while the immunofluorescence intensity of the control cell (i.e., Δ85-negative) represented the
background signal caused by non-specific staining, which can be subtracted from the other data. At 0.5 hr after release from the nocodazole treatment, the endogenous cyclin B1 had not been degraded. The immunofluorescence intensity of the control cell represented mainly the protein level of the endogenous cyclin B1. Thus, by comparing the average immunofluorescence intensity of Δ85-positive cells fixed at 3.5 hr with the immunofluorescence signal of the control cells at pro-metaphase (which was fixed at 0.5 hr), we could estimate the average protein level of GFP-cyclin B1-Δ85 in comparison to that of the endogenous cyclin B1. Since we can also measure the average GFP fluorescence of the GFP-cyclin B1-Δ85 fixed at 3.5 hr, we can determine the converting ratio between the fluorescence intensity of GFP and the protein level of GFP-cyclin B1-Δ85.

3.2 Molecular biology techniques

3.2.1 Fluorescent protein vectors

Green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria*, has proven to be a useful reporter for monitoring gene expression and protein localization *in vivo* and in real time. GFP expressed alone or in GFP fusion proteins, is superior to conjugated antibodies in FACS applications because there is no need to incubate the cells with a fluorescently tagged reagent and there is no background resulting from nonspecific antibody binding. Furthermore, GFP fluorescence is stable, species independent, and does not require any substrates or cofactors. The pEGFP protein fusion vectors (Figure 2-1) are versatile vectors that provide high-level expression in mammalian systems. In our study, we fused several
important cell cycle genes with GFP, CFP, YFP or DsRed respectively. pEGFP-N1 encodes a red shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm). The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences. Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (6) to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-N1 is between the immediate early promoter of CMV (Psz) and the EGFP coding sequences. Genes cloned into the MCS will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. A neomycin-resistance cassette (neo), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex thymidine kinase gene, allows stably transfected eukaryotic cells to be selected using G418.

pDsRed1-N1 encodes a novel red fluorescent protein that has been optimized for high expression in mammalian cells (excitation maximum = 558 nm; emission maximum = 583 nm). RFP was isolated from an IndoPacific sea anemone-relative, Discosoma sp. DsRed1’s coding sequence contains 144 silent base pair changes, which correspond to human codon-usage preferences for high expression in mammalian cells. Sequences upstream of DsRed1 have been converted to a Kozak consensus translation initiation site to increase translation efficiency in eukaryotic cells. The MCS is between the immediate early promoter of CMV (Psz) and the DsRed1 coding sequence. Genes cloned into the MCS as described below are expressed as fusions to the N-terminus of DsRed1.
Figure 3-1  Restriction map of the cyclin B1-GFP construct.

The human cyclin B1 gene was cloned behind the CMV promoter and upstream of EGFP between HindIII and BamHI sites in the MCS box of a pEGFP-N1 (Clontech) vector.
3.2.2 Fusion gene constructs

3.2.2.1 Cyclin B1-GFP and cyclin B1-DsRed

The human cyclin B1 gene was isolated from human bone marrow cDNA library. An HindIII site was introduced 5' to the start codon of human cyclin B1 gene and a BamHI site to the stop codon, by the by the polymerase chain reaction (PCR) using the following oligonucleotides: 5' CTCAGGCTTGGCATGGCGGCTCCGAGTCAC and 5' CCGGATCCCGACCTTTCTCCACAGGCTTGG. The PCR product was then cloned behind the CMV promoter and upstream of EGFP between HindIII and BamHI sites in the MCS box of a pEGFP-N1 or DsRed-N1 (Clontech) vector.

3.2.2.2 GFP-cyclin B1-Δ85 and CFP-cyclin B1-Δ85

Truncate cyclin B1-Δ85 was cut out from the full-length human cyclin B1 gene by KpnI and BamHI digestion and subcloned behind EGFP or ECFP at KpnI and BamHI sites in the MCS of pEGFP-C3 or pECFP-C3 vector. The newly generated EGFP-cyclin B1-Δ85 fusion gene used the stop codon provided on the vector sequence (Figure 3-2).

3.2.2.3 Securin-YFP

Human securin (hPTTG) gene was kindly provided by Francisco Ramos-Morales (Departamento de Microbiologia, Facultad de Biologia, Universidad de Sevilla, Spain). The gene was amplified by PCR using the following oligonucleotides:
Figure 3-2  Full-length cyclin B1 and cyclin B1-Δ85.

Comparison of the primary structure of wild-type human cyclin B1 with the truncate cyclin B1 used in this study. The stippled area indicates the location of the wild-type destruction box [210]. Cyclin B1-Δ85 lacks the 85 N-terminal amino acids of the wild-type protein.
5' CGGGGTACCATTGCTACTCTGATCTATGT GG and 5' GCCCCGCAGTAAT ATCTATGTCACAG CAAACAG. The PCR product was then cloned behind ECFP at KpnI and SacII sites in the MCS of pECFP-C1 vector. After we got EYFP-securin fusion gene, we found this fusion protein could not be degraded during the cell division because the N-terminal destruction box was fused with ECFP. So we cut out securin gene from pECFP-C1 vector with BamHI site first and fill in the sticky end with Klenow, then digested with XhoI. pEGFP-N1 vector was digested with EcoRI and XhoI site. Then the gene and vector was fused with T4 DNA ligase. The Construct was sequenced using Big dye terminator v2 kit and 3100 Genetic Analyzer (Applied Biosystems).

3.2.2.4 GFP-Mad2

Human Mad2 gene was kindly provided by Dr. Guowei Fang. The gene was amplified by PCR using the following oligonucleotides: 5' GAAGATCTCGCCA TGGCGCTGCAGCTC, 5' CGGAATTCCGGTCATTTGACAGGAATTTTGTAG. The PCR product was cloned behind EGFP at BglII and EcoRI sites of pEGFP-C3 vector (Clontech). In order to get ECFP-Mad2 and EYFP-Mad2 fusion constructs. Mad2 gene was cut out from EGFP-Mad2 with BglII first and filled in the sticky end with Klenow, then digested with EcoRI. pEYFP-C1 or pECFP-C1 vector was digested with XhoI first and filled in the sticky end with Klenow and then digested with EcoRI. The gene and vector was fused with T4 DNA ligase. All the construct was sequenced using Big dye terminator v2 kit and 3100 Genetic Analyzer (Applied Biosystems).
3.2.2.5 GFP-Mad1

Human Mad1 gene was also kindly provided by Dr. Guowei Fang. The gene was amplified by PCR using the following oligonucleotides: 5' CCCAAGCTTACCA TGGAAGACCTGGGGG and 5' CGGAATTCGTTTCACAAGGTGAGGAACCC. The PCR product was cloned behind EGFP at HindIII and EcoRI sites of pEGFP-C3 vector.

3.2.2.6 GFP-Plk1 and mutants

An XhoI site was introduced 5' to the human Plk1 and EcoRI site 3' to the stop codon by the PCR reaction using the following oligonucleotides: 5' CCGCTCGAGACCATGAGTGCAGTGACTG and 5' CGGAATTCGGGGAGGCCTTGA GACGGTTG. The PCR product was then cloned into pEGFP-C3 (V164A) vector. The construct was sequenced using Big dye terminator v2 kit and 3100 Genetic Analyzer (Applied Biosystems).

Site-directed mutagenesis in human Plk1 cDNA was carried out by using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). The T210D, K82R and N181A mutants of GFP-Plk1 was created by PCR using pairs of oligonucleotides with following sequences: T210D, 5' GAGAGGAAGAAGGACCTGTGTTGGAC and 5' GTCCACACAGGTCTTCTTCTCTCTC; K82R, 5' GTTCGCGGGGCTTGT GCCTAAG and 5' CCTAGGCACAATCTGCCCCGCAAC; N181A, 5' CTCAAG CTGGGCGCCCTTTTCTCTGAATG and 5' CATTCAAGAAAGGCGCCACGC TTGAG. All mutations were confirmed by sequencing.
Figure 3-3  Schematic representation of wild type and mutant Plk1.

Plk wide type and mutant Plk1 used in this study are depicted. The catalytic domain and polo-box are represented by gray box and deviant lined box, respectively. From top to the bottom: full-length wild type Plk1 (WT); kinase-defective mutant Plk1 (K82R); kinase-defective mutant (N181A); constitutive active mutant Plk1 (T210D).
3.2.3 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify a segment of DNA that lies between two regions of known sequence. A typical PCR reaction mainly contained the following steps. In a sterile 0.5-ml microfuge tube, the following was mixed in order: 10 × PCR reaction buffer (250 mM KCl, 100 mM Tris.Cl (pH 8.85 at room temperature), 50 mM (NH4)2SO4, 20 mM MgSO4), mixture of four dNTPs, each at a concentration of 1.25 mM, 5’ primer 5 pmol, 3’ primer 5 pmol, template DNA (100-200 ng, depending on the concentration of target sequences), dH2O to a final volume of 50 μl. The reaction mixture was heated for 2 minutes at 96°C to denature the double-strand DNA completely. While the mixture is still at 96°C, 1.5 μl of Pwo DNA polymerase (Roche Applied Science, Germany) was added. Then the amplification was carried out as described below. Typical conditions for denaturation, annulling, and polymerization were as follows: first cycle, denaturation 1 minute at 96°C, annealing 1 minutes at 45-55°C depends on the Tm of primer, polymerization 3 minutes at 72°C. The subsequent cycle repeated all these steps for more than 20 times. After the last cycle, polymerized 15 minutes at 72°C, withdraw a sample of the amplified DNA from the reaction mixture and analyzed it by gel electrophoresis.

3.2.4 Bacteria transformation

The appropriate pEGFP vector and cDNA fragment was fused to create an in-frame fusion. The ligation product was mixed with 100 μl of competent E.Coli DH5α cells and placed on ice for 30 minutes. Then the mixture was heated for 90 seconds at
42°C. The mixture was then left on ice for 5 minutes. Four hundreds μl of LB was immediately added to the bacteria and was then incubated with shaking at 37°C for 1-3 hours. Cell culture was spread on the selective LB plates (50 μg/ml kanamycin or 100 μg/ml ampicillin) and incubated overnight at 37°C. The transformants were then selected and confirmed by restriction enzyme digestion analysis.

3.2.5 Plasmid DNA purification

High Pure Plasmid Isolation Kit (Boehringer Mannheim) was used in this study to purify small scale plasmid DNA from bacteria. A single colony was selected from bacteria plate and incubated in 2-4 ml LB medium with antibiotics (100 μg/ml ampicillin or 50 μg/ml kanamycin) for 12 to 16 hours, cells were collected by centrifugation. The isolation protocol was provided by the manufacture. The total amount of plasmid DNA yielded according to this protocol was 3-6 μg for E.coli DH 5α cells. To obtain more amount of plasmid DNA, a QIAGEN Plasmid Midi Kit (QIAGEN) was used to prepare large scale plasmid DNA. Two ml of overnight culture from a single colony picked from a selective plate was transferred into 100 ml LB medium and cultured for 12 to 16 hours. The purification protocol was followed by the manufacture. Usually, the expected DNA yield was 300-500 μg from 100 ml of culture for high-copy plasmids and 20-100 μg for low-copy plasmids.

3.2.6 Introduction of plasmid into mammalian cells

There are many methods have been developed for introducing cloned eukaryotic DNAs into cultured mammalian cells, such as calcium phosphate, liposomes,
microinjection and electroporation. In this study, electroporation technique was mainly used to introduce the GFP-labeled fusion genes into mammalian cells. The protocol is described in Section 3.4.2.

For stable transformation, following 24-48 hours after transfection, the cells were split into various dilutions into fresh medium containing G418 (500 µg/ml, Gibco-BRL). G418 is an antibiotics, and can be used to isolate a single stable cell line expressing the fusion construct. The cells were feed with selective medium every 3-4 days until foci are identified. Colonies may be picked and transferred to new plates, grow cells to near confluence before expanding to larger plates. To maintain stable cell lines, the same concentration of G418 for selection should be added into the medium.

3.3 Imaging techniques

3.3.1 The real-time living cell imaging system

We have developed specialized methods to study in vivo signal transduction by combining advanced imaging techniques with newly developed molecular biology tools. Particularly, we labeled various signaling molecules with GFP (green fluorescent protein) by fusing their genes together and then monitor their movement within the living cell using state-of-the-art optical techniques. We have successfully used such techniques to obtain in vivo information on the translocation, activation and protein-protein interaction of several important signaling molecules in this study.

3.3.1.1 Assembly of the imaging system
In order to study the spatial and temporal dependent distribution, translocation and degradation of the signaling molecules, we used a custom-built imaging system to record fluorescent images in living cells. The assembly of our highly sensitive living cell imaging system is described as following:

A Zeiss Axiovert 35 inverted microscope was mounted on a vibration isolation table (TMC). A filter wheel changer with a shutter was mounted on the excitation light pathway. The filter wheel was connected to a controller (Lambda-10, Sutter Instrument Company, Novato, CA). The controller was connected to an IBM compatible PC through the parallel port. The filter position and the position of the shutter could be controlled from the front panel of the controller or from the imaging software.

The filter wheel has 10 positions for filter installation. Various excitation filters were installed on the filter wheel, such that the excitation wavelength could be changed by changing the filter position. Besides, Two neutral density filters can be slided in and out easily. The shutter was controlled by the computer so that the sample would not be exposed to excitation light between observations.

A micro-incubator (LU-CB-1, Medical System Corp., Greenvale, NY) was mounted on the stage of the microscope. The temperature of the micro-incubator was controlled by the temperature controller (TC-202, Medical System Corp., Greenvale, NY).

A digital CCD (charge-coupled-device) camera with cooling system (MicroMax, Princeton Instruments, Ltd., Trenton, NJ) was mounted on the binocular phototubes of the Zeiss microscope through a “C” mount. This digital CCD camera has an air cooling system, and can be cooled down to –40°C. It can be used for collecting fluorescent signals as well as transmitted light images, and has the
capacity to provide 12-bit gray level digital image which is equivalent to 4096 gray levels, giving much more flexibility than 8-bit images.

A SPOT camera (SPOT-JR, Diagnostic Instruments, Inc.) was also used in this study. This camera, supplied with a Kodak KAF - 400 CCD, provides a dual purpose 24 or 36 bit color / 8 or 12 bit monochrome system. Because the "SPOT Jr." still uses 3-shot digital technology in which the red, green and blue acquisition is taken separately, the resulting resolution is higher than many other digital cameras that take "1-shot".

3.3.1.2 Imaging acquisition

For imaging acquisition, a set of software called MetaMorph imaging system (Universal Imaging Corp., West Chester, PA) was used to control imaging acquisition for the digital camera through a device driver of the camera provided by the Universal Imaging Corp. The 12-bit digital image data were converted into 16-bit image by adding extra empty 4-bit on the top of the 12-bit data. The image data were stored as 16-bit on the hard disk of the computer. For outputting image to other imaging analysis software, the 16-bit images were converted into 8-bit images using MetaMorph.

Our imaging system has been integrated so that the temperature of the sample, the exposure and intensity of the excitation light, the excitation wavelength, and the image acquisition and recording can be fully automated. Using this fluorescence imaging system, we can trace the variations of the fluorescence probes in living cells continuously for a long period without disturbing normal cellular processes.
The living cells were maintained at 37°C and stable pH for a long period using the temperature controller. In order to reduce photo damage [211], the coverslip was mounted onto a chamber containing an observation medium (Hepes-buffered Dulbecco's MEM containing 4 mM glutathione, 1 mM L-ascorbic acid, 0.5 mM DTT, pH 7.4). Using neutral density filters with different T%, the excitation light intensity can be adjusted from 100% to 3% of the original intensity. In order to minimize photo damage to the cells, the shutter-gated excitation light was only opened during the period when an image was recorded (using 0.5 to 2 seconds exposure). The shutter then was closed until the next image recording.

A MetaMorph “journal” which is a composition of several commands was created to perform the data acquisition automatically. The journal consisted of the following steps: (1) Move the correct excitation filter to the excitation light path. (2) Open the shutter on the filter wheel changer. (3) Acquisition the image data. (4) Close the shutter. To perform a time-series observation, the journal was looped at adjusted time interval. The recorded images can be further processed off-line to enhance its quality.

### 3.3.1.3 Imaging analysis

The image data obtained by digital camera or other sources including confocal microscope were further processed and analyzed using various imaging processing software, including COMOS v7.0 (Bio-Rad), MetaMorph v3.0-4.6 (Universal Imaging Corp.), Confocal Assistant v4.0 (Bio-Rad), Adobe Photoshop v4.0-6.0 (Adobe Systems Inc.). COMOS and Confocal Assistant are downloaded from Bio-

The projection of z-series confocal image stack was done using COMOS. Image montages were made using Confocal Assistant, The montage images were converted into Bitmap images readable by Adobe Photoshop.

### 3.3.2 Confocal microscope observation

To study the distribution and translocation of some regulatory proteins in mitosis in mammalian cells, we used confocal microscopy instead of our custom-built imaging system. The reason is that some cell-cycle regulatory proteins are specifically localized in the chromosome region, the kinetochores or mitotic spindle, but the cells is not evenly thick, it’s difficult to get a clearly focused image with a conventional microscope. Therefore, we need the confocal microscope to examine the optical sections of the cell.

A laser-scanning confocal microscope MRC600 from Bio-Rad Labs (Hercules, CA) was used to study the fluorescence signals from living and fixed samples. The laser scanning module was adapted to a compound microscope (Zeiss Axioskop). The confocal microscope was equipped with a krypton/argon mixed gas laser, which is capable of providing three separated laser lines at different wavelength: 488 nm, 568 nm, and 657 nm. To lower the extensive exposure of laser light to the specimen, several neutral density filters (ND) were mounted on a filter wheel, which was installed on the light path of excitation laser. ND 2 to3, which was equivalent to 3 to 1% of the total laser transmission, was usually used. The scanning module has two photo-multipliers with independent pinhole sizes, “Gain”, and “Black Level” control.
allowing two-channel measurement. The objectives used were Zeiss Plan-Neofluar Plan-Neofluar 10×/0.30, Plan-Neofluar 40×/0.75, Plan-Neofluar 63×/1.25 oil, Plan-Neofluar 100×/0.30 oil. The focal depth can be controlled by a focus motor. The pixel size of the confocal image is dependent on the objective used and zoom factors.

