ANALYSIS OF AURORA B REGULATION AND SIGNALING

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ABSTRACT

Aurora B is a serine/threonine kinase that functions in a complex with two other chromosomal passenger proteins called INCENP and Survivin. Its function is implicated in a variety of processes related to mitosis, such as chromosome condensation, regulation of arm cohesion, spindle assembly, chromosome bi-orientation and cytokinesis. During the cell cycle, the level of this protein is tightly controlled and its deregulated abundance is suspected to contribute to aneuploidy. The cell cycle profile for Aurora B is reminiscent of those for substrates of the anaphase-promoting complex/cyclosome (APC/C), an ubiquitin ligase essential for mitotic progression. Here, we showed that Aurora B is a substrate of APC/C both in vitro and in vivo. Aurora B is efficiently ubiquitinated

in an in vitro reconstituted system by APC/C that had been activated by Cdh1. The recognition of Aurora B by APC/C^{Cdh1} is specific as it requires the presence of a conserved KEN-box motif at the amino terminus of Aurora B. Degradation of Aurora B at the end of mitosis requires Cdh1 in vivo as the reduction of Cdh1 level by RNA interference stabilizes Aurora B protein. We conclude that, as a key mitotic regulator, Aurora B is degraded by APC/C^{Cdh1} in late mitosis.

Aurora B lies at the heart of the cellular mechanism that resolves synthelic and merotelic attachments. A failure to eliminate such events results in gain or loss of chromosomes. Therefore, identifying the physiological substrates of Aurora B is of pivotal importance for research. We screened Aurora B substrates using an in vitro expression cloning system. However, the methodology we employed didn't lead to candidate substrates to be further validated by more rigorous in vivo approaches. The use of high concentrations of misfolded recombinant Aurora B was partially responsible for the loss of specificity. Therefore, purifying active recombinant Aurora B has become a primary goal for future biochemical and structural work. Two molecular chaperones Hsp90 and Cdc37 assist the folding of a variety of kinases in vivo, among which Aurora B is also a candidate. This gave us the final idea of expressing Aurora B-INCENP complexes in bacteria via the coexpression of Hsp90-Cdc37 molecular chaperones.

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2. Erdemir, T., Bilican, B., Öncel, D., Goding, C. R., and Yavuzer, U. (2002). DNA damage-dependent interaction of the nuclear matrix protein C1D with Translin-associated factor X (TRAX). J Cell Sci *115*, 207-216.

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LIST OF ABBREVIATIONS

Aim1	Absent in Melanoma 1
AMP	Adenosine Monophosphate
APC	Anaphase Promoting Complex
APC/C	Anaphase Promoting Complex/Cyclosome
Ase1	Anaphase Spindle Elongation 1
Ask1	Associated with Spindles and Kinetochores 1
ATP	Adenosine Triphosphate
Bub	Budding Inhibited by Benzimidazole
Cdc	Cell Division Cycle
Cdh1	Cdc20 Homolog 1
CENP-E	Centromeric Protein E
Cin8	Chromosome Instability 8
Cks1	Cyclin-dependent Kinase Subunit 1
Clb2	Cyclin-B like 2
Clb3	Cyclin-B like 3
Ctf19	Chromosome Transmission Fidelity 19
Dam1	Duo1 and Mps1 Interacting 1
Doc1	Destruction of Cyclin B1
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
eGFP	Enhanced Green Fluorescent Protein
EDTA	Diaminoethane Tetraacetic Acid
EGTA	Ethylene Glycol-bis(beta-aminoethyl-ether)-N,N,N',N' TetraAcetate
Esp1	Extra Spindle Pole 1
FACS	Fluorescence Activated Cell Sorter

GFP	Green Fluorescent Protein
GST	Glutathione-S-Transferase
HA	Hemagglutinin
Hct1	Homolog of Cdc Twenty 1
HDAC2	Histone Deacetylase 2
Hepes	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
Hsl1	Histone Synthetic-lethal 1
INCENP	Inner Centromere Protein
Ipl1	Increase-in-ploidy 1
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropylthio-b-D-galactosidase
KESTREL	Kinase Substrate Tracking and Elucidation
LB	Luria-Bertoni
MBT	Midblastula Transition
MCAK	Mitotic Centromere Associated Kinesin
MCC	Mitotic Checkpoint Complex
MCM7	Minichromosome Maintenance 7
MKlp1	Mitotic-kinesin-like Protein 1
MKlp2	Mitotic kinesin-like Protein 2
MPF	Maturation Promoting Factor
Mps1	Monopolar Spindle 1
Myt1	Membrane-associated, tyrosine and threonine-specific, Cdc2 inhibitory kinase 1
Ndc80	Nuclear Division Cycle 80
NP-40	Nonidet P-40
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