For living cell measurement, cells transfected with the fusion construct were grown on a coverslip. Then the coverslip was mounted on to a chamber containing an observation medium (Hepes-buffered MEM containing 4 nM glutathione, 100 M L-ascorbic acid, 0.5 nM DTT, pH 7.4). The cells were examined under the confocal microscope with a heating box to maintain the temperature at 37°C.

When doing two-channel measurement, the laser lines of 488 nm and 568 nm were used to observe the distribution of GFP-labeled protein and immunostaining patterns labeled with rhodamine or DsRed-labeled protein. The emitted signals were separated by the K1/K2 filter blocks and collected by the two photo-multipliers simultaneously. In order to monitor the temporal variation of the fluorescence signals, the “Gain” and “Black Level” of the photo-multipliers were fixed throughout each z-series or time-series measurement.

In order to improve the temporal resolution, a half-frame display box (384 × 256 pixels) and normal scan speed (512 lines/second) with a Kalman filter (average of 2-3 frames) were selected in most of the measurements.

The scanning and image collection were controlled by the computer software COMOS v7.0, through an IBM-PC compatible computer (AST Manhattan V series 5090). All images were saved in Bio-Rad PIC format.

3.3.3 Fluorescence microscopes and filter sets
Zeiss Axiphot, Axioskop, and Axivert 35 microscope (Carl Zeiss, Oberkochen, Germany) were used in this study. The objectives used were Zeiss Plan-Neofluar 10×/0.30 Ph1, Plan-Neofluar 20×/0.50 Ph2, Plan-Neofluar 40×/0.75 Ph2, Plan-Neofluar 40×/1.25 oil Ph3, Plan-Neofluar 63×/1.25 oil Ph3, Plan-Neofluar 100×/1.25 Ph3.

Several sets of fluorescence filters were used for examining various fluorophores. There were purchased from either Carl Zeiss (Germany) or Omega Optical Inc. (Brattleboro, VT). The following are the terms used to describe the fluorescence filters sets used in this study:

“DAPI filter” — Zeiss 48 79 02 (G365/FT395/LP420) or Omega XF05 (only use excitation filter 365HT25). For DAPI, Hoechst 33342, etc.

“FITC” filter” — Zeiss 48 79 09 (BP450-490/FT510/LP520). For fluorescein (FITC), GFP, YFP, etc.

“Rhodamine filter” — Zeiss 48 79 15 (BP546/FT580/LP590). For rhodamine, DsRed, etc.

“CFP/YFP FRET set” — Omega XF88 (440DF20/455DRLP/480DF30/535DF25). For CFP/YFP fusion protein. Emitter 480DF30 is for CFP and Emitter 535DF25 is for YFP.

3.3.3 Fluorescence dyes

The fluorescence dyes used in this study, such as fluorescein dextran (10 KD), rhodamine dextran (10 KD), and Hoechst 33342. DAPI, PI were purchased from Molecular Probes Inc. (Eugene, OR).
3.4 Electroporation techniques

In this study, we utilized a special method of electroporation to introduce plasmid DNA or cell-impermeable reagents into the cultured mammalian cells [79].

3.4.1 Electroporation apparatus

The radio-frequency (RF)-oscillating electric pulse generator was designed by Prof Donald C. Chang, and constructed by the Electronic Workshop of HKUST. The wavelength of the output electric pulses were monitored using a digital storage oscilloscope (Tektronix 2221A).

3.4.2 Electroporation with cell suspension

In this study, most of the fusion genes were transfected into cell suspension using electroporation method. Cells grown in the mid-log phase were trypsinized and then suspended in the Poration Medium (260 mM Mannitol, 5 mM sodium phosphate, 10 mM potassium phosphate, 1 mM MgCl₂, 10 mM Heps, 4 mM ATP; pH 7.3) in a concentration of 4×10⁵ per ml. One hundred μl of the cell suspension was mixed with 2-4 μg of plasmid DNA and put into a plastic cuvette with 1 mm gap of aluminum plates (Bio-Rad Labs). The cuvette was then placed into an electroporation chamber (Bio-Rad Labs) connected to the RF-oscillating electric pulse generator. Two trains of electric pulses were applied to the cell/DNA mixture suspension with an interval of 10 sec between each shock. After electroporation, cells were incubated in the Recovery Medium (Poration Medium supplemented with
2 mM MgCl$_2$ and 10% FBS) for 20 minutes and then returned to normal culturing. Cells were usually cultured for at least 20 hours for expressing the introduced fusion genes.

3.4.3 *In situ* electroporation with attached cells

Most of the cell-impermeant reagents were loaded into the attached cell by *in situ* electroporation. The advantage of this method is that: 1) the loading efficiency is much higher; 2) the cells is exposed to less damage without the trypsin treatment; 3) in the inhibitor experiments, the region outside the electroporation fields can used as an internal control in the same coverslip.

Cells grown on the coverslip were washed with Poration Medium (PM) before electroporation. The cell-impermeant reagent was mixed with rhodamine-dextran (10 KD) (100 μM) in PM. The coverslip was then mounted onto the special chamber of the electroporation apparatus. Fifty μl of PM mixture was applied to cover the cell monolayer. A set of electrodes was laid on top of the cell layer. Two trains of RF electric pulses (pulse amplitude 1.0 kV/cm, pulse width 2 ms, 10 pulses per train, 1 sec between pulses, 10 sec between trains) were applied to the electrodes. Only cells between the parallel electrodes were electroporated. After electroporation, cells were incubated in the recovery medium (RM) for 15 minutes before returning to normal culture medium for at least two hours. During electroporation, only the plasma membrane was permeabilized. Therefore, reagents contained in the PM were loaded only into the cytosol of the electroporated cells. Rhodamine-dextran was used here to indicate the cells with the reagent loading under a fluorescence microscope.
3.5 Materials

3.5.1 Chemicals

The major chemicals having been used in this study were listed in Table 3-1.

Table 3-1 List of chemicals

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<tr>
<td>Mouse anti-cyclin B, monoclonal</td>
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<td>Mouse anti-CaM KII, monoclonal</td>
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<td>Mouse anti-β-Tubulin, monoclonal</td>
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<td>Goat anti-rabbit IgG, HRP-conjugated</td>
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**Tissue culture**

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**Fluorescent dyes**

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<td>Propidium iodide</td>
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3.5.2 Solutions

(1) Poration Medium (PM)

Stock solution:

500 mM Na’PO₄, pH 7.3: 80 % Na₂HPO₄, 20 % NaH₂PO₄

500 mM K’PO₄, pH 7.3: 80 % K₂HPO₄, 20 % KH₂PO₄

Working solution:

260 mM Mannitol, 5 mM Na’PO₄, 10 mM K’PO₄, 1 mM MgCl₂, 10 mM Hepes

(2) Recovery Medium (RM)

Poration Medium + 10 % FBS + 2 mM MgCl₂

(3) 10×SDS-PAGE running buffer:

0.25 M Tris-base, 1.92 M glycine, 1% SDS

(4) Transfer buffer

38.6 mM glycine, 48 mM Tris-base, 20% methanol, 0.037% SDS

(5) Stripping buffer

100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris.HCl (pH 6.7)

(6) Lysis buffer

50 mM Tris (pH 7.7), 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 10% glycerol

2 mM EDTA, 20 µg/ml aprotinin, 50 mM NaF, 0.5 µM okadaic acid, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 mM Benzamidine, 10 µg/ml chymostatin,

100 µg/ml PMSF

(7) 50× TAE

242 g Tris base, 57.1 ml glacial astric acid, 100 ml 0.5 M EDTA per liter

(8) Living cell imaging observation medium

Hepes buffered (7.5 mM Hepes, pH 7.4) MEM, 4 mM glutathione (reduced),
1 mM L-ascorbic acid, 0.5 mM DTT

(9) 10× PBS

137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄

(10) Trypsin/EDTA

20 g/l EDTA, 2.5 % Trypsin in PBS

(11) Immunostaining fixation buffer

4% paraformaldehyde + 0.1 % glutaraldehyde in PBS
CHAPTER 4

Degradation Of Cyclin B Is Required For The Onset Of Anaphase In Mammalian Cells

4.1 Specific aim and rationale

Two types of enzymes have key roles in controlling chromosome behavior and its co-ordination with cell division: Cdc2/cyclin B complex and anaphase-promoting complex or cyclosome (APC/C). Cdc2/cyclin B complex triggers breakdown of the nuclear envelope and the formation of a mitotic spindle that can capture microtubules. Cyclin B degradation by the APC/C causes inactivation of Cdc2, which triggers cytokinesis and is a prerequisite for a new round of chromosome duplication [117].

It is well know that cyclin B must be degraded to inactive MPF (M-phase-promoting factor) in order to exit mitosis [88, 90, 212-217]. But is inactivation of MPF required for the metaphase to anaphase (M/A) transition? There have been many conflicting reports on this topic [12, 133, 212, 216-222]. The prevailing view was that inactivation of MPF is not required for the onset of anaphase [1, 106]. It was demonstrated in an in vitro study using Xenopus egg extract that degradation of cyclin B (and thus the inactivation of MPF) was not required for the separation of sister chromatids [218]. A similar conclusion was reached in the in vivo study of budding yeast which reported that the destruction of MPF activity is not required for the M/A transition [219]. Recently, Stemmann et al [133] extended the in vitro study of Holloway et al [218] and found that when a high concentration of nondestructable cyclin B was added to the Xenopus extract, sister chromatid separation could be
blocked. They attributed this blockage to an inhibitory phosphorylation of separase which might be caused by high kinase activity of MPF. Their findings now raise a very important question: In an \textit{in vivo} vertebrate system, is cyclin B required to be degraded before the onset of anaphase?

In this study, we utilized living cell imaging techniques to answer this question. In order to measure the dynamic change of the cyclin B level within a single living cell, we fused the wild type and nondestructable form of cyclin B1 (which is the major form of mitotic cyclin in mammalian cells) [223] with fluorescence proteins such as GFP [224] and DsRed [225]. The protein stability of cyclin was then correlated with several key cellular events, including sister chromatid separation and the degradation of securin. To ensure the general applicability of our results, we used both transformed cells (HeLa) and non-transformed cells (PtK2) as model systems. Our experiments were designed to clarify two major points: (1) In mammalian cells, will the nondegradable cyclin B prevent the onset of anaphase? (2) Is the cell cycle arrest caused by the nondegradable cyclin B an artifact, or, is it physiologically relevant?

4.2 Results

4.2.1 The expression and distribution of securin-YFP in mammalian cells

The first securin to be identified were the Cut2 and Pds1 proteins from fission yeast and budding yeast respectively. Both proteins are degraded at the metaphase-anaphase transition via ubiquitin-mediated proteolysis in a process that involves the activity of APC. The human securin turned out to be identical to the protein encoded
by Pituitary Tumor Transforming Gene (hPTTG) [45], which is abundantly expressed in several neoplasms and its over-expression leads to transformation of NIH3T3 cells. In order to study the temporal and spatial distribution of human securin in mitosis in living cells, we fused human securin (hPTTG) gene with the pEYFP-N1 vector, then introduced the securin-YFP fusion gene into HeLa cells or PtK2 cells by electroporation. Then we examined the expression level of securin-YFP in comparison to the endogenous hPTTG protein using Western blotting analysis, the result was shown in Figure 4-1A. HeLa cell extract, HeLa cells expressed securin-GFP and HeLa cells expressed securin-YFP were stained with anti-GFP and anti-PTTG antibody respectively. The endogenous hPTTG was about 22 Kda, it was only detected by anti-PTTG antibody, the fusion protein is about 49 Kda, which was detected by both anti-GFP and anti-PTTG antibody. This result indicated that the securin-YFP fusion gene can be used to monitor the endogenous securin.

We examined the localization of securin-YFP in living HeLa and PtK2 cells, the localization of human securin is cell-cycle dependent (Figure 4-1B). During interphase, securin-YFP fusion protein were evident throughout the cell, but it was more concentrated in the nucleus than in the cytoplasm (Panels a & e). The fusion protein was well visualized from prophase (Panels b & f), prometaphase (Panels c & g) to metaphase (Panels d & h), it was distinct from chromosomes but remained elsewhere. Immunostaining results showed that the colocalization of human securin with microtubule asters in prophase and prometaphase. During anaphase and telophase, securin-YFP disappeared from the cells.
Figure 4-1  The expression and distribution of securin-YFP in HeLa cells.

(A) Expression of human securin-YFP in HeLa cells examined by Western blotting analysis. Cell lysate from HeLa cells (Lane 1 & 4), HeLa cells transfected with securin-YFP (Lane 2 & 5) or securin-GFP (Lane 3 & 6) were subjected to Western blotting assay. The antibodies against hPTTG and GFP were then used to detect the specific proteins. (B) The distribution of securin-YFP in HeLa cells. Fluorescent images of securin-YFP fusion protein and fluorescent images of nucleus which was stained with Hoechst are shown. Scale bar, 10 μm.
4.2.2 The temporal and spatial proteolysis of securin-YFP in mitosis in living mammalian cells

Using securin-YFP as a fluorescence marker, we can monitor the spatial and temporal dynamic proteolysis of human securin during the metaphase-anaphase transition in a single living cell. We examined the proteolysis of securin-YFP in both HeLa and PtK2 cells using confocal microscope. It was found that, like yeast system, securin was degraded before the sister-chromatid separation (Figure 4-2).

By using time-lapse fluorescence microscopy, we examined when and where securin-YFP begin to degrade. We chose PtK2 cells as our model system because they are flat, typically contain 11 large chromosomes and have been used extensively in studies of spindle checkpoint. Securin-YFP fusion gene was introduced into PtK2 cells by electroporation, then we examined the proteolysis of the fusion protein in mitotic PtK2 cells. As shown in Figure 4-3B, securin-YFP had a slightly higher concentration on mitotic spindle in prometaphase and early metaphase. In mid metaphase, the fluorescence throughout the cell decreased rapidly to reach close to background level when the sister-chromatid began to separate. We measured the change in fluorescence of the total cellular securin-YFP throughout mitosis (Figure 4-3A) in four different cells. It was found that the fluorescence of securin-YFP rose to peak from prometaphase to metaphase. Degradation of the fusion protein began at early metaphase and it decreased rapidly and reached close to background levels by the time of anaphase began. The rapid decrease in securin-YFP fluorescence in mitosis represented the proteolysis of endogenous securin. Our measurements showed that like budding yeast, the degradation of securin occur before the sister-chromatid separation in mitosis in mammalian cells. We also measured the dynamic
Figure 4-2  Confocal microscopy examination of the degradation of securin-YFP in HeLa and PtK2 cells.

Human securin-YFP was introduced into HeLa (A) or PtK2 cells (B) by electroporation. After 24 hours of expression, the fusion protein was examined by confocal microscopy with FITC filter. The fluorescent images were taken at the indicated time points. Scale bar, 10 μm.
Figure 4-3  Real time visualization and measurement of human securin-YFP proteolysis.

(A) Proteolysis of human securin-YFP in PtK2 cells during mitosis. PtK2 cells expressed securin-YFP fusion protein were measured at the indicated time points in a set region (the whole cell). Levels of fluorescence were normalized to the maximum value at late prometaphase or early metaphase. The decrease of fluorescence during mitosis accurately reflected changes of fusion protein. Stages of mitosis are shown at the top of the figure. P/M, prometaphase; M, metaphase; A, anaphase; T, telophase. These data are representative of four different cells in 10 separate experiments. (B) Phase contrast images and YFP fluorescence images of a representative PtK2 cell that expressed human securin-YFP fusion protein going through mitosis. All fluorescence images were taken with the same exposure time with a cooled CCD camera. Scale bar, 10 μm.
proteolysis of securin-YFP in HeLa cells and the results were very similar.

4.2.3 The expression and distribution of cyclin B1-GFP in mammalian cells

The prevailing model of late mitotic events is that Pds1/Cut2 (securin) is degraded before cells enter anaphase, cyclin B is degraded later in mitosis. Immunostaining studies on animal cells have been interpreted as showing that cyclin B is degraded at the metaphase-anaphase transition or later [90, 92, 226]. However, immunostaining has inherent flaws as a quantitative technique, because when cells are fixed and permeabilized, any protein that is not crosslinked into insoluble materials will be lost. Immunofluorescence method can only give a qualitative measure of when a protein is lost from a cell, and does not pinpoint the time when its destruction begins.

We fused human cyclin B1 gene with pEGFP-N1 vector (Clontech) and then introduced the fusion gene into HeLa cells and allowed it to be expressed, the gene product, cyclin B1-GFP fusion protein can be observed easily using a fluorescence microscope equipped with a FITC filter. Figure 4-4 showed the distribution of cyclin B1-GFP in HeLa cells (A) and PtK2 cells (B). Cyclin B1-GFP was distributed more or less evenly in the cytoplasm in interphase. Panel a-d showed the distribution of GFP labeled cyclin B1 in interphase and mitosis. It can be seen that most of the cyclin B1 was excluded from the nucleus in interphase; As cells entered late G2, some amount of cyclin B1 start to enter into the nucleus. Then the fusion protein concentrated at the nucleus at the prophase. After nuclear envelope breakdown, cyclin B1-GFP bound to the mitotic apparatus, particularly to the spindle fibers and spindle poles. Cyclin B1-GFP also transiently associated with condensed chromosomes at the beginning of metaphase. When cells entered anaphase, cyclin B1-GFP was disappeared from the cells.
Figure 4-4  The distribution of cyclin B1-GFP in HeLa cells and PtK2 cells.

Cyclin B1-GFP fusion gene was transfected into HeLa cells (A) or PtK2 cells (B) by electroporation. The fluorescence images of cyclin B1-GFP fusion protein (a-d) and Hoechst staining (e-h) are shown. Distribution of cyclin B1-GFP in interphase (a-b, e-f) and mitosis (c-d, g-h) are shown. Scale bar: 10 μm.
To verify that the distribution of cyclin B1-GFP was similar to that of the endogenous cyclin B, we used antibody against cyclin B to do immunostaining and the results were shown in Figure 4-5A. Comparing the images in the two-side panels, the distribution pattern of cyclin B1-GFP protein was very similar to that of the endogenous cyclin B. Using Western blotting analysis, we examined the level of cyclinB1-GFP in comparison to the endogenous cyclin B and the result was shown in Figure 4-5B. *Lane a* was the control HeLa cell extract, which was stained with anti-cyclin B antibody and only one band was detected. The estimated molecular weight (62 KDa) indicated it is endogenous cyclin B. *Lane b* was extracts from HeLa cells that expressed cyclin B1-GFP fusion gene stained with anti-cyclin B antibody, the major band was the same as that represented by the endogenous cyclin B, the minor band had an approximate molecular weight of 89 KDa, which was close to the expected molecular weight of cyclin B1-GFP fusion protein. This band was also stained positively with anti-GFP antibody (*lane c*), indicating that it was indeed the fusion protein. It is clear from *lane b* that the amount of cyclin B1-GFP was much less than that of the endogenous of cyclin B. We estimated that the population of these two proteins was close to 1:10 ratio.