Pds1	Precocious Dissociation of Sister Chromatids 1
Plk1	Polo-related Kinase 1
PMSF	Phenylmethylsulfonyl Fluoride
RENT	Regulator of Nucleolar Silencing and Telophase
RNAi	RNA Interference
RNAse A	Ribonuclease A
Rpm	Rotation per minute
Scc1	Sister Chromatid Cohesion 1
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interference RNA
Skp2	S-phase kinase Associated Protein 2
SPB	Spindle Pole Body
Spc34	Spindle Pole Component 34kDa
UBC	Ubiquitin Conjugating Enzyme
Xkid	Xenopus Kinesin-like DNA Binding Protein

CHAPTER ONE

INTRODUCTION

Historical Perspectives on Cell Cycle

Cell cycle is the set of events that leads to the duplication of a cell. Early light microscopic studies recognized that cell division was preceded by mitosis, during which cells condensed their chromosomes, aligned them on a microtubular spindle and segregated sister chromatids to opposite poles of the cell. Interphase, the interval between succeeding mitoses, remained a mystery until DNA was discovered to be the source of information stored in chromosomes. Chromosome duplication was then detected and shown to occur during a narrow window of time during interphase (Howard and Pelc, 1951) which split interphase into three intervals: G1, the gap between mitosis and the onset of DNA replication; S phase, the period of DNA synthesis; and G2, the gap between S and M phase.

Our initial understanding of the cell cycle stemmed from two different approaches. The genetic approach to the cell cycle evolved in the early 1970s when Hartwell and his colleagues attempted to understand cell cycle by analyzing mutations that arrested the cell cycle of the budding yeast at specific points (Hartwell, 1978); the same approach was applied to the fission yeast by Nurse and

his colleagues (Lee and Nurse, 1988). These investigators have isolated a large collection of cell division cycle (*cdc*) mutants that arrest the cell cycle at a specific point when grown at the restrictive temperature (Culotti and Hartwell, 1971; Hartwell, 1971a; Hartwell, 1971b; Hartwell, 1973; Hartwell et al., 1970; Nasmyth and Nurse, 1981; Nurse et al., 1976). The most extreme models of these processes suggested that they would resemble the metabolic pathways where the initiation of each step in the pathway would be dependent on the completion of the preceding step. The result of their investigations was to define cell cycle as a set of dependent reactions. For example, cells must finish DNA replication before they could enter mitosis. Although enormously useful as a starting point for identifying gene functions that are essential at specific points in the cell cycle, this large collection of *cdc* mutants had limited utility for those interested in the control processes regulating the rate of progression through the cell division cycle. The breakthrough came when a second class of cell cycle mutants was identified in the fission yeast (Nurse, 1975; Thuriaux et al., 1978). These mutants designated as *wee* (refers to the small size and Scottish origin) advanced into mitosis at a reduced cell size and defined the rate-limiting components of the controls regulating the initiation of mitosis. These initial observations led to the discovery and intensive study of a small class of genes classified as the rate-limiting inducers or inhibitors of mitosis.

The biochemical approach to the cell cycle came through the examination of natural cell cycle arrests occurring during oocyte maturation and the examination of the agents that induced the embryonic cell cycle to proceed. The pioneering work of Masui and Markert identified an activity in the cytoplasm of unfertilized eggs that are naturally arrested at the metaphase of second meiotic division (Masui and Markert, 1971). The transfer of egg cytoplasm into a fully grown oocyte, which is naturally arrested in G2 preceding the first meiotic division, induced the recipient to undergo meiotic maturation. This activity was named maturation promoting factor (MPF) and was later found to be present in the meiotic and mitotic states of all examined eukaryotic cells (Gerhart et al., 1984; Nelkin et al., 1980; Sunkara et al., 1979; Tachibana et al., 1987; Weintraub et al., 1982). The biochemical approach was championed by researchers who favored marine and amphibian embryos which offered a convenient system for studying cell cycle because they were specialized for rapid, nearly synchronous cell cycles that proceeded without any detectable G1 or G2 phases until there is an abrupt change to a more complex and asynchronous cell cycle (Newport and Kirschner, 1984). The result of further investigations using egg extracts that would undergo multiple rounds of cell cycle in vitro defined the cell cycle as a biochemical machine that oscillated between two states, mitosis (high MPF) and interphase (low MPF) (Murray and Kirschner, 1989; Murray et al., 1989); and the oscillations were independent of the completion of many of the cell cycle events

such as DNA replication (Kimelman et al., 1987) and spindle assembly (Gerhart et al., 1984).

Some 20 years after these separate lines of investigation had begun, a unified view of the eukaryotic cell cycle emerged when the key components of the embryonic and somatic cell cycles were demonstrated to be the products of homologous genes. In their discovery of *CDC28* protein kinase, fortune favored yeast geneticists; of the forty or so cell cycle genes identified on the basis of mutant cells arresting uniformly at particular cell stages, *cdc28* mutants were considered exceptional because they were the only ones defective in START (a point in late G1 when haploid cells become refractory to cell cycle arrest caused by pheromone and no longer require nutrients for the completion of the mitotic cell cycle) despite the normal rates of protein and RNA synthesis (Reid and Hartwell, 1977). Meanwhile, better evidence for an important regulatory role has emerged from the distantly related fission yeast in which there were alleles of the $cdc2^{+}$ gene that advanced the timing of mitosis (Nurse and Thuriaux, 1980). The discovery that $cdc2^{+}$ and *CDC28* can each complement defects in the other and they encode homologous kinases was therefore of fundamental importance in bringing together two different schools of cell cycle genetics (Beach et al., 1982). Biochemical and genetic searches converged with the discovery that the activity called maturation-promoting factor (MPF) capable of causing frog extracts to enter M phase was composed of $p34^{cdc2}$, a *Xenopus* homolog of the $cdc2^{+}$ gene of

the fission yeast and the *CDC28* gene of the budding yeast (Dunphy et al., 1988; Gautier et al., 1988).

Initially, MPF was defined by its ability to bypass the protein synthesis requirement for inducing meiosis I and its activity responded to many factors besides Cdc2 kinase including a phosphatase, activated *ras* genes, activated tyrosine kinases, the serine/threonine kinase c-Mos (and v-Mos), and Cyclins A and B. Several of these meet the criterion that they will act in the presence of protein synthesis inhibitors (Hunt, 1991). It would be potentially misleading and unfortunate for the biochemists to use marine and amphibian eggs without the good evidence that came from yeast genetics since biochemistry didn't provide attractive opportunities for the initial molecular identification of the regulatory elements. And in addition, it was necessary for the biochemists to turn to cell-free extracts in order to test the role of various cell cycle control proteins.

Mitosis

In this introductory review of mitosis beyond explaining various mitotic regulators which can limit mitosis in different circumstances, my aim is to describe the architecture where the central players act. Since the goal of modern cell biology is to explain the structure and behavior of the whole cell in terms of the biochemical properties of its individual components, I will extend the simple picture of mitosis to the general question of how all these processes are

coordinated in space and time to achieve the segregation of sister chromatids into two daughter cells with stunning precision.