So the results in Figure 4-5 clearly suggested that the distribution of cyclinB1-GFP was very similar to that of the endogenous cyclin B. And in our system, the expression of cyclin B1-GFP fusion gene was not too high, so that the fusion protein was significantly less than the level of endogenous cyclin B. This allowed us to use cyclin B1-GFP to monitor the dynamic of the cyclin B turn over without affecting the behavior of the progression of cell division.
Figure 4-5  The distribution and expression of cyclin B1-GFP in comparison to endogenous cyclin B.

(A) Comparison of the distribution of cyclin B1-GFP and endogenous cyclin B by immunostaining in HeLa cells. Fluorescent images of cyclin B1-GFP proteins (a, c, e) and fluorescent images of endogenous cyclin B (b, d, f) in fixed HeLa cells as revealed by immunostaining using anti-cyclinB antibody (Transduction Lab) are shown. Images are recorded using a confocal microscope. Scale bar: 10 μm.  (B) Expression of cyclinB1-GFP in HeLa cells examined by western blotting. Cell lysate from HeLa cells transfected with cyclin B1-GFP gene was subjected to a western-blot assay. The antibodies against cyclin B and GFP were then used to detect the specific proteins. Lane a, extracts of normal HeLa cells; Lane b and c, extracts of HeLa cells transfected with cyclin B1-GFP. Lane a and b were probed by anti-cyclin B; Lane c was probed by anti-GFP antibody.
4.2.4 The temporal and spatial proteolysis of cyclin B1-GFP in mitosis in living mammalian cells

Using cyclin B1-GFP fusion protein, we were able to study the temporal and spatial proteolysis of cyclin B in a single living cell during mitosis. We introduced cyclin B1-GFP fusion gene into HeLa or PtK2 cells and then used time-lapse fluorescence microscope to study the degradation of cyclin B in mitosis in living cells.

Figure 4-6 showed the degradation of cyclin B1-GFP in a representative HeLa and PtK2 cell undergoing mitosis. It was very clear that cyclin B1-GFP fusion protein mainly concentrated at the mitotic apparatus and condensed chromosomes during prometaphase and early metaphase. When all the chromosomes aligned at the metaphase plate, the fluorescence decreased rapidly. When the sister-chromatid began to separate, the fluorescence disappeared from the cell. Using living cell imaging system, we measured the fluorescence intensity of cyclin B1-GFP protein in a living cell as the cell progress from prometaphase to metaphase, and then to anaphase, and continue to G1 and the next cell cycle. The measurements were shown in Figure 4-6A and C. It was clear that the cyclin B1-GFP fluorescence began to decrease rapidly about 18-40 minutes (n = 25) before the metaphase-anaphase transition in HeLa cells and about 20-45 min (n = 6) in PtK2 cells. Based on living cell measurement on 23 HeLa cells, we calculated that 94.2% of cyclin B1-GFP had been destroyed before the onset of anaphase. In all the cells that we measured, we found that the onset of cyclin B1-GFP degradation correlated with the time when the last chromosome aligned on the metaphase plate. These results suggested cyclin B1 was degraded by the Cdc20-activated APC in mammalian cells.
Figure 4-6  Time course of the degradation of cyclin B in a single living cell.

(A) and (C), Fluorescence measurements of cyclin B1-GFP in HeLa or PtK2 cells. The fluorescence was measured in a set region at the indicated times, interval, 3 minutes. Levels of fluorescence were normalized to the maximum value. (B) and (D). Visualization of human cyclin B1-GFP during mitosis in a living HeLa cell (B) or PtK2 cell (D). HeLa cells or PtK2 cells were introduced with cyclin B1-GFP fusion gene by electroporation. GFP fluorescence images and phase contrast images of a cell expressed cyclin B1-GFP fusion protein undergoing mitosis were recorded at the indicated time points. Scale bar, 10 μm.
4.2.5 The timing of cyclin B degradation during mitosis is similar to that of securin and is before the onset of anaphase

During mitosis, both cyclin B and securin are known to be degraded by APC through a ubiquitination proteasome system [106, 117]. APC can be activated by binding to Cdc20 during metaphase, or become activated later through the binding of Cdh1/Hct1 [114, 118, 119]. It is well known that securin is degraded exclusively by the Cdc20-activated APC [7, 13]. Using living cell imaging techniques, we measured both time courses of cyclin B1 degradation and securin degradation in the same HeLa cell. At early metaphase, both DsRed-labeled cyclin B1 and YFP-labeled securin were clearly visible in the cell (Figure 4-7A). Their protein levels began to decrease at early metaphase and vanished almost completely before the onset of anaphase. Figure 4-7C showed the results of a quantitative analysis of the temporal dependence of the degradation of cyclin B1 and securin. Their time courses were very similar. The same experiment was repeated in the PtK2 cells. Again, we found that both cyclin B and securin were degraded before the metaphase-anaphase transition (Figure 4-7B & D). We noticed that cells over-expressing both the cyclin B and securin genes spent a longer time period in the metaphase in comparison to the control cells. This is probably due to the fact that these over-expressed gene products are substrates of APC and thus may compete with the endogenous substrate proteins and delay their degradation.

4.2.6 Onset of anaphase is blocked in the presence of nondegradable cyclin B in mammalian cells
Figure 4-7  Temporal dependent proteolysis of securin and cyclin B1 in single living cells

(A) and (B) Temporal dependent proteolysis of DsRed-labeled cyclin B1 (red) and YFP-labeled securin (green) in a living HeLa cell (A) or a PtK2 cell (B). The fluorescence images, as well as the phase contrast images of the same cell were recorded at various time intervals starting from prometaphase. Scale bar, 10 μm. The normalized fluorescence intensities of cyclin B1-DsRed and securin-YFP in a HeLa (C) and a PtK2 (D) cell were plotted as a function of time. Scale bar, 10 μm.
Previously, it was reported that nondegradable form of cyclin B could cause mitotic arrest in mammalian cells. For example, overexpressing nondegradable cyclin B2 mutant Arg32 (to serine) caused HeLa cells to arrest in a pseudomitotic state, the arrested cells displayed multiple mitotic spindles [90]. Expression of cyclin BΔ90 causes exaggerated chromosome separation and inhibits cytokinesis in NRK cells [221]. To investigate whether the degradation of cyclin B is required for the onset of anaphase, we deleted the N-terminal destruction box of cyclin B1 gene (Figure 3-2) and fused the mutant gene with pEGFP-C3 vector. Like the full length cyclin B, cyclin B1-Δ85 can bind with Cdk1 to form an active MPF complex and maintain its kinase activity [210, 212]. When we transfected HeLa cells with GFP-cyclin B1-Δ85, a large percentage of cells were found in the M-phase, while cells transfected with GFP alone was not significantly different from the untransfected control cells. As shown in Figure 4-8, only 6 % cells in mitosis in control cells (GFP alone) while 25.2 % cells were arrested in M-phase when cells expressed GFP-cyclin B1-Δ85. The blockage became more significant after fusion protein expressed for 48 hours, only 11.2 % cells in mitosis in control group. However more than 55 % cells were arrested in mitosis when cells overexpressed GFP-cyclin B1-Δ85.

Previous studies found that the failure of cyclin B to degrade causes cells to arrest in telophase with separated and condensed chromatids [90, 221]. However, from our observation, we found that most of the cells that overexpressed GFP-cyclin B1-Δ85 were arrested before the anaphase. To confirm our findings, we did chromosome spread experiment. HeLa cells transfected with GFP-cyclin B1-Δ85 were synchronized at prometaphase by nocodazole treatment. After release from nocodazole block for 0.5 hour and 3.5 hours, we prepared chromosome spreads from
Figure 4-8  Overexpressing nondegradable form of cyclin B1 (Δ85) cause mitotic arrest in HeLa cells.

HeLa cells were transfected with GFP along, GFP-cyclin B1 and GFP-cyclin B1-Δ85 by electroporation. After fusion protein expressed for 24 h, 36 h and 48 h, cells were fixed and stained with Hoechst 33342. The number of mitotic cells was determined by fluorescence microscope.
these cells and stained with kinetochore antibody, anti-ANA, and the results were shown in Figure 4-9. The metaphase cells had paired sister chromatids, anaphase cells showed single chromatid and separated kinetochores. When cells overexpressed GFP-cyclin B1-Δ85, most of the cells had paired sister chromatids (Figure 4-9A). These observation suggested that nondegradable cyclin B inhibited sister chromatids separation. We also counted the cell number with paired chromatids or single chromatid, the statistical analysis was showing in Figure 4-9B, the results demonstrated that overexpressing GFP-cyclin B1-Δ85 blocked the separation of sister chromatids. Our findings that the onset of anaphase is blocked in the presence of nondegradable cyclin B1 would imply that mammalian cells cannot enter anaphase when the MPF activity remains high.

4.2.7 Blockage of anaphase onset is due to the failure of removing cohesin rather than preventing APC activation

One important question arises. That is, is the mitotic arrest caused by the active MPF due to its direct effects on the securing/separase/cohesin system, or, is it because a high level of MPF may prevent the activation of APC? To answer this question, we co-transfected cells with GFP-cyclin B1-Δ85 and cyclin B1-DsRed. Using the green and red fluorescence channels, we measured the temporal dependent changes of both wild type and truncated cyclin B1 in the same HeLa cell during mitosis. We found that cyclin B1-Δ85 was not degraded and the cell cycle was blocked at the M/A transition (Figure 4-10A). The full-length cyclin B1, on the other hand, was found to be destroyed when the cell entered metaphase (Figure 4-10B & C), similar to what happened in the control cells. Then we examined whether securin, the other substrate
Figure 4-9  Sister chromatids separation was blocked in the presence of nondegradable cyclin B1-Δ85.

(A) HeLa cells were transfected with or without GFP-cyclin B1-Δ85. After fusion protein expressed for about 16 to 18 hours, cells were arrested in M-phase by nocodazole treatment. At 0.5 h and 3.5 h after releasing from nocodazole treatment, chromosome spreads of HeLa cells were prepared as described in Materials and Methods (Section 3.1.5). DNA staining of chromatids by Hoechst (Panels 1-3). Kinetochores of the same chromatids were revealed by immunostaining using an antibody ANA-C (Panels 7-9). Merged images of panels 1, 2 & 3 and panels 4, 5 & 6 respectively. Scale bar, 2 μm.  (B) Percentage of cells showing paired or unpaired chromatids at 0.5 h and 3.5 h after nocodazole release (based on the measurement of 7,298 cells). Percentage of cells having de-condensed nuclei were counted but not plotted.
of Cdc20-APC, was degraded or not in the presence of nondegradable cyclin B1-Δ85. We transfected CFP-labeled cyclin B1-Δ85 and securin-YFP into HeLa cells, then measured the temporal dependent changes of both securin and cyclin B1-Δ85. We found that like the full-length cyclin B1, securin was also degraded when the cell entered metaphase (Figure 4-11). Apparently, the presence of the nondegradable cyclin B1 did not prevent the activation of APC.

Hence, the blockage of anaphase onset by the nondegradable cyclin B1 must be due to a direct effect of the active MPF on the securing/separase/cohesin system. Indeed, when we stained the DNA and the kinetochores of the paired sister chromatids in the metaphase-arrested cells in the presence of cyclin B1-Δ85, we observed that the paired chromatids were not separated, implying that the cohesin protein holding them together must remain intact (Figure 4-9). This result suggests that in the mammalian cells, even after APC is fully activated, the active MPF could still prevent the removal of cohesin from the chromosomes.

4.2.8 A low amount of nondegradable cyclin B1 is sufficient to block the metaphase-anaphase transition

Our finding that over-expressing the nondegradable cyclin B1 gene resulted in blocking the onset of anaphase could have two alternative interpretations: (1) The endogenous MPF must be inactivated before the cell can enter the anaphase, or (2) a hyperactive MPF may artificially block the M/A transition. To support the first interpretation, one must show that the protein level of cyclin B1-Δ85 required to block the anaphase onset is lower than that of the endogenous cyclin B1. Otherwise, the blockage of the anaphase onset by the cyclin B1-Δ85 is likely to be an artifact.
Figure 4-10  Full-length cyclin B1 is degraded in the presence of nondegradable cyclin B1.

(A) Time-dependent measurement of GFP-cyclin B1-Δ85 in a PtK2 cell. The morphology of its chromosomes and its phase images were recorded at the same time. The cell was clearly arrested at metaphase. (B) Time-dependent changes in the protein distribution of cyclin B1-DsRed and GFP-cyclin B1-Δ85 co-expressed in a HeLa cell. Morphology of chromosomes and phase images were also shown. Scale bar, 10 μm. (C) The fluorescence intensities of cyclin B1-DsRed and GFP-cyclin B1-Δ85 co-expressed in the same HeLa cell was plotted as a function of time.
Figure 4-11 Securin is degraded in the presence of nondegradable cyclin B1.

(A) Time-dependent measurement of securin-YFP and CFP-cyclin B1-Δ85 in a HeLa cell. The morphology of chromosomes and its phase images were recorded at the same time. Scale bar, 10 μm. (B) The fluorescence intensities of securin-YFP and CFP-cyclin B1-Δ85 co-expressed in the same HeLa cell was plotted as a function of time.
due to an unphysiologically high level of MPF activity. Hence, we have conducted two independent experiments to test this point. The first one was Western blotting analysis. Using an anti-cyclin B1 monoclonal antibody (GNS-11, from PharMingen), we probed protein blots obtained from two random populations of cells expressing the GFP-cyclin B1 and GFP-cyclin B1-Δ85 genes separately (Figure 4-12A). It is evident that this antibody clearly recognized both the endogenous cyclin B1 and the GFP labelled cyclin B1 (or cyclin B1-Δ85). To compare the sensitivity of this antibody in recognizing the full-length and truncated cyclin B1, we re-probed the same membrane with an anti-GFP antibody. As shown in Figure 4-12A, we found that the intensity ratio between the GFP-cyclin B1 and GFP-cyclin B1-Δ85 bands were similar using either the anti-GFP or the anti-cyclin antibody (The intensity ratio obtained from GNS-11 and anti-GFP was 2.1 and 1.9, respectively). These results suggested that this anti-cyclin B1 antibody was equally sensitive in recognizing the full-length or truncated cyclin B1.

Using this antibody, we conducted Western blotting analysis to measure the relative amount of GFP-cyclin B1-Δ85 (in comparison to the endogenous cyclin B1) in cell extracts obtained at 0.5 and 3.5 hours after release from nocodazole arrest. The results are shown in Figure 4-12B. Unlike the situation in a random cell population, the protein level of endogenous cyclin B1 in the M-phase cells was very high, and thus the relative concentration of cyclin B1-Δ85 in mitotic cells was far less than that of the endogenous cyclin B1 (Figure 4-12B). These results suggest that a low concentration of nondegradable GFP-cyclin B1-Δ85 was sufficient to block a large percentage of cells from entering the anaphase.

Since not every cell may express the GFP-cyclin B1-Δ85 gene efficiently in a cell population, the protein level in a single cell may be higher than the average
Figure 4-12  Anti-cyclin B1 antibody has the same sensitivity to wild type and truncated cyclin B1.

(A) Western blotting analysis of cells extracts obtained from two random populations of cells expressing either cyclin B1-GFP (lane 1) or GFP-cyclin B1-Δ85 (lane 2). It is evident that the probing antibody (GNS-11, PharMingen) recognizes both the endogenous cyclin B1 (band "C") as well as the GFP-labeled full-length (band "A") or truncated cyclin B1 (band "B"). The same membrane was re-probed using anti-GFP antibody to compare the sensitivity of the two antibodies. (B) Synchronized HeLa cells with (lane 3 & 4) or without (lane 1 & 2) expressing the GFP-cyclin B1-Δ85 gene (for 24 hours) were harvested at 0.5 and 3.5 h after nocodazole release. Cell extracts were analysed by Western blotting and probed with anti-cyclin B1. It is evident that the endogenous cyclin B protein was degraded quickly following the nocodazole release while the GFP-cyclin B1-Δ85 protein was not. The incomplete degradation of cyclin B1 at 3.5 h was due to imperfect cell synchronization of the nocodazole treatment.
protein level indicated in a cell extract. Thus, one could still question whether the protein level of cyclin B1-Δ85 required to block the anaphase onset within a single cell is indeed lower than that of the endogenous cyclin B1. To answer this question, we devised an imaging method to measure the protein level of cyclin B1-Δ85 in individual cells. We first synchronized the HeLa cells at early M-phase using a nocodazole treatment. Cells fixed at 0.5 and 3.5 hours after release from nocodazole arrest were immunostained with the GNS-11 antibody. Typical results are shown in Figure 4-13A. At 0.5 h after release from nocodazole treatment, cells usually were at the pro-metaphase and the endogenous cyclin B1 was not degraded. At 3.5 h after nocodazole release, the endogenous cyclin B1 was destroyed. Under this condition, the immunofluorescence intensity represented mainly the nondegradable cyclin B1-Δ85. Thus, by comparing the immunofluorescence intensities before and after the degradation of the endogenous cyclin B1, one can estimate the protein level of cyclin B1-Δ85 in comparison to that of the endogenous cyclin B1 (Figure 4-13B). Under this experimental condition, we estimated that the average protein level of cyclin B1-Δ85 within a metaphase-arrested cell was about 61% of the endogenous cyclin B1 found at the prometaphase (based on measurement of 1822 cells).

Finally, we examined the relationship between the level of nondegradable cyclin B1 contained in an individual cell and its probability of metaphase arrest (Figure 4-13C). The relative protein level was determined from the fluorescence intensity of GFP emitted from the GFP-cyclin B1-Δ85 protein, which was then compared with the endogenous cyclin B1 protein level based on the immunostaining analysis (for details, see Materials and Methods, Section 3.1.6). We found that a very low level of nondegradable cyclin B (equivalent to about 20% of the endogenous cyclin B1 at prometaphase) is sufficient to block the M/A transition (Figure 4-13C).
### 0.5 hr after nocodazole release

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### 3.5 hr after nocodazole release

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Figure 4-13  A low level of nondegradable cyclin B1 is sufficient to block the metaphase-anaphase transition.