The onset of mitosis was found to be regulated by $p34^{cdc2}$ family of kinases which is activated at both mitotic and meiotic M-phase and acts as a global signaling system which affects essentially all aspects of the cell (Nurse, 1990). Its activation required its interaction with cyclins, a class of proteins which vary in level during the cell cycle and changes in the phosphorylation state of the kinase. During interphase, Cyclin B accumulates above a threshold level and binds to Cdc2 (Solomon et al., 1990). There are two different Cyclin B proteins in mammalian cells. Cyclin B2 is a non-essential protein that associates with the Golgi apparatus (Jackman et al., 1995). Cyclin B1 is an essential protein that is responsible for most of the other actions of Cdc2 in the cytoplasm and nucleus (Brandeis et al., 1998). Binding to Cyclin B1 induces both threonine and tyrosine phosphorylation of the previously unphosphorylated Cdc2, rendering it inactive (Solomon et al., 1990). The inactive complex is also phosphorylated at Thr-161 and phosphorylation on a homologous site appears to be universally required for the activation of cyclin-dependent kinases (Fesquet et al., 1993; Poon et al., 1993); however, this phosphorylation is not sufficient to overcome the inhibitory effect of tyrosine-15 phosphorylation and that of Thr-14 (The amino acid positions cited refer to human $p34^{cdc2}$, unless specified otherwise.) (Solomon et al., 1993; Solomon et al., 1992). Phosphorylation of Cdc2 at Thr14 and Tyr15 is

catalyzed by the protein kinases Wee1 which phosphorylates Tyr15 only (McGowan and Russell, 1993) and Myt1 which phosphorylates both Thr14 and Tyr15 and is found in the cytoplasm throughout the cell cycle (Liu et al., 1997; Mueller et al., 1995). Cdc2 activation at the onset of mitosis results from the concurrent inhibition of Wee1 and Myt1 and the stimulation of the dual-specificity protein phosphatase Cdc25C which dephosphorylates both Thr14 and Tyr15. Activation of Cdc25C requires the phosphorylation of several sites on its amino-terminal domain which is catalyzed by at least two kinases, one of which appears to be the Polo-related kinase (Plk) (Kumagai and Dunphy, 1996). Activating phosphorylation of Cdc25C is also carried out by the Cdc2/Cyclin B1 complex itself (Hoffmann et al., 1993). The ability of Cdc2 to activate its own activator provides the potential positive feedback loop in Cdk1 activation, whereby partial activation of Cdc25C perhaps by Plk1 could lead to the complete and overwhelming Cdc2 activation. This positive feedback loop may be enhanced by the ability of Cdc2 to phosphorylate and inactivate Wee1 (Kumagai and Dunphy, 1995; Patra et al., 1999). Historically, Wee1 which was originally identified in the fission yeast as a critical negative regulator of mitosis (Russell and Nurse, 1987) was shown to be the kinase that phosphorylates Cdc2 on Tyr15 (Parker et al., 1992); however, Wee1 localizes to the nucleus (Heald et al., 1993) while Cdc2/Cyclin B1 is found entirely in the cytoplasm, either in a soluble form or associated with microtubules and centrosomes during interphase (Bailly et al.,

1992; Ookata et al., 1993; Pines and Hunter, 1991). Experiments have later shown that Cyclin B1 continuously shuttles into and out of the nucleus and Cdc2/Cyclin B1 is primarily cytoplasmic because its rate of export is greater than its rate of import (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). In late prophase, a fraction of Cdc2/Cyclin B1 remains associated with the duplicated centrosomes while the majority is rapidly translocated to the nucleus due to multiple phosphorylations of Cyclin B1 in its cytoplasmic retention sequence which triggers a decrease in export and an increase in import, after which the nuclear envelope breaks down (Hagting et al., 1999).

The realization that a single switch controls mitosis onset has important consequences for understanding downstream events. An interphase cell faces several structural problems at the onset of mitosis. The unipolar cytoskeleton must be transformed into the bipolar arrangement that foreshadows the organization of two new unipolar daughters. The onset of prophase is characterized by a rapid transition from few relatively long microtubules that stretch to the cell periphery to many short microtubules near the centrosomes. This new steady state is the metaphase configuration, which represents the most stable global organization of cytoskeletal elements after the initial events of prophase.

During the first half of M phase, duplicated chromosomes condense and via their kinetochores become attached to the mitotic spindles emanating from organizing centers known as centrosomes at opposite poles of the cell. The

chromosomes then move to the metaphase plate, which bisects the spindle, through a series of coordinated movements known as chromosome congression. Sister chromatids remain attached to each other throughout this phase, and a key transition occurs when the sisters split and move to opposite poles due to two types of force exerted by the spindle: the attraction of sister kinetochores toward opposite poles and the repulsion of the poles themselves.