(A) Immunostaining pattern of cyclin B and distribution of GFP-cyclin B1-Δ85 in HeLa cells with or without expressing the GFP-cyclin B1-Δ85 gene. DNA in the same cells was visualized by staining with Hoechst dye. (B) Relative immunofluorescence intensity if an individual HeLa cell stained with an antibody against cyclin B1. The signal represented the combined protein levels of the endogenous cyclin B1 and the overexpressed cyclin B1-Δ85 (if any). Background due to non-specific staining was corrected. (C) Percentage of the cells arrested before M/A transition as a function of the protein level of the over-expressed GFP-cyclin B1-Δ85 at 3.5 h after release from the nocodazole treatment.
These results strongly suggest that the blockage of metaphase to anaphase transition by the expression of cyclin B1-Δ85 was not due to an artifact caused by a hyper-activity of MPF. Instead, our findings suggest that the endogenous MPF must be inactivated before the cell can enter the anaphase.

4.3 Discussions

A key step in mitosis is the separation of sister chromatids at the metaphase-anaphase transition. This step is known to be regulated by the activation of APC (anaphase promoting complex) [2], which triggers the removal of cohesin at the chromosome through the degradation of securin and the activation of separase [40-42, 47]. Previously, it was not clear whether this step also requires the inactivation of MPF by destroying cyclin B (Ghiara et al., 1991; Luca et al., 1991; Gallant et al., 1992; Luo et al., 1994). In a well-known in vitro study using Xenopus egg extract, it was shown that addition of nondegradable cyclin B arrested the cell cycle mainly at anaphase instead of metaphase, implying that cyclin B degradation is not required for sister chromatid separation [218]. Similarly, expression of nondegradable cyclin B was found to arrest cells in anaphase or telophase in budding yeast or fission yeast [217, 219]. It was thus concluded that "MPF inactivation is required for the transition of anaphase, but rather that MPF must be inactivated for the cell to complete cytokinesis and return to the interphase state" [227]. This view has become the standard model in later years [2, 106] (Figure 4-14A).

In reviewing the literature, we noticed that there appears to be difference in the temporal pattern of cyclin B degradation between the vertebrate cells and yeasts. In vertebrated cells, most studies reported that cyclin B starts to degrade before the M/A
transition [90, 92, 222, 226], while in yeast, destruction of certain mitotic cyclins (e.g., clb2) was initiated during anaphase and might persist throughout the G1 phase [219, 228]. Thus, it is possible that cyclin B may play a different role in the regulation of M/A transition in vertebrate cells in comparison to yeasts. In this study, we used single cell measurements to test such a possibility. Our results suggest that the regulation of M/A transition in mammalian cells is indeed different from the standard model that was based on studies in yeasts and *Xenopus* egg extract (see Figure 4-14B). First, we observed that in HeLa and PtK2 cells, the time course of cyclin B1 destruction was very similar to that of securin; both of them were degraded before the onset of anaphase. Second, in the presence of nondegradable cyclin B1, the cell cycle was clearly blocked before the M/A transition (Figures 4-8, 4-9). Such a blockage was independent of the activation of APC, since degradation of the full-length cyclin B1 and securin progressed normally during this metaphase arrest (Figure 4-10, 4-11). Third, we showed that such a blockage was not an artifact due to the hyperactivity of MPF. When we measured the protein level of GFP-cyclin B1-Δ85 in individual single cells, we found that a very low level of nondegradable cyclin B1 (about 20% of the endogenous cyclin B1 at prometaphase) was sufficient to inhibit the separation of sister chromatids in HeLa cells (Figure 4-12, 4-13).

These results suggest that unlike yeast, mammalian cells in metaphase must destroy the mitotic cyclin (and thus inactivate MPF) before it can activate the cohesin cleavage system (Figure 4-14B). This model is not only consistent with many earlier studies suggesting that mitotic cyclin in metazoans was destroyed at late metaphase [90, 92, 226, 229], it is also supported by a recent study by Stemmann et al who re-examined the *in vitro* study of *Xenopus* extract [133]. They found that when the amount of nondegradable cyclin B added to the extract was increased, sister
chromatid separation was blocked. They attributed this blockage to an inhibitory phosphorylation of separase by the active MPF.

The only major study that appeared to be in conflict with the model presented in Figure 4-14 is that by Wheatley et al, who micro-injected mRNA of a nondegradable form of cyclin B (cyclin BΔ90) into prometaphase NRK cells and found that expression of cyclin BΔ90 did not block chromatid separation but inhibited cytokinesis [221]. One possible explanation of their results is that since they injected mRNA instead of mature proteins, it might require certain time for the cyclin BΔ90 to be translated and become mature. Even after maturation, it also takes time for the cyclin BΔ90 to bind to Cdk1 and go through the proper phosphorylation and dephosphorylation to activate it. Thus, it is highly possible that in their system, Cdk1 was not yet activated by the cyclin BΔ90 during the M/A transition. Instead, Cdk1 was activated later and thus was able to block cytokinesis.

At the completion of this work, we noticed that a new study on securin proteolysis in living cells has just been published [230]. Their observations on the time course of securin degradation were essentially consistent with ours. In the same paper, the authors also reported preliminary observations that after securin degradation, sister chromatids in PtK1 cells could separate when a “low level” of nondegradable cyclin B1 was expressed. They, however, also observed that in a few cells that expressed “high levels” of nondegradable cyclin B1, metaphase was blocked or delayed for at least one hour. Since they did not actually determine the protein levels of nondegradable cyclin B1 in their cells, it is difficult to compare their results with ours. Nevertheless, their observations are not inconsistent with our model presented in Figure 4-14.
Figure 4-14  Models of signaling control on the metaphase-anaphase transition

(A) The standard model appearing in the current literature [227]. (B) A new model suggested for mammalian somatic cells. In mitosis, cyclin B1 must be degraded by the Cdc20-activated APC. Such an action will turn off the MPF activity and allow sister chromatids to be separated.
In summary, we present evidence in this study that the control mechanism of M/A transition in mammalian cells is different from that of yeasts (Figure 4-14A). Destruction of mitotic cyclin is through the ubiquitination by APC which can be activated by either binding with Cdc20 or Cdh1 [106]. In yeasts, it is known that the mitotic cyclins can be degraded by either Cdc20- or Cdh1-activated APC [106]. Results of this study suggest that in mammalian cells, cyclin B1, like securin, must be destroyed by the Cdc20-activated APC. Such an action will turn off the MPF activity and allow separase to be activated, which in turn removes cohesin from the sister chromatids and allows the onset of anaphase.
CHAPTER 5

Ca\textsuperscript{2+} Signaling Is Involved In The Metaphase-Anaphase Transition

In Mammalian Somatic Cells

5.1 Specific aims and rational

In recent years, there has been considerable progress in understanding the molecular mechanisms that govern cell cycle progression. Two types of enzymes play very important roles in controlling mitotic entry and exit. One is the cyclin-dependent protein kinases (CDKs), a member of a conserved family of serine/threonine protein kinases. Mitotic Cdk1/cyclin B triggers nuclear envelope breakdown, chromosome condensation and formation of mitotic spindle. The other is anaphase-promoting complex (APC/Cyclosome), it's a multimeric ubiquitin ligase. APC-dependent destruction of anaphase inhibitors and other regulators initiate the sister chromatids separation, spindle disassembly and cytokinesis [105, 106, 117]. In addition, there is strong evidence indicating that CDKs can also be regulated by other signaling pathways. For example, during meiosis in many egg cells, including the egg of amphibians and most of the mammalian eggs. Unfertilized eggs were arrested at the second metaphase of meiosis II and this metaphase arrest can be removed only during fertilization. And it is well known that the critical event in fertilization is the elevation of calcium signal [50, 231], which will trigger the removal of metaphase arrest at meiosis II and also trigger the degradation of cyclin B. This calcium signal in regulating the progression of meiosis is mainly due to parallel mechanism. One is that the calcium signal is supposed to trigger the degradation of CSF (cytostatic facor)
[232], and also the activation of ubiquitin system, which will trigger the degradation of cyclin B [73, 181, 233].

The interesting question here is in mitosis, whether a similar calcium signal dependent regulation of the metaphase-anaphase transition and cyclin B degradation is involved. In the literature, there has been study suggesting that calcium signals may be involved in the metaphase-anaphase transition, but so far the evidence has not been very clear. For example, some reports demonstrated a [Ca$^{2+}$]$_{o}$ transient could trigger the onset of anaphase in PtK1 cells and Swiss 3T3 fibroblasts [54, 76]. While other reports suggested that calcium changes are smaller or absent and may not be directly related to the triggering of the anaphase onset [53]. On the other hand, there has been no experimental evidence indicating that in mitosis, cyclin B degradation was regulated by the calcium signal system.

In this study, we would like to explore two key questions by examining: (1) Is Ca$^{2+}$ signal involved in regulating M/A transition in mammalian cells? (2) Why blocking Ca$^{2+}$ signal can prevent M/A transition in somatic cells? What's the mechanism? Our approach was to obtain experimental evidence from both in vitro and in vivo studies. In the first part of this study, we assayed progression of mitosis and time course of cyclin B degradation with or without calcium signal inhibitors using Western blotting analysis. In the second part, using cyclin B1-GFP and securin-YFP fusion proteins, we measured the time-dependent proteolysis of cyclin B1 and securin under the treatment of calcium signal inhibitors. Third, using pEYFP-tubulin probe to monitor the dynamic change of mitotic spindle, we examined the effects of various calcium signaling inhibitors on the stability of mitotic spindle structure.
5.2 Results

5.2.1 The metaphase-anaphase transition in mitotic HeLa cells was blocked by inhibiting the calcium signal

To study the requirement of calcium signal at specific point of cell division, we examined the effects of the calcium chelator, BAPTA/AM, on the progression of mitosis in HeLa cells. BAPTA is a calcium chelator that specifically inhibits the [Ca$^{2+}$] elevation, but not the basal level [234]. In this experiment, HeLa cells were arrested at prometaphase by nocodazole treatment. Then with the removal of nocodazole, cells will be released from nocodazole block and the cell division are allowed to progress. As shown in Figure 5-1A, at one hour after released from nocodazole block, the majority of the cells was already progressed beyond the metaphase-anaphase transition. At two hours, less than 20% of the cells remained before the metaphase-anaphase transition. By contrast, if we applied 10 μM BAPTA/AM into the cells released from nocodazole block, the majority of the cells was prevented to pass the metaphase-anaphase transition. For example, as can be seen in Figure 5-1A, at one hour, about 80% of the cells remained at metaphase, and even two hours after release from the nocodazole block, about 70% of the cells were still prevented from entry into the metaphase-anaphase transition. These results suggested that the progression of the metaphase-anaphase transition in HeLa cells was very sensitive to the inhibition of the calcium signal by the BAPTA/AM treatment. The previous study in our lab also found that Trp-peptide (Ac-RRKWQKTGHAVRAIG RL-NH2), a specific inhibiting peptide of CaM [80, 179], could also block the metaphase-anaphase transition in HeLa cells. As shown in Figure 5-1B, only 27.3% of injected cells remained at metaphase when injected with
A

% of arrested cells

Time after loading

0 h 1 h 2 h 0 h 1 h 2 h
Control 10 uM BAPTA/AM

B

% of arrested cells

Trp peptide

Control (Tyr peptide) 2 mM 5 mM 10 mM
Figure 5-1 The effects of calcium signaling inhibitors on the metaphase-anaphase transition.

(A) Effects of calcium chelator BAPTA/AM on the mitotic progression in HeLa cells. HeLa cells released from nocodazole treatment were loaded with or without the calcium chelator BAPTA/AM (10 µM). Cells were fixed and stained with Hoechst 33342 after loading for 0 h, 1 h and 2 h. The percentage of mitotic cells were counted by nuclear morphology. (B) Effects of CaM inhibitory peptide Trp-peptide on the mitotic progression. HeLa cells were microinjected with Trp-peptide (2 mM, 5 mM, 10 mM). Tyr-peptide is a negative control. The percentage of mitotic cells were counted based on nuclear morphology.
2 mM Trp-peptide. But when injected with 5 mM and 10 mM Trp-peptide, the inhibitory ratio increased to 50.9% to 66.5%. Meanwhile the Tyr-peptide (Ac-RKRYQKTGHAVRA IGRIL-NH2, an analogue of Trp-peptide, but no inhibitory effect) microinjection only made 20.5% of injected cells stayed at metaphase. These results suggested that calcium regulates the metaphase-anaphase transition mediated through CaM.

5.2.2 The degradation of cyclin B was blocked or delayed by inhibiting the calcium signal

Previously, we have demonstrated that both cyclin B and securin are degraded before the onset of anaphase. Here, we found evidence that not only the inhibiting of the calcium signal can block the metaphase-anaphase transition, the inhibition of the calcium can also block the degradation of cyclin B. We can analyze the turn over of cyclin B after release from nocodazole block using Western blotting analysis. As shown in Figure 5-2A, when HeLa cells were released from nocodazole treatment, cyclin B was degraded very quickly. At one hour after release, more than 50% of cyclin B has been degraded, and by two hours, only a very little cyclin B (less than 20%) was detected. By contrast, if BAPTA/AM was applied into the cell medium, the degradation of cyclin B was greatly slowed down or prevented. Even 4 hours after released from nocodazole block, there was still roughly 50% of the cyclin B not degraded. A similar phenomenon was also seen with the application of KN-93, which specifically blocks the activation of CaMKII. KN-93 can significantly delay the degradation of cyclin B following the release of nocodazole treatment. The dynamics of cyclin B degradation under these three conditions (control, BAPTA/AM, KN-93) were summarized in Figure 5-2B, it was very clear that the degradation of
Figure 5-2  The degradation of cyclin B was blocked or delayed by inhibiting the calcium signal.

(A) Western blotting analysis of the cyclin B protein level from cell extracts that were prepared at the indicated time released from nocodazole. The calcium chelator, BAPTA/AM (10 μM) or the CaMKII inhibitor, KN-93 (10 μM) was applied to the medium of synchronized cells after released from nocodazole. Samples were prepared at the indicated time points and analyzed both cyclin B and Cdc2 protein level. (B) The protein levels of cyclin B and Cdc2 were analyzed by measuring the intensity of each band using Eagle Eye software. One hundred percent cyclin B1 protein level represents the protein level of cyclin B1 at 0 h after the cells were released from nocodazole block.
cyclin B was significantly different from the control groups and groups treated with either BAPTA/AM or KN-93. These results suggested that calcium signal is important for regulating the degradation of cyclin B in mitosis.

5.2.3 Single cell measurement showed that the degradation of cyclin B during mitosis is affected by the BAPTA treatment

In vitro examination has shown that the metaphase-anaphase transition and the degradation of cyclin B can be blocked or delayed by inhibiting the calcium signal. Using the fusion protein of cyclin B1-GFP, we were able to study the dynamic of cyclinB degradation and the activation of APC in a single living cell during mitosis. Figure 5-3A shows the typical result of two measurements with or without BAPTA treatment. In the control cell, we can see that cyclin B1-GFP started to degrade at the early metaphase, when the cell entered anaphase, cyclin B1-GFP was totally disappeared from the cell. In contrast, the degradation of cyclin B1-GFP in cell treated with BAPTA was very different, cyclin B1 was not degraded in the cell. Even two hours after the cell entered mitosis, the majority of cyclin B1 was still there and the cell was arrested before anaphase. Sample images of two representative cells under control treatment or BAPTA treatment were shown in Figure 5-3B and C respectively. We measured cyclinB1-GFP protein level in more than 100 cells under different concentrations of BAPTA/AM treatment, the dosage-dependency was summarized in Figure 5-3D. Under 10 μM BAPTA/AM treatment, about 75% of cells were arrested before M/A transition. Under 50 μM BAPTA/AM treatment, the degradation of cyclin B1-GFP and the metaphase-anaphase transition were completely blocked.
Figure 5-3  Time course of the degradation of cyclin B in a single cell and the effects of the calcium chelator BAPTA/AM.

(A) Visualization and measurement of human cyclin B1-GFP during mitosis in living HeLa cells. HeLa cells were introduced with cyclin B1-GFP fusion gene, and BAPTA/AM was loaded into medium after expression for 24 hours. The fluorescence was measured in a set region at the indicated time pointes (The time interval was 3 minutes). (B) GFP fluorescence images and phase contrast images of a HeLa cell expressed cyclin B1-GFP fusion protein undergoing mitosis. (C) Degradation of cyclin B1-GFP was blocked after the application of BAPTA/AM (25 μM). Scale bar, 10 μm. (D) Dosage-dependent effects of BAPTA/AM on the degradation of cyclin B1-GFP and cell division process. BAPTA/AM was applied into the medium for 1 h, and selected cells in prometaphase and measured the fluorescence change within 2-3 hours. More than 100 cells were measured.
5.2.4 Inhibition of CaMKII activity also affect the degradation of cyclin B in HeLa cells

To investigate the calcium signaling pathway that affects the degradation of cyclin B, we examined the effects of specific CaMKII inhibitors. It has been suggested from early studies on egg cells that calcium signals may regulate the degradation of cyclin B through CaMKII in meiosis [181], we would like to see if CaMKII can also play a similar role in regulating the degradation of cyclin B in mitosis in mammalian somatic cells. In this study, we used KN-93, (2-[N-2-hydroxyethyl]-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) [235], to block the activity of CaMKII in HeLa cells. A similar chemical, called KN-92, (2-[N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) [173], is known to have much weaker effect on blocking the activity of CaMKII. It can be used as a control. Results of two representative cells under the treatment of KN-92 or KN-93 were shown in Figure 5-4A. Under the treatment of KN-92, cyclin B1-GFP was degraded at the late stage of metaphase, just like what we observed in control cells. By contrast, with the same concentration of KN-93, the degradation of cyclin B1 was completely blocked. Figure 5-4B & C showed the sample images of cells under KN-92 or KN-93 treatment. In KN-92 treated cell, we can see that cyclin B1-GFP was degraded before the separation of sister chromatids and the cell divided just like normal. Under the treatment of KN-93, cyclin B1 was not degraded and cell was arrested before the metaphase-anaphase transition. Figure 5-4D showed the comparison of the effect of KN-92 and KN-93. Under 10 μM or 20 μM KN-92 treatment, the percentage of cells that undergo normal cell division was similar to that of the control; while under the treatment of
Figure 5-4 Time course of the degradation of cyclin B in a single cell and the effects of the specific CaMKII inhibitor KN-93.

(A) HeLa cells were introduced with cyclin B1-GFP fusion gene by electroporation. After expression for 24 hours, KN-92 or KN-93 (20 µM) was loaded into the medium. The fluorescence change of cyclin B1-GFP was measured at the indicated time points (The time interval was 3 minutes). Levels of fluorescence were normalized to the maximum value. (B) GFP, Hoechst and phase contrast images of HeLa cells expressed cyclin B1-GFP fusion protein which were treated with 20 µM KN-92 or KN-93 (C). Scale bar, 10 µm. (D) Comparison of the effects of KN-92 and KN-93 on the degradation of cyclin B1-GFP and cell division. In most of the control cells and KN-92 treated cells, cyclin B1-GFP was degraded and cells divided, while in KN-93 treated cells, cells were arrested and cyclin B1 degradation was also blocked.
KN-93, the majority of cells were blocked or delayed from undergoing cell division. These results suggested that like meiosis in egg cells, the effect of calcium signal on the degradation of cyclin B is likely to be through the activation of CaMKII.

5.2.5 Degradation of securin was also blocked by inhibiting the calcium signal

Using our living cell image technique, we have demonstrated that both cyclin B1 and securin are degraded by Cdc20-activated APC before the onset of anaphase. We found that in mammalian cells, the degradation of cyclin B and the metaphase-anaphase transition were blocked by inhibiting the calcium signal. The question here is that is the degradation of securin also blocked by inhibiting the calcium signal? To answer this question, we used YFP-labeled securin to measure the dynamic proteolysis of securin in a single living cell treated with calcium signaling inhibitors. Two representative cells under the treatment of BAPTA/AM and KN-93 were shown in Figure 5-5. Different from control cell (Figure 4-3), under the treatment of BAPTA/AM (20 μM), securin-YFP was not degraded and the metaphase-anaphase was also blocked. The same phenomenon can be seen in KN-93 (20 μM) treated cell. We measured the fluorescence change of securin-YFP in control, BAPTA/AM, and KN-93 treated cells. As shown in Figure 5-5C, securin-YFP was degraded before the onset of anaphase in control cell, while under the treatment of BAPTA/AM or KN-93, there were no significant decrease of the fluorescence. These results confirmed that calcium signaling inhibitors can block or delay the degradation of both cyclin B and securin, thus block the metaphase-anaphase transition in mammalian cells.
Figure 5-5  Time course of the degradation of securin in a single cell and the effects of BAPTA/AM or KN-93.

(A) YFP, Hoechst and phase contrast images of HeLa cells expressed securin-YFP fusion protein which were treated with 20 μM BAPTA/AM or 20 μM KN-93 (B). (C) HeLa cells were introduced with securin-YFP fusion gene by electroporation. After expression for 24 hours, BAPTA/AM (20 μM) or KN-93 (20 μM) was loaded into the medium. The fluorescence change of securin-YFP was measured at the indicated times. Levels of fluorescence were normalized to the maximum value.
5.2.6 High concentration of BAPTA/AM can disrupt mitotic spindle structure

In the past several years, results from many studies have indicated that both the metaphase-anaphase transition and the degradation of cyclin B require the activation of APC (anaphase-promoting complex). Although several lines of evidence suggest that calcium signaling may be involved in triggering APC activation, it is still not clear what is the mechanism of calcium signal in regulating APC activation. In this study, we established a stable cell line that expressed YFP-tubulin and then used YFP-tubulin to monitor the dynamics of microtubule during mitosis and examine the effects of various calcium signaling inhibitors on microtubule structure in mitosis in living cells.

First, we examined the effects of the calcium chelator BAPTA/AM on mitotic spindle structure. As shown in Figure 5-6A, under normal conditions, the bipolar spindle structure is very clear and all the chromosomes are aligned across the middle of the spindle. The paired sister chromatids separate synchronously at early anaphase and then move toward the poles. By late anaphase, the spindle poles have moved further apart, increasing the separation of the two groups of chromosomes. Cytokinesis is completed at late telophase and the midbody persisted between the daughter cells. Very interestingly, when cells were treated with the calcium chelator BAPTA/AM, mitotic spindle structure was disrupted, and the effects were dosage-dependent. Under the treatment of lower concentration (10 μM) of BAPTA/AM, most of cells (91.9%) still maintained the normal mitotic spindle (Figure 5-6B). Under the treatment of higher concentration (20 μM, 35 μM), mitotic spindle structure in most of the cells were disrupted (90.5%, 100% respectively). We found
Figure 5-6  The effects of BAPTA/AM on mitotic spindle structure in mitosis.

(A) pEYFP-tubulin (Clontech) was stably transfected into HeLa cells, cells were treated with 10 μM (B) or 20 μM (C) BAPTA/AM, and the fluorescence images of pEYFP-tubulin were taken at the indicated time points using confocal microscope. (D) PtK2 cells were transfected with pEYFP-tubulin by electroporation, cells were treated with 20 μM BAPTA/AM, and the fluorescence images were taken at the indicated time points. Scale bar, 10 μm. (E) Dosage-dependency of the effects of BAPTA/AM on mitotic spindle. HeLa-YFP-tubulin cells were treated with BAPTA/AM (10, 20, 35 μM) for 2.5 hours, then cells were fixed and the cell number with normal mitotic spindle structure was counted.
that the effect of the calcium chelator BAPTA/AM on mitotic spindle is not unique in HeLa cells; other cell types have the similar behavior, such as PtK2 cells (Figure 5-6D).

5.2.7 Cell-impermeant BAPTA, the CaM inhibitory peptide and the IP₃R antagonist heparin also affect mitotic spindle structure

Is it possible that the effects of high concentration BAPTA/AM on mitotic spindle an artifact? To exclude this possibility, we examined the effect of cell-impermeant BAPTA on microtubule. Three mM BAPTA was loaded into cells using in situ electroporation method. We found that, under the treatment of cell-impermeant BAPTA, the normal mitotic spindle structure was also disrupted and cells were arrested before the metaphase-anaphase transition (Figure 5-7A).

The Trp peptide, a specific inhibiting peptide of CaM (downstream receptor of calcium), was found to have the similar effect on mitotic spindle. The previous study in our lab have demonstrated that after microinjection with the Trp-peptide, the mitotic spindle was also disrupted into many small pieces and the localization of cyclin B at spindle poles disappeared. Another way of preventing calcium signals is to block the release of calcium from internal stores using heparin, an IP₃R antagonist [167]. One mg/ml heparin was loaded into HeLa-YFP-tubulin cells by in situ electroporation. It was found that under the treatment of heparin, the normal mitotic spindle structure was disrupted, like BAPTA and trp peptide treated cells (Figure 5-7B). All these results suggested that [Ca²⁺]ᵢ is important for maintaining the integrity of mitotic spindle.
Figure 5-7  The effects of cell-impermeant BAPTA and the IP$_3$R antagonist heparin on mitotic spindle structure

(A) pEYFP-tubulin was stably transfected into HeLa cells. Three mM cell-impermeant BAPTA or 1 mg/ml heparin (B) was loaded into cells using in situ electroporation method. The fluorescence images of pEYFP-tubulin were taken at the indicated time points using confocal microscope after loading for 30 minutes. Scale bar, 10 μm.
5.2.8 CaMKII inhibitor KN-93 inhibit the metaphase-anaphase transition without affecting mitotic spindle structure

We have examined the effects of CaMKII inhibitor KN-93 on the progression of mitosis and the degradation of cyclin B. Both Western blotting analysis and living cell measurement showed that like the calcium chelator BAPTA, KN-93 could also severely delay or block the degradation of cyclin B and the metaphase-anaphase transition. Does KN-93 also affect the mitotic spindle structure in mitosis? The results are shown in Figure 5-8. When we treated stable cell lines with 20 μM KN-93, cell division process was blocked at metaphase (Figure 5-8B). However, we noticed that the mitotic spindle structure was normal, like control cells (Figure 5-8A). This result suggested that KN-93, as well as low concentration BAPTA/AM can inhibit the activation of APC without affecting mitotic spindle structure. Besides spindle checkpoint pathway, calcium may regulate the activation of APC through other pathways.

5.3 Discussions

Observations from many early studies suggested that the metaphase-anaphase transition and cyclin B degradation was dependent on the activation of calcium signal during meiosis II [75, 181, 236]. The clearest example of calcium’s involvement in anaphase onset comes from fertilization in Xenopus oocytes. Fertilization is accompanied by a large calcium transient that leads first to cyclin destruction [72, 73], presumably through activation of the APC/proteasome pathway [74]. These experiments indicate that, at least in the special case of fertilization, a calcium signal can lead to stimulation of APC activity and exit from meiosis.
Figure 5-8 The CaMKII inhibitor KN-93 block the metaphase-anaphase transition without affecting mitotic spindle.

(A) HeLa-YFP-tubulin cells were pre-incubated with 20 μM KN-93 for 30 minutes, and the fluorescence images of pEYFP-tubulin were monitored at the indicated time points using confocal microscope. (B) The fluorescence images of pEYFP-tubulin (green) and Hoechst 33342 (blue) were recorded using inverted fluorescence microscope equipped with a cooled CCD camera at the indicated time points. Scale bar, 10 μm.
There has been longstanding evidence that calcium may also regulate anaphase onset at mitosis in somatic cells. In 1983, Izant showed that anaphase in PtK1 cells could be advanced by microinjecting calcium and delayed by injecting calcium-EGTA buffers. They concluded that an increase in $[\text{Ca}^{2+}]_i$ during metaphase might stimulate anaphase onset but did not act directly on kinetochores. The development of the fluorescent calcium indicator fura 2 [51] allowed the detection of brief calcium increases at anaphase onset in mammalian cells [52, 76]. However, these calcium transients were shown to occur several minutes before anaphase onset was detected [172]. They could be suppressed by removing serum or calcium from the extracellular medium without affecting the progression of mitosis in Swiss 3T3 cells [53]. Tombes and Borisy (1988) also showed that a serum-independent steady rise in $[\text{Ca}^{2+}]_i$ accompanied anaphase onset. Indeed, EGTA and BAPTA blocked anaphase onset only in calcium-free medium, a condition in which the sustained $[\text{Ca}^{2+}]_i$ increase was suppressed. The dissociation of calcium spikes and anaphase onset was confirmed in REF52 cells and Swiss 3T3 fibroblasts [54]. Serum removal and permeant BAPTA abolished the calcium spikes without preventing anaphase onset. Photolysis of a caged calcium chelator to generate a calcium pulse, an approach used successfully to induce premature nuclear envelope breakdown (NEB), did not alter the timing of the metaphase-anaphase transition when delivered at metaphase in these fibroblasts [54]. Present evidence in mammalian cell lines thus leaves the question of calcium's involvement at anaphase unresolved, though it is evident that a calcium signal is essential for entry into mitosis [56, 173].

In this study, we used both \textit{in vitro} and \textit{in vivo} methods to investigate the role of calcium signaling in the metaphase-anaphase transition. Our results demonstrated that calcium signaling is indeed involved in the metaphase-anaphase transition in
mitosis in mammalian cells. Our conclusions are:

(1) Western blotting results demonstrated that both the calcium chelator BAPTA and CaMKII inhibitor KN-93 could block or severely delay the metaphase-anaphase transition and the degradation of cyclin B.

(2) Using cyclin B1-GFP to monitor the activation of APC in single living cells, we found that calcium signaling is required for the activation of APC in mitosis in mammalian cells.

(3) The calcium signaling pathway during the metaphase-anaphase transition is through the activation of Ca\(^{2+}\)/CaM-dependent protein kinase II (CaMKII).

(4) Using pEYFP-tubulin fluorescence probe, we examined various calcium signaling inhibitors on mitotic spindle structure during mitosis. We found that high concentration BAPTA/AM, CaM inhibitory peptide and the IP3R antagonist heparin could disrupt mitotic spindle structure thus block cell division process, indicating that the [Ca\(^{2+}\)]\(_i\) gradient may regulate the integrity of spindle microtubule. However, low concentration BAPTA/AM or inhibiting CaMKII activity with KN-93 does not affect mitotic structure, but the metaphase-anaphase transition was blocked, indicating that calcium signaling may regulate APC activity through different pathways, one is through spindle checkpoint, the other one is not.

Although several lines of evidence suggest that calcium signaling may be involved in triggering APC activation, it is still not clear what is the relationship between calcium signals and the activation of APC. APC is controlled by a network of regulatory factors (Kramer, 1998; Morgan, 1999; Fang, 1999; Hershko, 1999). Recent studies suggested that both Cdk1 and Polo-like kinase can phosphorylate APC core subunits thus regulating APC activity [118]. On the other hand, spindle-assembly checkpoint is clearly positioned to be a key regulator of APC activation.
and anaphase initiation [106]. The major function of the mitotic spindle is to ensure that when cells divide, each daughter faithfully receives one copy of each chromosomes. The spindle checkpoint arrests the cell cycle in metaphase by blocking activation of the Cdc20-APC. Mad2 is an essential component of the spindle-checkpoint system, in both yeast and animal cells, Mad2 associates with Cdc20 and APC. This association is required for blocking APC-Cdc20 ubiquitin ligase activity which then prevents proteolysis of securin/Pds1 and cyclin B. In this study, we found that blocking calcium signal pathway at different steps will have different effects on spindle checkpoint and the metaphase-anaphase transition. From our observations, we speculate that there could be two possible pathways of how calcium signaling regulate the activation of APC during the metaphase-anaphase transition (Figure 5-9).

Firstly, several lines of evidence supported that chromosome separation is triggered by a sudden change in spindle calcium concentration and the elevated $[\text{Ca}^{2+}]$, appeared uniformly distributed over the entire spindle and central region of the cell (Poenie et al., 1986). Ultrastructural studies on a variety of cell types reveal that the endoplasmic reticulum (ER) may extensively align, or interdigitate with the kinetochore spindle fibers (Hepler P.K, 1989). $[\text{Ca}^{2+}]$, gradients may regulate spindle microtubule equilibrium and directe chromosome movement during mitosis. But calcium may affect microtubules indirectly, for example, by regulating the phosphorylation of microtubule-associated proteins. Blocking $[\text{Ca}^{2+}]$, elevation with either calcium chelator or the IP$_3$R antagonist heparin causes the disruption of mitotic spindle structure. When this happens, kinetochores cannot acquire spindle microtubules, and spindle-checkpoint protein Mad2 still accumulates at unattached
kinetochores. Thus, calcium signal may regulate spindle checkpoint indirectly by maintaining the integrity of mitotic spindle structure.

Secondly, recent studies found that Polo-like kinase can phosphorylate APC core subunits (Cdc27, Cdc16 and Tsg24). It has been shown in Xenopus egg extracts that the addition of catalytic inactive Xenopus Plk (Plix) mutant to egg extract blocks their calcium-dependent exit from mitosis. In the presence of Plix mutant, both endogenous cyclin B and cut2 were not degraded (Descombes et al., 1998). This result suggested that calcium signal might activate APC through polo-like kinase by phosphorylating some subunit of APC. We speculate that the Polo-like kinase is the downstream target of CaMKII.
Figure 5-9  The functional roles of Ca$^{2+}$ in the regulation of the metaphase-anaphase in mitosis in mammalian cells.

Hypothesis of the regulation of the metaphase-anaphase transition in mitosis in mammalian cells, which is summarized from our results. In this hypothesis, both securin and cyclin B are degraded by APC-Cdc20 before the metaphase-anaphase transition. Various calcium signaling inhibitors, including the IP$_3$R antagonist heparin, calcium chelator BAPTA, CaM inhibitory peptide and CaMKII inhibitor KN-93 can block or delay the metaphase-anaphase transition and the degradation of cyclin B through different pathways. One is spindle-assembly checkpoint, the other is not. Based on the literauters, we suspected that the other possibility is Plk1, but there is no direct evidence in mammalian cells.
CHAPTER 6

Studying The Signaling Molecules Involved In The APC Activation

6.1 Specific aims and rationale

The metaphase to anaphase transition is controlled by ubiquitin-mediated degradation process. Many cellular proteins are degraded by this pathway, such as anaphase inhibitors and mitotic cyclins. APC-dependent proteolysis requires Cdc20 and Cdh1, which function as rate-limiting and cell-cycle stage-specific activators of the APC. Cdc20 initiates APC-dependent degradation at the metaphase-anaphase transition [7, 12, 13, 117]. By mediating the degradation of securin and mitotic cyclins, Cdc20 has a central role in controlling entry into and exit from anaphase.

The APC has emerged as the key target of a surveillance mechanism known as the "mitotic checkpoint". This system transiently blocks cell-cycle progression beyond metaphase until sister kinetochores of all duplicated chromosomes have been attached to microtubules from opposite spindle poles. Components of the mitotic checkpoint are highly conserved in yeast and vertebrates, including Mad1, Mad2, Mad3 (mitotic arrest deficient), Bub1, Bub3 (budding uninhibited by benzimidazole) and BubR1 [19-25]. In vertebrates, these proteins selectively accumulate at unattached kinetochores and disappear from them upon microtubule attachment. The checkpoint component Mad2 inhibits activation of the APC by interacting with Cdc20 [29-31, 237]. Mad2-Cdc20 interaction is required for blocking APC-Cdc20 ubiquitin ligase activity, which then prevents proteolysis of securin and cyclin B. Studies on Mad2, Cdc20 and the APC have established a general model for how the occupancy of kinetochores by spindle microtubules monitors the spindle assembly
checkpoint, but it remains unclear how Mad2 is recruited to unattached kinetochores for its activation and how mad2 is released from kinetochores. And it is very important to examine in the same cell the quantitative relationship between the depletion of Mad2 at kinetochores and cyclin B/securin degradation.

The Polo-like protein kinases (Plks) are a conserved family of enzymes that play a variety of roles in the passage of cells through M phase [99, 100]. For example, the activation of Cdc25C at the entry of mitosis, the spindle assembly and cyclin B degradation. Recent study showed that both Cdk1-cyclin B and polo-like kinase directly phosphorylated the APC, this joint activation may be due to the complementary phosphorylation of different APC subunits by the two protein kinases.