It was initially thought that the key transition might be triggered by the inactivation of Cdc2, initiated by the rapid degradation of Cyclin B which was first identified in sea urchin eggs on the basis of its periodic abundance during early cleavage divisions (Evans et al., 1983). The theory was based on the observation that mutated versions of Cyclin B lacking the destruction box, a short consensus sequence located within the N-terminal 90 amino acids of Cyclin A and B, caused cycling *Xenopus* egg extracts to become arrested in mitosis in a state resembling metaphase characterized by high kinase activity (Murray and Kirschner, 1989). However, the close examination of the mitotic arrest later revealed that sister chromatid separation occurred normally, with the arrest occurring in telophase (Holloway et al., 1993). Additional evidence was presented in the budding yeast where the overexpression of a wild type or stable B-type mitotic cyclin (CLB2) caused cells to arrest with elongated mitotic spindles and segregated chromosomes (Surana et al., 1993). Therefore, it was concluded that

the inactivation of Cdc2 activity was required for exit from mitosis not for the metaphase to anaphase transition.

In contrast, an N-terminal fragment of Cyclin B which competes for the degradation machinery but can not activate Cdc2 delayed sister chromatid separation in a dose-dependent manner suggesting the proteolytic system that degrades cyclin also recognized other proteins whose destruction is required for the sister chromatid segregation (Holloway et al., 1993). Several lines of evidence suggested that cyclin proteolysis is mediated by the ubiquitin pathway, which was implicated in the turnover of cellular proteins. Cyclin B-ubiquitin conjugates could be observed in mitotic *Xenopus* extracts (Glotzer et al., 1991); methylated ubiquitin, an inhibitor of polyubiquitin chain formation, blocked the degradation of Cyclin A and B in clam egg extracts (Hershko et al., 1991); and mutations in subunits of the 26S proteasome in yeast, a complex required for the proteolysis of ubiquitinated proteins, arrested the cell cycle in metaphase with elevated levels of B-type cyclins and Cdc2 activity (Ghislain et al., 1993; Gordon et al., 1993); and interestingly, at least one proteasome mutant could be suppressed by deletion of CLB2, the major B-type cyclin in the budding yeast (Friedman and Snyder, 1994).

One attractive hypothesis was to postulate that non-cyclin proteins could play a role in holding sister chromatids together until they are degraded. This idea was favored when genes involved in sister chromatid separation such as Cdc23

and Cdc16 were also found to be required for B-type cyclin proteolysis initiated at the onset of anaphase (Irniger et al., 1995). Around the same time, human homologs of Cdc16 and Cdc27 which were previously shown to form a complex with Cdc23 in yeast were found to be required for anaphase progression in mammalian cells (Tugendreich et al., 1995); and these two proteins were identified as the constituents of a 20S complex fractionated from mitotic *Xenopus* extracts which can support the ubiquitination of Cyclin B (King et al., 1995). This newly identified complex from there on will be referred as the anaphase promoting complex (APC) since the components of APC were essential for anaphase progression in multiple organisms. A similar activity was independently purified from clam extracts and was called as the cyclosome (Hershko et al., 1994; Sudakin et al., 1995).

Ubiquitin is a small polypeptide that was originally isolated by Goldstein and coworkers in the purification of polypeptide hormones from the thymus (Goldstein et al., 1975). Subsequently, it has been detected in all cells examined, including those of vertebrate or invertebrate animals, plants and yeast. The enzymes of the ubiquitin system were first defined as eluates from an ubiquitin-affinity column (hence the letter E in their name) (Hershko et al., 1983). The ubiquitin-activating enzyme (E_1) was first bound to the ubiquitin-Sepharose in the presence of ATP as a thiol ester intermediate, and was then eluted with AMP and pyrophosphate. A second enzyme (E_2) later designated as the ubiquitin-carrier