In this study, we are also interested in examining those important signaling molecules involved in the metaphase-anaphase transition, such as spindle checkpoint proteins, Mad2 and Mad1 and the Polo-like kinase 1. Firstly, using living cell imaging technique, we examined the quantitative relationship between the depletion of Mad2 and the degradation of cyclin B in a same living cell. Second, using GFP-Mad2 fusion construct, we examined the possibility that calcium signaling affect the activation of APC through spindle checkpoint. Third, we investigated the effects of wild-type and mutant Plk1 on mitotic progression and tried to find out whether calcium signaling can affect the activation of APC through Plk1.

6.2 Results

6.2.1 GFP-Mad2 localizes to unattached kinetochores and spindle poles in early mitosis
To study the distribution and dynamics of Mad2 in single living cells, we fused human Mad2 gene to the C-terminal of pEGFP-C3 vector. Then, we transfected GFP-Mad2 fusion gene into HeLa cells or PtK2 cells by electroporation. After fusion gene expressed for more than 24 hours, we examined the localization pattern of GFP-Mad2 using confocal fluorescence microscopy. The fluorescence was diffused throughout the cytoplasm in interphase and prophase cells (data not shown). After nuclear envelope breakdown, GFP-Mad2 localized to unattached kinetochores. We can see a lot of bright dots in chromosome region. As shown in Figure 6-1, GFP-Mad2 localized to unattached kinetochores in early prometaphase, and disappeared from kinetochores upon chromosome attachment to the spindle during late prometaphase and early metaphase. GFP-Mad2 also localized to the spindle poles and along the spindle microtubules during prometaphase, but was not evident at the kinetochores, spindle fibers or spindle poles in anaphase. Consistent with the localization in HeLa cells, we found that GFP-Mad2 also localized to unattached kinetochores and disappeared upon chromosome attachment in PtK2 cells.

To see if Mad2 colocalizes to kinetochores in mitotic cells, we did immunofluorescence staining. HeLa cells expressed GFP-Mad2 were fixed and then stained with anti-kinetochore auto-antibody (ANA-C, from Sigma). We then collected Z-series optical sections of cells in different stage, the fluorescence images of GFP, anti-ANA and merged images are shown in Figure 6-2. In prometaphase cells, after nuclear envelope breakdown, GFP-Mad2 localized strongly to kinetochores (a). In addition, we found that GFP-Mad2 also localized to the spindle poles during prometaphase and early metaphase (b). When chromosomes achieved bipolar attachment to the spindle during late prometaphase and early metaphase, we can see that the bright green dots decreased significantly, which means that some of
Figure 6-1  The localization of GFP-Mad2 in living HeLa and PtK2 cells.

(A) GFP-Mad2 fusion gene was introduced into HeLa cells by electroporation. After the fusion protein expressed for more than 24 hours, the fluorescence images of GFP-Mad2 were recorded using Bio-Rad confocal microscope. (a) and (b) Early prometaphase. GFP-Mad2 localized to unattached kinetochores. There are a lot of bright dots in the chromosome region. (c-e) Mid prometaphase. Kinetochore fibers are visible. GFP-Mad2 localized to unattached kinetochores, along the spindle fibers and spindle poles. (f) Late prometaphase. When kinetochores attach to the microtubule, most of the GFP-Mad2 disappeared from kinetochores. (B) The localization of GFP-Mad2 in PtK2 cells. The cells were in early metaphase, and most of the kinetochores have been attached to the microtubules. GFP-Mad2 also localized to the spindle poles.
Figure 6-2 GFP-Mad2 colocalizes to kinetochores in early mitosis.

HeLa cells expressed GFP-Mad2 fusion gene were fixed with cold methanol and then stained with human anti-kinetochoore autoantibody (ANA-C, Sigma) followed by anti-human secondary antibody (Rhodamine). The fluorescence images GFP-Mad2 (green) and ANA-C (red) were captured using a Bio-Rad confocal microscope. Z-series optical sections of fluorescence images were overlaid with computer assistance. Colocalizations are shown in yellow. Samplings of cells in different phases of the cell cycle are shown: early prometaphase (a), mid-prometaphase (b), early metaphase (c), late metaphase (d), anaphase (e) and telophase (f). Examples shown are representative of quadruplicate preparations from three independent experiments.
the GFP-Mad2 has already disappeared from kinetochores (c). After all the chromosome aligned at the metaphase plate, GFP-Mad2 was not evident on kinetochores, spindle microtubules, or at the spindle poles during late metaphase (d), anaphase (e) and telophase (f).

6.2.2 GFP-Mad2 colocalizes with Cdc20 in mitosis

Recently, a lot of in vitro studies demonstrated that the checkpoint protein Mad2 and the mitotic regulator Cdc20 can form a complex with the APC to control anaphase initiation (Fang, 1998; Sudakin et al., 2001; Zhang et al., 2001). It has been found that Mad2 binds to a small fragment of Cdc20, amino acids 122 to 145. APC binding region overlaps with the Mad2 binding motif. This predicts that Mad2 may compete with APC for the shared binding region in Cdc20. To examine the interaction of Mad2 and Cdc20 in mitosis, we expressed GFP-Mad2 in HeLa cells and immunostained with anti-p55CDC (human Cdc20) antibody. The results are shown in Figure 6-3. At mitosis, both GFP-Mad2 and Cdc20 accumulated at the kinetochores from late prophase to early metaphase, and was also present at the spindle poles and along kinetochore fiber microtubules. In anaphase and telophase cells, the kinetochore-bound Mad2 and Cdc20 were diminished. The staining of Cdc20 in G1 phase cells was very weak, indicating that Cdc20 was degraded in early G1 phase. From the merged images showed here, we found that Mad2 and Cdc20 are highly colocalized in mitosis. These results are consistent with the previous immunoprecipitation results that Cdc20 and Mad2 form complex in mitosis.
HeLa cells expressed GFP-Mad2 were stained with anti-p55CDC antibody. Fluorescence images of GFP-Mad2 (green), anti-p55CDC (red) and Merged images are shown. Mad2 and p55CDC are highly colocalized throughout mitosis. The left column shows GFP-Mad2 (green), the middle column shows anti-p55CDC (red), the right column shows merged images (yellow). At prophase and prometaphase (a-b), both GFP-Mad2 and p55CDC had strong kinetochore labeling, the signal was also present at the spindle poles and along kinetochore microtubules. At late prometaphase and early metaphase (c-d), both of them still had strong signal at kinetochores, but the bright dots decreased because of the attachment of kinetochores to the microtubule. From anaphase to telophase (f), kinetochore labeling of GFP-Mad2 and p55CDC diminishes and is lost by the end of telophase.
6.2.3 Visualization of Mad2 dynamics in living HeLa cells

In order to study the dynamics of GFP-Mad2 in living cells, we transfected GFP-Mad2 into HeLa cells. After the fusion protein expressed for more than 24 hours, the localization of Mad2 and mitotic progression were observed using confocal fluorescence microscope. Figure 6-4 showed a typical cell that was undergoing mitosis. GFP-Mad2 localized strongly to kinetochores in late prophase and early prometaphase (Figure 6-4a & 4b). The fluorescence remained localized to kinetochores until their chromosomes achieved bipolar attachment to the spindle during late prometaphase (Figure 6-4c) and early metaphase (Figure 6-4d), and was frequently observed along spindle microtubules. The fluorescence was undetectable on kinetochores, spindle microtubules, or at the spindle poles during late metaphase (Figure 6-4e), anaphase and telophase (Figure 6-4f).

We measured the fluorescence change of GFP-Mad2 in more than 10 cells that were undergoing cell division process. There was no significant decrease of the fluorescence. This result suggested that during mitosis, GFP-Mad2 has a cell-cycle dependent translocation from kinetochores to cytoplasm, while the protein level is stable. We also found that when cells over-expressed GFP-Mad2, the mitotic progression become a little longer compared with control cells. In our experiments, the fluorescence was not so strong in most of the cells we observed, thus the overexpression had less effect on mitotic progression.

6.2.4 The dynamics of Mad2 and the proteolysis of cyclin B1 in mitosis in single living cells
Figure 6-4  The dynamic redistribution of GFP-Mad2 in a living HeLa cell.

GFP-Mad2 was introduced into HeLa cells by electroporation. After fusion protein expressed for more than 24 hours, the fluorescence images were taken at the indicated time points. GFP-Mad2 localizes to unattached kinetochores in prophase (a) and early prometaphase and to the spindle poles in prometaphase (b). Fluorescence depletes from kinetochores upon their attachment to the spindle in late prometaphase (c & d) and is undetectable on aligned chromosomes (e). Fluorescence of fusion protein is not present at kinetochores or spindle poles during anaphase and telophase (f). Scale bar, 10 μm.
The current model proposes that spindle assembly checkpoint inactivation occurs after depletion of Mad2 from all kinetochores. But so far there is no direct evidence to demonstrate this model *in vivo*. Thus it is very important to examine the quantitative relationship between the depletion of Mad2 at kinetochores and cyclin B degradation in the same cell.

DsRed-labeled cyclin B1 and GFP-Mad2 were transfected into HeLa cells by electroporation to allow fusion gene expression. Then using confocal microscope, we measured the temporal- and spatial-dependent redistribution of GFP-Mad2 and the proteolysis of cyclin B1-DsRed in the same single living cells. A representative cell was shown in Figure 6-5. We measured the fluorescence of cyclin B1-DsRed and GFP-Mad2 respectively. At the beginning of measurement, the cell was in prometaphase, some of the GFP-Mad2 localized to unattached kinetochores. With the depletion of GFP-Mad2 from attached kinetochores, the fluorescence of cyclin B1-DsRed began to decrease. Our measurement showed that there was no significant decrease of the fluorescence of GFP-Mad2, indicating that the Mad2 protein level remained stable during mitosis. Our results demonstrated that the depletion of Mad2 from kinetochores correlates with the initiation of cyclin B proteolysis.

A catalytic model for the role of Mad2 in the generation of the anaphase delaying signal has therefore been proposed (Figure 2-5) [238]. In this model, the checkpoint component Mad2 inhibits activation of the APC by interacting with Cdc20 [28, 29, 31]. It is dynamically associated with unattached kinetochores, and unattached kinetochores on chromosomes provide sites for the activation of Mad2. Activated Mad2 then binds to Cdc20-bound APC and inhibits the ubiquitination activity of the APC, thus prevents the degradation of cyclin B. After microtubules have attached to all the kinetochores, sites for Mad2 activation are no longer
Figure 6-5  The dynamics of GFP-Mad2 and the proteolysis of cyclin B1-DsRed in the same living cell.

(A) GFP-Mad2 and cyclin B1-DsRed were introduced into HeLa cells by electroporation. The temporal- and spatial-dependent redistribution of Mad2 and proteolysis of cyclin B1 were recorded using confocal microscopy. Scale bar, 10 μm. 

(B) The fluorescence of GFP-Mad2 and cyclin B1-DsRed were measured using MetaMorph software.
available, which eventually leads to APC activation by Cdc20 and triggering of anaphase onset.

6.2.5 The localization of GFP-Mad1 in living mammalian cells

Mad1 was found to be a nuclear phosphoprotein in yeast [239], and its function in the mitotic checkpoint is dependent on its association with the Mad2 protein. Interestingly, this association persists throughout the cell cycle [240]. In *Xenopus* homologues, Xmad1 [19] and Xmad2 [26] have been shown to be essential for the mitotic checkpoint in egg extracts. Both Xmad1 and Xmad2 accumulate at unattached kinetochores where they are thought to be participate in generating the inhibitory signal to block anaphase.

In order to study the localization and the dynamics of Mad1 in mammalian cells, we fused human Mad1 gene to the C-terminus of pEGFP-C3 vector, then transfected GFP-Mad1 fusion gene into HeLa cell by electroporation. The localization of GFP-Mad1 was examined by confocal microscope (Figure 6-6). The fluorescence are concentrated in the nucleus in interphase (data not shown). In prophase and early prometaphase, GFP-Mad1 can be seen usually in discrete packets, along spindle microtubules and localize to the kinetochores and spindle poles. Kinetochore localization of GFP-Mad1 becomes apparent in prometaphase, which follows nuclear envelope breakdown. GFP-Mad1 remains at kinetochores until they align at the spindle equator in metaphase. From metaphase through telophase, Mad1 is no longer detectable at kinetochores.

Then, we did immunofluorescence staining to examine the colocalization of Mad1 to kinetochores. HeLa cells expressed GFP-Mad1 were fixed and then stained
Figure 6-6  The localization of GFP-Mad1 in living HeLa cells.

GFP-Mad1 fusion gene was introduced into HeLa cells by electroporation. After the fusion protein expressed for more than 24 hours, the fluorescence images of GFP-Mad1 were recorded using Bio-Rad confocal microscope. Early prometaphase (a-b): GFP-Mad1 localize to unattached kinetochores, as well as spindle fibers and spindle poles, there’re a lot of bright dots in the chromosome region. (c-d) Mid prometaphase (c) and late prometaphase (d): kinetochore fibers are visible, GFP-Mad1 only localize to unattached kinetochores, while those aligned at the metaphase plate have lost it. The specific localization at kinetochores are lost from late metaphase (e) to anaphase (f) and cytokinesis.
Figure 6-7  GFP-Mad1 colocalizes to kinetochores in early mitosis.

GFP-Mad1 was introduced into HeLa cells by electroporation and allowed the fusion protein expressed for more than 24 hours. Cells were fixed with cold methanol and then stained with human anti-kinetochore autoantibody (ANA-C, Sigma) followed by anti-human secondary antibody (Rhodamine). The fluorescence images GFP-Mad1 (green) and ANA-C (red) were captured using Bio-Rad confocal microscope, Z-series optical sections of fluorescence images were overlaid by computer assistance. Co-localizations are shown in yellow. Samplings of cells in different phases of the cell cycle are shown: early prometaphase (a), late prometaphase (b), early metaphase (c), and telophase (d).
with anti-kinetochore auto-antibody (ANA-C, from Sigma). We then collected Z-series optical sections of cells in different stage, the fluorescence images of GFP, anti-ANA and merged images are shown in Figure 6-7. In prometaphase cells, after nuclear envelope breakdown, GFP-Mad1 localized strongly to kinetochores. We found that, like GFP-Mad2, GFP-Mad1 also localized to the spindle poles during prometaphase and early metaphase. When chromosomes achieved bipolar attachment to the spindle during late prometaphase and early metaphase, GFP-Mad1 has already disappeared from kinetochores. When all the chromosome aligned at the metaphase plate, GFP-Mad1 was not evident on kinetochores, spindle microtubules, or at the spindle poles during late metaphase, anaphase and telophase.

These results are consistent with the previous work in PtK1 cells and *Xenopus* egg extracts, which demonstrated that both Mad1 and Mad2 accumulate at unattached kinetochores in early mitosis by using immunoprecipitation or immunostaining method. In this study, we used state-of-the-art optical techniques to demonstrate that both Mad1 and Mad2 localize to unattached kinetochores in early mitosis in HeLa cells.

### 6.2.6 Visualization of Mad1 dynamics in living cells

Previous study in *Xenopus* egg extracts demonstrated that Mad1 is essential for establishing and maintaining the spindle checkpoint. Like Mad2, Mad1 localizes to the nuclear envelope and the nucleus during interphase, and to those kinetochores that are not bound to spindle microtubules during mitosis. Adding an anti-Mad1 antibody to egg extracts inactivates the checkpoint and prevents Mad2 from localizing to unbound kinetochores. In the presence of excess Mad2, neither
chromosomes nor Mad1 are required to activate the spindle checkpoint, suggesting that the physiological role of Mad1 is to recruit Mad2 to kinetochores that have not bound microtubules. In this study, we also examined the dynamics of GFP-Mad1 in single living cells. Figure 6-8 showed two typical cells that undergoing mitosis. GFP-Mad1 strongly localized to unattached kinetochores in late prophase and early prometaphase. The fluorescence remained there until the chromosomes achieved bipolar attachment to the spindle during late prometaphase and early metaphase and was frequently observed along spindle microtubules. The fluorescence was undetectable on kinetochores, spindle microtubules, or at the spindle poles during late metaphase, anaphase and telophase.

The fluorescence intensity of GFP-Mad1 was measured in more than 10 cells that were undergoing cell division process, we found that there was no significant change of the fluorescence, indicating that the expression level of Mad1 is stable during mitosis.

6.2.7  The dynamics of Mad1 and the proteolysis of cyclin B1 in mitosis in a single living cell

Recently, it has been demonstrated that spindle checkpoint protein Mad1 recruits Mad2 to unattached kinetochores and is essential for Mad2-Cdc20 complex formation in vivo. Here, we quantified the dynamics of Mad1 and the proteolysis of cyclin B in mitosis in the same living cell. A representative cell was shown in Figure 6-9. In prometaphase (t = 0 min), GFP-Mad1 localized to unattached kinetochores, cyclin B1-DsRed concentrated to condensed chromosomes and mitotic spindle, especially the spindle poles. With the attachment of sister kinetochores to
Figure 6-8  Real-time dynamics of GFP-Mad1 in a living HeLa cell.

GFP-Mad1 was introduced into HeLa cells by electroporation to allow gene expression. The fluorescence images were taken at the indicated time points. GFP-Mad1 localized to unattached kinetochores in prophase and early prometaphase and to the spindle poles in prometaphase. Fluorescence depletes from kinetochores upon their attachment to the spindle in late prometaphase and is undetectable on aligned chromosomes. Fluorescence of fusion protein is not present at kinetochores or spindle poles during anaphase and telophase.
microtubules from opposite poles at early metaphase (t = 14 min), most of the GFP-Mad1 disappeared from the chromosome region, cyclin B1-DsRed began to degrade. At late metaphase, all the sister chromatids attach to the microtubules, the specific localization of Mad1 disappeared (t = 34 min). We measured the fluorescence of GFP-Mad1 and cyclin B1-DsRed during mitosis. Like GFP-Mad2, the protein level of GFP-Mad1 is almost constant during mitotic progression, it means that GFP-Mad1 translocates from kinetochores to cytoplasm after the spindle assembly checkpoint is inactivated. The destruction of cyclin B1 occurs after the attachment of sister kinetochores to microtubules.

6.2.8 High concentration of BAPTA/AM can affect the release of Mad2 from unattached kinetochores

From the results in Chapter 5, we have demonstrated that under the treatment of high concentration of BAPTA/AM or the CaM inhibitory peptide, the mitotic spindle structure was disrupted (Figure 5-6), and the degradation of cyclin B was also inhibited or severely delayed. We suspected that calcium may affect the activation of APC through mitotic spindle checkpoint. Since Mad2 is the major component of spindle checkpoint, here we used GFP-Mad2 fusion protein to examine whether BAPTA/AM can affect the redistribution of MAD2 in mitosis. The result was shown in Figure 6-10A. High concentration of BAPTA/AM (20 μM) was applied into HeLa cells expressed GFP-Mad2 fusion protein, then we chose cells in early mitosis to examine the effects of BAPTA/AM. At the very beginning, there was a clearly localization of GFP-Mad2 in unattached kinetochores, like control cells. Under normal conditions, cell will go through the cell division process within 20-45 min.
Figure 6-9  The dynamics of GFP-Mad1 and the proteolysis of cyclin B1-DsRed in the same living cell.

(A) GFP-Mad1 and cyclin B1-DsRed were introduced into HeLa cells by electroporation. The temporal and spatial-dependent translocation of Mad1 and proteolysis of cyclin B1 were recorded using confocal microscopy. (B) The fluorescence of GFP-Mad1 and cyclin B1-DsRed were measured as a function of time. Scale bar, 10 μm.
In BAPTA/AM treated cell, we found that the cell was blocked before the metaphase-anaphase transition and there was still a small amount of Mad2 remained at kinetochores (Figure 6-10A). This result confirmed our speculation that calcium may affect the activation of APC through spindle checkpoint.

### 6.2.9 Low concentration of BAPTA/AM and KN-93 does not affect the release of Mad2 from unattached kinetochores

When we checked the effect of low concentration of BAPTA/AM and the CaMKII inhibitor KN-93 on mitotic spindle. We found that they could block the metaphase-anaphase transition without affecting the mitotic spindle structure (Figure 5-8). So we suspected that calcium can affect the activation of APC through another pathway instead of the spindle checkpoint. To confirm our speculation, we expressed GFP-Mad2 in HeLa cells and treated cells with the CaMKII inhibitor KN-93 or low concentration of BAPTA/AM. We found that in KN-93 treated cells (Figure 6-10B), GFP-Mad2 concentrated on unattached kinetochores at prometaphase. When sister kinetochores attached to the microtubules, cell entered metaphase, and the localization of GFP-Mad2 at kinetochores was not detectable. The dynamic distribution of GFP-Mad2 was the same as in control cells, but the mitotic progress was blocked at metaphase. We co-transfected GFP-Mad2 and cyclin B1-DsRed into HeLa cells to examine the distribution of GFP-Mad2 and destruction of cyclin B1 in a same cell under the treatment of low concentration of BAPTA/AM (Figure 6-10C). The effect of low concentration of BAPTA/AM was very similar to KN-93, it also blocked the degradation of cyclin B without affecting the release of Mad2 from kinetochores. All these results demonstrated that calcium signal can affect the
Figure 6-10  The effects of BAPTA/AM and KN-93 on the distribution of GFP-Mad2 in mitosis in living cells.

(A) GFP-Mad2 was introduced into HeLa cells by electroporation. After the fusion protein expressed for more than 24 hours, cells were treated with 20 µM BAPTA/AM, or 20 µM KN-93 (B). The fluorescence images of GFP-Mad2 were recorded as a function of time with Bio-Rad confocal microscope. Scale bar, 10 µm.

(C) Cyclin B1-DsRed and GFP-Mad2 were co-transfected into HeLa cells. Cells was treated with 10 µM BAPTA/AM, and the fluorescence images of cyclin B1-DsRed and GFP-Mad2 were captured with confocal microscopy.
metaphase-anaphase transition through parallel pathways, one is spindle checkpoint, the other is not.

6.2.10 The expression and localization of GFP-Plk1 in mitosis in living HeLa cells

Previous immunofluorescence studies has revealed striking association of human Plk1 with different elements of the mitotic spindle apparatus [158, 241], consistent with the view that this kinase may perform multiple important functions throughout cell division. To understand better the physiological significance of the various localizations observed for Plk1, we would ultimately like to study the dynamic behavior of this kinase in living cells. We therefore constructed a GFP-Plk1 fusion gene and expressed in HeLa cells by transient transfection. The expression of the fusion protein was examined by western blotting analysis. The results were shown in Figure 6-11. HeLa cells and HeLa cells transfected with GFP alone or GFP-Plk1 were collected and lysed after the fusion protein was expressed for 24 hours. The fusion protein was determined with both anti-Plk1 antibody and anti-GFP antibody. Lane a was the control HeLa cell extract, which was stained with anti-Plk1 antibody. Only one band was detected, and the estimated molecular weight (68 Kd) indicated that it was the endogenous Plk1. Lane b was extracts from HeLa cells that expressed the GFP vector alone stained with anti-Plk1 antibody. Lane c was extracts from HeLa cells expressed GFP-Plk1 fusion protein stained with anti-Plk1. The lower band was the same as that represented by the endogenous Plk1, and the upper band had an approximately molecular weight of 95 KDa, which was close to the expected molecular weight of GFP-Plk1 fusion protein. This band was also stained positively
Figure 6-11  The expression of GFP-Plk1 in HeLa cells.

HeLa cells were transfected with GFP vector alone or GFP-Plk1 fusion construct. After expressed for 24 hr, cells were collected and lysed. The cell extracts were subjected to SDS-PAGE then analyzed by Western blotting. Control HeLa cells and HeLa cells transfected with GFP and HeLa cells transfected with GFP-Plk1 were probed with both anti-Plk1 (Santa Cruz, 1: 500 dilution) and anti-GFP (Mol Probe, 1:5000 dilution) antibodies.
with anti-GFP antibody (lane c), indicating that it was indeed the fusion protein.

Then we examined the localization of this fusion protein in living HeLa cells. The results were shown in Figure 6-12. In interphase cells expressing GFP-Plk1, diffused labeling was observed throughout the cell; most prominently, however, a closely spaced pair of bright dots near the nucleus could be seen in virtually all cells. These dots were supposed to be centromosomes. When the cell entered mitosis, GFP-Plk1 remained associated with centrosomes (spindle poles) up to metaphase. But during anaphase, fluorescence became prominent in the midzone of the spindle. Then, intense fluorescent staining was seen in the region of the contractile ring during cytokinesis, and, at the end of mitosis, within the postmitotic bridge.

Interestingly, we found that there are some dot-like distribution of GFP-Plk1 on mitotic chromosomes. To investigate whether GFP-Plk1 colocalizes to kinetochores, we stained cells with specific anti-kinetochore antibody, anti-ANA-C. It was found that the fluorescence of GFP-Plk1 and chromosomal staining with anti-ANA-C showed an extensive degree of overlap, indicating GFP-Plk1 indeed associated with the kinetochore/centromere region (data not shown). Thus, the localization data described here for GFP-Plk1 are in excellent agreement with those obtained previously for endogenous Plk1.

6.2.11 The dynamics of GFP-Plk1 in mitosis in living cells

Both Western blotting analysis and localization results have demonstrated that GFP-Plk1 was a good fluorescent marker for endogenous Plk1, we can use it to monitor the dynamics of Plk1 in mitosis in intact living cells. Figure 6-13 showed a representative HeLa cell that expressed GFP-Plk1. From prophase to prometaphase,
Figure 6-12 The localization of GFP-Plk1 in HeLa cells.

The localization of GFP-Plk1 in living HeLa cells. HeLa cells were transfected with GFP-Plk1, the fluorescent images of GFP-Plk1 (a-d, i-l) and Hoechst 33342 (e-h, m-p) were shown here. Interphase cells (a, b, e, f): GFP-Plk1 diffused in the cytoplasm, but concentrated on the centromere. Prometaphase cells (c-d, g-h, l & m): GFP-Plk1 is localized predominantly at spindle poles, but discrete dots can also be seen within the spindle. Metaphase cells (j & n): GFP-Plk1 associated with spindle poles. Anaphase cells (K & o): GFP fluorescence can be seen in the midzone. In addition, clusters of fluorescence dots are associate with chromosomes. Telophase cell (l & p): GFP-Plk1 “lights up” the postmitotic bridge. Note that the central midbody is even more intensely labeled than the flanking regions.
GFP-Plk1 concentrated in spindle poles and discrete chromatin-associated dots (data not shown), immunofluorescence staining has demonstrated that it was kinetochores. When all the chromosomes aligned at the metaphase plate, GFP-Plk1 mainly remained associated with spindle poles and spindle microtubules. When cell entered anaphase, GFP-Plk1 became prominent in the midzone of the spindle as well as the spindle poles. Then, fluorescence was concentrated in the region of the contractile ring during cytokinesis, and, at the end of mitosis, within the midbody.

We also noticed that after cell division, the fluorescence of GFP-Plk1 had a significant decrease. Previous studies found that Plk, like other mitotic regulators, is targeted for destruction at the end of mitosis through the ubiquitin-proteasome mediated degradation pathway [242].

6.2.12 Dominant negative Plk1 can cause mitotic arrest in HeLa cells

Previous study showed that Plk1 is specifically phosphorylated during mitosis, phosphatase treatment reduces mitotic Plk1 activity to interphase levels. Plks share substantial sequence similarity throughout the N-terminal catalytic domains, and they display a highly conserved sequence motif (the polo-box) in the C-terminal non-catalytic domains [243]. In this study, we explored the consequences of overproducing wide-type, catalytically inactive or hyperactive Plk1 on cell cycle progression in cultured mammalian cells. We constructed two dominant negative mutants and a constitutive active mutant. The catalytic inactive GFP-Plk1 (K82R or N181A), carrying a point mutation at a critical amino acid (the K82R mutant, or the N181A mutant), served as a dominant negative mutant. For the constitutive active GFP-Plk1 (T210D), the specific activity with substitution of aspartate for
Figure 6-13  The dynamics of GFP-Plk1 in mitosis in living HeLa cells.

HeLa cells were transfected with GFP-Plk1 to allow fusion gene expression. The fluorescence images of GFP-Plk1, Hoechst 33342 and phase contrast images were taken at the indicated time points. Scale bar, 10 μm.
threonine-210 is increased several fold relative to wide type. HeLa cells were transiently transfected with plasmids encoding GFP-tagged wild-type, constitutive active (T210D) or dominant negative (K82R or N181A) Plk1 under the control of the cytomegalovirus (CMV) promoter. Cells were fixed and analyzed by fluorescence microscopy after expressed for 28 and 40 hours. The results were shown in Figure 6-14A. At 28 hours after transfection, the percentage of mitotic cells were similar for all groups. And we also noticed that the distribution pattern of wilde type or mutant Plk1 were very similar. At 40 hours after transfection, a significant proportion of the cells expressing either K82R or N181A mutants displayed an apparently mitotic cells (31%, 29.4%) compared to wide type (12%) or constitutive active Plk1 (T210D) (13.5%). The results showed that dominant negative Plk1 can cause mitotic arrest in HeLa cells.

Then, using synchronized cells, we confirmed this result. In Figure 6-14B, HeLa cells transfected with either dominant negative Plk1 (K82R) or GFP vector alone were synchronized at prometaphase and then released from nocodazole block. At 0 hour, more than 90% of cells were in mitotic stage in both control and K82R groups. With the removal of nocodazole, cells entered anaphase and finished cytokinesis within 1-2 hours. However, when cells overexpressed dominant negative Plk1 (K82R), there was a significant number of cells that were arrested in mitosis, about 2-3 fold compared with control cells.

6.3 Discussions

The metaphase to anaphase transition is a point of no return: the duplicated sister chromatids segregate to the future daughter cells, and any mistake in this process
Figure 6-14  Dominant-negative Plk1 induces mitotic arrest in HeLa cells.

(A) HeLa cells were transfected with wide type, T210D, K82R, or N181A GFP-Plk1. At 28 and 40 hours after transfection, cells were fixed and stained with Hoechst, the mitotic cells were counted based on Hoechst staining and cell morphology. (B) HeLa cells transfected with GFP vector alone or dominant-negative K82R GFP-Plk1 were treated with nocodazole for 10 hours. Mitotic cells were collected and released from nocodazole block. Cells were fixed and counted after released from nocodazole for 0, 1, 2 and 3 hours.
may be deleterious to both progeny. The M/A transition is controlled by the APC, many cellular proteins are degraded by this pathway, such as securin and cyclin B. The APC has emerged as the key target of a surveillance mechanism known as the “spindle assembly checkpoint”. This system transiently blocks cell-cycle progression beyond metaphase until sister kinetochores of all duplicated chromosomes have been attached to microtubules from opposite spindle poles. Components of checkpoint proteins in yeast and animal cells is highly conserved, including Mad1, Mad2, Mad3, Bub1, Bub2, Bub3 and BubR1. How does mitotic checkpoint inhibit anaphase initiation? Numerous studies suggested that in several organisms, Mad2 associates with Cdc20 and the APC [29-31, 125, 126, 237]. There is compelling evidence that the Mad2-Cdc20 interaction is required for blocking APC-Cdc20’s ubiquitin ligase activity, which then prevents proteolysis of securin and cyclin B. The finding that Mad2 tetramers are more potent than monomers in blocking cyclin degradation in Xenopus extracts suggests that inhibition of APC activity might involve a structural rearrangement of the Mad2-Cdc20 complex [29]. One of the puzzling aspects of the checkpoint control is that checkpoint proteins are involved in both blocking cell cycle progressing and sensing microtubule attachment at kinetochores. In vertebrate cells, Mad1, Mad2, Bub1, BubR1 and Bub3 selectively accumulate at unattached kinetochores and disappear from them upon microtubule attachment. When all kinetochores have acquired microtubules, the inhibitory signal ceases and APC-Cdc20 becomes active. How is the spindle checkpoint turned off when all the chromosomes are properly attached? There has been much controversy over this issue. There are two models: (1) the “attachment” model, in which full occupation of kinetochores by bound spindle microtubules switches off the checkpoint [244, 245]; and (2) the “tension model”, in which proper tension exerted upon kinetochores due
to bipolar microtubule attachment is responsible [246, 247]. Attachment of kinetochores to spindle microtubules probably involves kinesin- or dynein-like microtubule motors. When a pair of kinetochores become attached to microtubules from two opposite spindle poles, tension develops across the sister kinetochores [248-250]. Tension is generated by the mitotic force that tends to pull the chromatids toward two opposite spindle poles against the glue (cohesin) that holds sister chromatids together [248, 251, 252]. The role of tension in spindle assembly checkpoint signaling is not easy to distinguish from that of attachment, however, as application of tension on kinetochores can enhance both the stability of individual microtubule attachments and the overall occupancy of kinetochores [253-257].

Studies of Mad2, Cdc20 and the APC have established a general model for how the occupancy of kinetochores by spindle microtubules monitors the spindle assembly checkpoint, but it remains unclear how Mad2 is recruited to unattached kinetochores for its activation and how activated Mad2 is released from kinetochores. And it is also important to examine in the same cell the quantitative relationship between the disappearance of Mad2 at kinetochores and cyclin B degradation. In this studies, we used GFP-fusion technique and living cell imaging technique to explore those unresolved questions. Our conclusions are as follows:

(1) Using GFP-Mad2 and GFP-Mad1 fusion genes, we examined the localization and dynamic redistribution of Mad2 and Mad1 in mitosis in intact living cells. We found that GFP-Mad2 localize to unattached kinetochores and spindle poles at early mitosis, then disappear from kinetochores when all the chromosomes align at the metaphase plate. The localization and dynamics of Mad1 are very similar to that of Mad2.
(2) We measured the proteolysis of cyclin B1 and dynamic redistribution of Mad2 or Mad1 by co-expression GFP-labeled Mad2 or Mad1 and DsRed-labeled cyclin B1 in the same cells. We found that the initiation of cyclin B1 degradation is correlated with the release of Mad2 or Mad1 from kinetochores. These results indicate that APC is activated after spindle checkpoint is turned off.

(3) We examined the effect of different calcium signaling blockers on the dynamic distribution of Mad2 in living cells. It showed that high concentration of BAPTA/AM not only disrupts mitotic spindle structure, but also affects the release of Mad2 from kinetochores, indicating that calcium can regulate the activation of APC through spindle assembly checkpoint. However, low concentration of BAPTA/AM and the CaMKII inhibitor KN-93 neither damage mitotic spindle structure nor affect the release of Mad2 from kinetochores, indicating that calcium can affect the activation of APC through another pathway.

(4) Using GFP-Plk1 fusion protein, we examined the dynamic distribution of Plk1 in mitosis. It showed that GFP-Plk1 associates with centrosomes, the equatorial spindle midzone and the postmitotic bridge of dividing cells. In addition, we also studied the effect of dominant-negative Plk1 (K82R or N181A) on mitotic progression, we found that dominant-negative Plk1 causes mitotic arrest in HeLa cells.

In this study, we examined the important checkpoint proteins, Mad2 and Mad1 in regulating the proteolysis of cyclin B1. Our results are consistent with the recent model. Unattached kinetochores act as catalytic sites for the activation of Mad2, and activated Mad2 then inhibit the activation of APC by interacting with Cdc20. We examined the localization of Cdc20 and found that this protein is also localized to the kinetochores throughout mitosis (Figure 6-3). The colocalization indicate that Mad2
and Cdc20 can bind together at kinetochores. After all the chromosomes are properly attached by kinetochore microtubules and aligned at the metaphase plate, active Mad2 is not longer generated, resulting in the activation of APC and the proteolysis of cyclin B and securin.

In this study, we also tried to study the protein-protein interaction using FRET (fluorescence resonance energy transfer) technique. We generated two fusion genes, CFP-Mad2 and YFP-Mad2, and then co-expressed these two fusion proteins in HeLa cells. We will try to determine whether Mad2 protein can self-interact at kinetochores to regulate the activation of APC.