protein or the ubiquitin-conjugating enzyme (UBC) was bound to the ubiquitin-affinity column when E_1 and ATP were present and was eluted with a thiol compound at high concentration. The third enzyme (E_3) termed as ubiquitin ligase was adsorbed to the ubiquitin-affinity column by noncovalent interactions and could be eluted with high salt or increased pH. Ubiquitin, a small highly conserved protein, is first activated at its COOH-terminus Gly via ATP to a high energy thiol ester intermediate in a reaction catalyzed by the ubiquitin-activating enzyme, E_1. Ubiquitin is subsequently transesterified to one member of a family of E_2 enzymes. Finally, ubiquitin is transferred from the E_2 to an ε-NH_2 group of lysine residue of the target protein, either directly or with the assistance of an ubiquitin ligase (E_3). An E_3 is generally required for the formation of the multiubiquitin chains on the substrate, a step that facilitates the efficient recognition of the substrate by the proteasome. In addition to facilitating multiubiquitination of substrates, E_3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as some E_3s have been shown to directly bind substrates (Ciechanover, 1994). The ability of APC/C to complement recombinant E_1 and UBC4 (one of the E_2s identified in *Xenopus*) indicated that it functioned as an ubiquitin ligase (King et al., 1995).

If anaphase was regulated by a single inhibitor, one would expect that in its absence, APC/C function would no longer be required for anaphase initiation. The product of the budding yeast *PDS1* gene (for precocious dissociation of sister

chromatids) was shown to fit this criterion. The initial *pds1-1* allele was identified by its inviability after transient exposure to microtubule inhibitors and its precocious dissociation of sister chromatids in the presence of these microtubule inhibitors (Yamamoto et al., 1996). A significant fraction of *pds1cdc16* and *pds1cdc23* double mutant cells at the nonpermissive temperature arrested not prior to anaphase, as do the single *cdc* mutants, but rather at the end of mitosis (Yamamoto et al., 1996). This suggested that Pds1 is an anaphase inhibitor whose degradation via APC/C is required for anaphase initiation. Hence, in the absence of Pds1, the requirement for APC/C in anaphase onset is alleviated. It was later shown to be the postulated anaphase inhibitor whose degradation via APC/C (Anaphase Promoting Complex/Cyclosome) is required for anaphase initiation (Cohen-Fix et al., 1996).

Chromosome duplication during S phase produces sister chromatids that are held together by specific chromosomal proteins called cohesins (Guacci et al., 1997; Michaelis et al., 1997). Loss of cohesion was thought to be responsible for the separation of sister chromatids during anaphase disjunction of sisters since a cohesin called Scc1 disappeared from chromosomes at the metaphase to anaphase transition (Michaelis et al., 1997). A key question was what caused the sudden disappearance of Scc1 and how Pds1 blocked sister chromatid separation. Pds1 was found to be tightly associated with a protein called Esp1 (Ciosk et al., 1998). When G1 cells of an *esp1-1* strain were incubated at the restrictive temperature,

they duplicated DNA and formed mitotic spindles; they then failed to segregate chromosomes but nevertheless proceeded with cyclin destruction, cytokinesis and genome reduplication (McGrew et al., 1992). The persistence of sister chromatid cohesion in *esp1-1* mutants depended on Scc1 since Scc1 associated with chromatin failed to dissociate following the destruction of Pds1 (Ciosk et al., 1998). The strict dependence of sister separation on both Esp1 activity and Pds1 destruction suggested that Esp1 might be a sister-separating protein whose activity is blocked by its association with Pds1. These data implied that Anaphase Promoting Complex mediates sister chromatid separation by liberating the "sister-separating" Esp1 protein from an inhibitory embrace by its guardian Pds1. It was later shown that Esp1 causes the dissociation of Scc1 from chromosomes by stimulating its cleavage by proteolysis and was shown to be the protease that cleaves Scc1 (Uhlmann et al., 1999).

APC/C activity is regulated in the cell cycle and increases in late mitosis, primarily because of changes in its association with the activating subunits Cdc20 and Cdh1 (Fang et al., 1998b; Kramer et al., 1998). In the budding yeast, all known APC/C substrates (mitotic cyclins Clb2 and Clb3, Pds1 and Ase1) are unstable during G1 phase because of continuous APC/C-dependent proteolysis (Visintin et al., 1997). Genetic evidence has led to the hypothesis that Cdc20 promotes the degradation of Pds1, whereas Hct1 (Cdh1 homolog in the budding yeast) directs destruction of the major mitotic cyclin Clb2 (Schwab et al., 1997;