Combined the results from Chapter 5 and Chapter 6, we found evidence that calcium signaling is involved in the regulation of the metaphase-anaphase transition in mammalian somatic cells. High concentration of BAPTA/AM, as well as the CaM inhibitory peptide can disrupt mitotic spindle structure and also affect the turn off the spindle checkpoint thus prevent the activation of APC. Low concentration BAPTA/AM and the CaMKII inhibitor KN-93, however, neither affect the mitotic spindle stability nor affect the turn off of spindle checkpoint. The linkage between CaMII and the activation of APC is still unknown. Recent study showed that in Xenopus egg extracts the activity of Plk1 is absolutely required for the Ca$^{2+}$-induced transition of M-phase-arrested extract to interphase (Descombes, 1998). So based on the literatures and our results, we think that one possible pathway is Ca$^{2+}$ → CaM → CaMKII → Plk1 activation → Cdc27-p, and the Plk1 could be directly activated by CaMKII or through Plk kinase. We will examine this hypothesis by using in vitro kinase assay in the future.
CHAPTER 7
General Discussions And Conclusions

In this project, we have conducted a series of experiments to study the signaling transduction pathway in regulating the metaphase-anaphase transition in mitosis in mammalian cells. The major approaches we used are advanced imaging techniques with newly developed molecular biology tools, particularly the GFP-fusion technique, combined with immunofluorescence, electroporation, western blotting and other biological and biochemical assays. Results of the current study suggest the following conclusions.

7.1 Is Cdk1 inactivation required for the onset of anaphase in mammalian cells?

A key step in mitosis is the separation of sister chromatids at the metaphase-anaphase transition. This step is known to be regulated by APC, which triggers the removal of cohesin at the chromosomes through the degradation of securin and the activation of separase. The standard model based on the study of embryonic cells and yeast system suggested that “MPF inactivation is not required for the transition of anaphase, but rather MPF must be inactivated for the cell to complete cytokinesis and return to the interphase state”. In this study, we mainly focused on studying the degradation of securin and cyclin B in intact living cells. We also quantified the protein level of nondegradable cyclin B1 (Δ85) compared with endogenous cyclin B. Our results suggested that Cdk1 inactivation is required for the onset of anaphase in mammalian cells. Our proposed model was shown in Figure 4-14.
7.1.1 The temporal and spatial control of the degradation of securin and cyclin B in mitosis

The essential role of protein degradation in mitosis was first inferred from the discovery of the mitotic cylins that are degraded in each mitosis [258]. Subsequently, other key regulators have been identified whose destruction is also required for progress through mitosis. Most prominent amongst these is securin, an inhibitor of sister chromatid separation [43, 44, 218]. Securin binds and inactivates separase, a protease that cleaves the Scc1 cohesin subunit responsible for sister chromatid cohesion [33, 40-43, 45, 47]. Thus, one key to understanding mitosis is to determine how the right protein is degraded at the right time. One of the important players that mediate mitosis-specific proteolysis is the APC that acts as an ubiquitin ligase and the Cdc20 and Cdh1 proteins that are required for the APC to recognize its substrates [5, 6, 106]. APC-Cdc20 and APC-Cdh1 appear to have different substrate specificities [7]. APC-Cdc20 recognizes proteins that contain a destruction box (D-box), a loosely conserved nine amino acid motif with the consensus RxxLxxxxN, whereas APC-Cdh1 is able to recognize proteins with either a D-box or a KEN box [259]. In yeast cells, securin (Pds1/Cut2) and a fraction of the major mitotic B-type cyclin, Clb2, are degraded before anaphase, but a significant proportion of Clb2 remains to be degraded later in mitosis [260-262]. In this study, we have quantified the disappearance of fluorescence labeled securin and cyclin B1 and shown that their degradation begins at metaphase. We have shown that the timing of securin and cyclin B destruction is controlled by the spindle checkpoint. Thus, the properties of securin destruction resemble those of cyclin B1, indicating that protein degradation is temporally controlled in the cell division cycle.
The cell division cycle has to be regulated in both time and space. Evidence is now accumulating to suggest that the spatial organization of cell-cycle regulators is also important in the temporal control of the cell cycle. Subcellular localization could well provide spatial dimension to APC-substrate targeting. In vertebrate cells, APC subunits are concentrated at the kinetochores, spindle poles and along the spindle itself [263, 264]. For example, Cdc27 and Cdc16, the components of the APC, colocalize to the centrosome and the mitotic spindle at all stages of the cell cycle. Tsg24, another subunit of the APC, is a centromere-associated protein in mammalian cells. Tsg24 and Cdc27 can bind to isolated mitotic chromosomes. Cdc20, is found at the kinetochores throughout mitosis [125] (Figure 6-3). The spindle checkpoint proteins, Mad2, Mad1, Bub1 and BubR1 also localize to kinetochores (Figure 6-2, 6-6). The substrates of the APC, cyclin B and securin concentrate on condensed chromosomes and the mitotic spindle in early mitosis. Based on the localization of the APC, Cdc20, Mad/Bub proteins and the localization of cyclin B and securin, we can imagine that this subcellular localization pattern play important role in the control of APC-substrate targeting or other aspects of late mitotic regulation. There is intriguing evidence that the degradation of cyclinB is initiated at the spindle poles [222, 265] based on the living cell visualization. Cyclin B-GFP associated with the spindle disappears toward the end of metaphase in a wave that spreads from the spindle poles to the spindle equator. When the spindle-associated cyclin B-GFP is almost undetectable, cells enter anaphase, and the remaining cytoplasmic cyclin B-GFP disappears over the next few minutes. Why the disappearance of cyclin B initiates at the spindle poles and spreads toward the spindle equator? Perhaps polo-like kinases are involved in this process. These kinases have multiple functions during the cell cycle [99, 243], and they are located at multiple sites in the cell,
including the centrosomes at metaphase and the spindle midbody during late mitosis [159, 266-268]. These kinases can activate the APC/C [13, 120, 269], and they can associate with a kinesin-like protein [200] [159]. Perhaps a centrosomal polo kinase initially activates the APC/C at the centrosome, and a kinesin then transports the kinase toward the spindle equator, enabling the kinase to activate a wave of cyclin B destruction.

Recently, much attention has focused on the role of unattached kinetochores in activating the spindle assembly checkpoint. Our results suggested that the spindle pole may also play an important part in regulating the degradation of cyclin B. We noticed that the Mad2 protein is found both on unattached kinetochores and on centrosomes in mammalian cells [270]. Kinetochores provide a catalytic site for Mad2 activation, perhaps the Mad2 protein can travel along the microtubules within a spindle, communicating the status of the kinetochores to the spindle poles, explaining why an unattached kinetochore can block the exit from mitosis within its own spindle.

### 7.1.2 Inactivation of cyclin B-Cdk1 is required for sister chromatid separation

In the previous study, it was believed that the first step in mitotic exit is sister chromatids separation, which is triggered by APC-dependent destruction of “anaphase inhibitors” (or securins). The second step is the degradation of mitotic cyclins, which is required for spindle disassembly and cytokinesis. The order of events is determined by sequential changes in APC substrate targeting. The APC targets anaphase inhibitors first, after which inhibitors of late mitotic events (cyclins) are destroyed [106]. Several studies in embryonic cells and yeast system showed that expression of nondegradable cyclin B cause mitotic arrest not in metaphase but
in telophase, the destruction of cyclin B is necessary for exit from mitosis, but is not for sister chromatid separation [12, 212-214, 216-219, 228, 229]. In mammalian somatic cells, immunostaining results showed that cyclin B1 degraded at the metaphase-anaphase transition or later [92, 226]. Expression of nondegradable cyclin B2 in HeLa cells caused cells to arrest in pseudomitotic state, and the arrested cells displayed multiple mitotic spindle [90]. Microinjection of cyclin B Δ90 mRNA into prometaphase NRK cells caused exaggerated chromosome separation and inhibits cytokinesis. The persisted CDK1 activity has little or no effect on anaphase A or on the rate of anaphase B [221]. So the standard model is that CDK1 inactivation is not required for anaphase onset but for the exit from mitosis.

However, our lab and other groups [222] measured the precise timing of cyclin B1 destruction in mammalian somatic cells (HeLa, PtK2) expressed with a cyclin B1-GFP fusion protein and reached the somewhat startling conclusion that cyclin B1 destruction is initiated at the beginning of metaphase and is nearly complete when anaphase begins. These results seem most consistent with the notion that general activation of the Cdc20-APC occurs at the beginning of metaphase, resulting the simultaneous destruction of securin and cyclin B1. We also found that overexpression nondegradable cyclin B1Δ85 cause metaphase arrest in HeLa cells, while it does not affect the degradation of full-length cyclin B1 and securin. By quantifying the expression level of nondegradable cyclin B1Δ85 compared with endogenous cyclin B1, we estimated that only a small amount of nondegradable cyclin B1Δ85 can inhibit the separation of sister chromatids. Our results indicating that degradation of cyclin B should be required for the onset of anaphase (Figure 4-14). Recent study in Xenopus egg extracts had very similar results to ours. They found that high Cdc2 activity inhibits sister chromatid separation and separase.
Figure 7-1  A model for the dual inhibition of separase in metaphase and its inactivation at anaphase onset.
activity. An inhibitory phosphorylation site (Ser1126) in separase has been identified, Ser1126 was efficiently phosphorylated by Cdc2-cyclin B1 and MAPK (ERK2) but not at all by CaMKII, Plks, or aurora A kinase in vitro [133]. Based on these results, a revised model has been proposed. Before anaphase onset, separase is subjected to a 2-fold inhibition: on one hand, there is the established inhibition of separase by association with the inhibitor securin; on the other hand, there is a hitherto unknown inhibitory phosphorylation, which is due to the high Cdc2/cyclin B1 activity at this stage of the cell cycle. According to this model, securin degradation on its own is not sufficient to activate separase. Before sister chromatid separation can take place, the inhibitory phosphorylation has to be removed away. APC causes destruction of cyclin B1, thereby causing a drop of Cdc2 activity, thus resulting in dephosphorylation and activation of separase. The activated separase cleaves cohesin subunit, Scc1 and sister chromatid separates. In this model, the separation of sister chromatids requires the degradation of anaphaseinhibitors as well as the inactivation of Cdk1 (Figure 7-1). The conclusion from these results is that at least two mechanisms prevent separase activation while chromosomes are in the process of bi-orienting. During this period, the mitotic checkpoint, and possibly other mechanisms, prevents the APC from destroying both securin and cyclin B. The persistence of either protein keeps separase inhibited, one by causing its phosphorylation at Ser1126 and the other by binding to and inhibiting the protease domain. The discovery of this mechanism clearly raises the question of why the cell uses two apparently redundant mechanisms to control separase. Is control by both mechanisms simply more robust or might there exist circumstances when one but not the other mechanism is called into play? Whatever the answer, the eukaryotic cell clearly places a high premium in controlling this critical step.
7.2 How does calcium signaling regulate the metaphase-anaphase transition in mitosis in mammalian cells?

Results from several earlier studies suggested that a Ca$^{2+}$ signal is involved in regulating chromosome separation during the transition between metaphase and anaphase [72, 75, 271]. Using GFP-cyclin B1 fusion protein, we have demonstrated that cyclin B1 was degraded by Cdc20-activated APC during the metaphase-anaphase transition. Thus, we can use this fusion protein to monitor the activation of APC in intact living cells and to explore the effects of different calcium signaling blockers on the activation of APC. In this study, we tried to determine the functional roles of Ca$^{2+}$ signaling in regulating the metaphase-anaphase transition in mitosis in mammalian cells based on our findings in this research.

7.2.1 The activation of APC depends on calcium signaling

The clearest sample of calcium's involvement in anaphase onset comes from fertilization in Xenopus oocytes. Fertilization is accompanied by a large of calcium transient that leads first to cyclin destruction [72, 73], presumably through activation of the APC/proteasome pathway [74]. The calcium signal also inactivates the mos/MAP kinase pathway, so preventing any subsequent effects of mos on later mitosis. These experiments indicate that, at least in the special case of fertilization, a calcium signal can lead to stimulation of APC activity and exit from meiosis. Exit from meiosis can be prevented by peptide inhibitors of the calcium binding protein calmodulin and, independently, its associated kinase, CaM kinase II, indicating that calcium signal cause meiosis exit by activating CaMKII [72, 181].
Calcium signals at mitosis are less dramatic than the large calcium waves of fertilization. The clear difference between mitosis and release from meiotic arrest are that mitosis requires no obvious external triggers and possesses no obvious internal arrest mechanism comparable to mos/MAP kinase. The phosphoinositide messenger system shows periodic activation during the sea urchin embryo cell cycle, with a peak of InsP₃ production that coincides with anaphase onset [167]. This evidence demonstrates that a pattern of calcium signals can be generated endogenously, the InsP₃ antagonist heparin prevents separation of the chromosomes at anaphase [75].

Cell cycle calcium transients were first measured in the early cell cycles of sea urchin embryos [51] and then in mammalian cells in culture [52, 76]. A set of experiments demonstrated that the calcium signal associated with the metaphase-anaphase transition of mitosis causes chromatid disjunction [75]. Preventing the increase in [Ca²⁺]ᵢ prevented the separation of chromosomes and artificially increasing [Ca²⁺]ᵢ in embryos at anaphase led to chromosome separation. Elongation of the mitotic spindle, decondensation of chromatin and reformation of the nuclear envelope occurred more or less normally in embryos in which the metaphase-anaphase calcium signal and chromatid disjunction were blocked, indicating that separation of the sister chromatids at anaphase was the major function of the calcium signal. The most obvious and striking is that calcium transients are not always recorded during mitosis, particularly in mammalian cells in culture [53, 54]. Recently, Whitaker's group used FRET (fluorescence resonance energy transfer) method to detect the [Ca²⁺]ᵢ at metaphase-anaphase transition in HeLa cells, but the result is negative [165]. These conflicting data does not necessarily mean that Ca²⁺ is not involved. In a series of studies we have shown that the degradation of cyclin B and the metaphase-anaphase transition were severely blocked or delayed by calcium
signaling blockers, such as Ca\textsuperscript{2+} chelator BAPTA, CaM inhibitory peptide, and the CaMKII inhibitor KN-93. Our results strongly suggested that the activation of APC is dependent on a calcium signaling.

7.2.2 Calcium signal regulates the APC activity through parallel pathways

One of the key questions of this study is how the calcium signaling regulates the activity of APC. We feel that there could be two possible pathways and examined each of them in this study. Our results demonstrated that at least one pathway is right, that is Ca\textsuperscript{2+} affects APC through regulating the spindle checkpoint.

1 Ca\textsuperscript{2+} affects APC through spindle checkpoint?

Several lines of evidence supported that chromosome separation is triggered by a sudden change in spindle calcium concentration and the elevated [Ca\textsuperscript{2+}], appeared uniformly distributed over the entire spindle and central region of the cell [76]. Ultrastructural studies on a variety of cell types reveal that the endoplasmic reticulum (ER) may extensively align, or interdigitate with the kinetochore spindle fibers [70]. Ca\textsuperscript{2+} regulate the assembly/disassembly of microtubules and possibly the transition from metaphase to anaphase. A series of studies have shown that the anaphase spindle apparatus and chromosome motion are exquisitely sensitive to Ca\textsuperscript{2+} levels modulated within the physiological range [272]. But calcium may affect microtubules indirectly, for example, by regulating the phosphorylation of microtubule-associated proteins. CaM, the major intracellular calcium receptor, was
found to distribute in the spindle poles during mitosis, and it may control microtubule polymerization equilibrium [80, 187].

In this study, we found evidence that blocking [Ca\(^{2+}\)], elevation with high concentration calcium chelator BAPTA/AM, the CaM inhibitory peptide or the IP\(_3\)R antagonist heparin causes the disruption of mitotic spindle structure, thus inhibits the activation of APC to trigger the degradation of cyclin B. Mad2 is the major component of mitotic spindle checkpoint, we examined the effects of different calcium signaling blockers on the localization of Mad2. We found that under the treatment of high concentration BAPTA/AM, kinetochores cannot acquire spindle microtubules, Mad2 remains to unattached kinetochores. Under this condition, spindle checkpoint is always on, thus APC-Cdc20 complex cannot be activated. These results demonstrated that calcium may affect the activation of APC through spindle checkpoint pathway.

(2) \(\text{Ca}^{2+}\) affects APC through Polo-like kinase (Plk1)?

It has been shown in *Xenopus* egg extracts the activity of Plk1 is absolutely required for the Ca\(^{2+}\)-induced transition of M-phase-arrested extract to interphase [273]. The addition of a catalytically inactive Plx1 mutant to M-phase-arrested egg extracts blocked their Ca\(^{2+}\)-induced release into interphase. The proteolytic destruction of several targets of the APC and the inactivation of the Cdk1 were prevented, but it can be restored upon the addition of recombinant Plx1. On the other hand, it has been demonstrated that Plk phosphorylate and activates the bacterially expressed APC components Cdc16 and Cdc27 *in vitro*, which suggests that Plk may directly regulate the APC [118, 120].
When we examined the effect of low concentration BAPTA/AM and CaMKII inhibitor KN-93 on mitotic spindle structure and distribution of Mad2. Very interestingly, we found that they had no any effect. We think there is another possible pathway that links calcium to polo-like kinase to regulate the activation of APC in mitosis. That is $\text{Ca}^{2+} \rightarrow \text{CaM} \rightarrow \text{CaMKII} \rightarrow \text{Plk1 activation} \rightarrow \text{Cdc27-p} \rightarrow$ APC activation, and the Plk1 could be directly activated by CaMKII or through Plk1 kinase (Plkk). In the future, we will use purified protein to examine whether CaMKII can phosphorylate Plk1 in vitro. And then, we will test whether constitutive active Plk1 can rescue the inhibitory effect of KN-93 in living cells.

7.3 Summary

In this project, we explored the signaling transduction pathway in the metaphase-anaphase transition in mitosis in mammalian cells. By using living cell imaging techniques, we examined the temporal- and spatial- proteolysis of two important regulatory proteins involved in sister chromatids separation, cyclin B and securing. We found that Cdk1 inactivation is required for the onset of anaphase. Based on our findings, we proposed a hypothesis that both the degradation of anaphase inhibitors (securin) and the inactivation of Cdk1 are required for sister chromatids separation in mammalian somatic cells. By examining the calcium signaling pathway involved in the metaphase-anaphase transition, we found evidence that calcium signaling is involved in regulating the activation of APC via $\text{Ca}^{2+}/\text{CaM}$-dependent protein kinase II. Calcium may play its role through parallel pathways, our results showed that one of the pathway is spindle checkpoint. We also investigated other regulatory proteins involved in metaphase-anaphase transition, including Mad2, Mad1 and Plk1. We
found that the dynamic redistribution of Mad2/Mad1 from kinetochores to cytoplasm correlates with the initiation of cyclin B proteolysis. Overexpressing dominant negative Plk1 can cause mitotic arrest in HeLa cells.
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