Visintin et al., 1997). It is noteworthy that this substrate specificity was seen in cells arrested in S phase by hydroxyurea in which Pds1 and Clb2 are normally stable, but not in M phase during which Cdc20 is actually capable of inducing the degradation of Pds1 and Clb2 when overexpressed (Schwab et al., 1997). These studies provided a scheme for APC/C-driven progression through mitosis. The simple model of Clb2 degradation targeted by Hct1 was later replaced with a biphasic inactivation model because Hct1-mediated Clb2 proteolysis occurred only after APC/C^{Cdc20} consigns Pds1 for degradation. *cdc20Δpds1Δ* mutants arrested in telophase with high Cdc28-Clb2 kinase activity and the induction of Cdc20 allowed the cells to exit mitosis (Lim et al., 1998). The mechanism that ensured the correct timing of these events evolved when it was shown that phosphorylation of Hct1 by the mitotic kinase renders it inactive (Zachariae et al., 1998) and dephosphorylation by Cdc14 restored its activity (Jaspersen et al., 1999). Cdc14, a dual specificity phosphatase, itself appeared to be spatially regulated in that it is sequestered to the nucleolar RENT (regulator of nucleolar silencing and telophase) complex during G1 to anaphase (Shou et al., 1999). During late anaphase, Cdc14 is dispersed from the RENT complex resulting in the activation of Hct1, which in turn triggered Clb2 proteolysis. Hence, it is the antagonistic action of the mitotic kinase and the Cdc14 phosphatase on Hct1 that may determine the correct timing of Clb2 proteolysis. However, this scenario required that, by the time cells reach late telophase, the balance is decisively

tipped in favor of net dephosphorylation of Hct1. This would be achievable if the rate of Cdc14-mediated dephosphorylation were inherently higher than the phosphorylation by the mitotic kinase Cdc28-Clb or if APC/C^{Cdc20} caused the destruction of a protein that normally inhibits Hct1 and thereby paves the way for rapid activation of Hct1.

Spindle assembly checkpoint

Accurate chromosome replication and segregation require mechanisms that can detect errors in these processes and initiate two responses: repair systems that correct the errors, and delay mechanisms that prevent the cell cycle from proceeding while the repairs are in progress. The combined detection and delay systems are known as cell cycle checkpoints or feedback controls. Experimentally, feedback controls were identified by finding conditions that overcome cell cycle arrest which results from the failure to complete a particular step in the cell cycle. The mitotic arrest caused by microtubule polymerization inhibitors suggested that the existence of a spindle assembly checkpoint that monitors the status of the spindle and regulates the metaphase to anaphase transition. The ability of a single improperly oriented chromosome (Rieder et al., 1994) or partially defective centromeres (Spencer and Hieter, 1992) to delay anaphase suggested that this checkpoint can detect subtle as well as gross defects in the structure of the spindle. Genetic analysis of the budding yeast mutants that fail to arrest their cell cycles at mitosis in response to the loss of microtubule

function, has allowed the identification of components of the spindle assembly checkpoint (Hoyt et al., 1991; Li and Murray, 1991). The *bub* (budding uninhibited by benzimidazole) and *mad* (mitotic arrest deficient) mutants were isolated from collections of mutants that failed to form colonies in the presence of low doses of benomyl, a microtubule polymerization inhibitor. Another checkpoint component was identified in the budding yeast when *mps1* (monopolar spindle) mutation, unlike other mutations involved in SPB duplication (Winey and Byers, 1993), failed to arrest in mitosis (Weiss and Winey, 1996).

Feedback controls work by generating an inhibitor of the cell cycle progress in response to the failure to complete an event, rather than producing an activator of progression in response to the completion of the event. This becomes more evident when the case of spindle assembly is considered. If the kinetochores that are not attached to the spindle produce an inhibitor, monitoring the attachment of the last kinetochore to the spindle requires only that the cell distinguish between the presence and absence of the inhibitory activity. Direct support for the existence of an inhibitory signal from kinetochores came from experiments on mammalian tissue culture cells (Rieder et al., 1995). Anaphase will not begin until the last kinetochore attaches to the spindle, even if attachment takes many hours. This delay can be abolished if the last unattached kinetochore is destroyed by a laser microbeam.

The spindle assembly checkpoint defined by the *bub* and *mad* mutants made it possible to determine which defects arrest cells in mitosis by activating the checkpoint and which induce arrest by other means. All of the defects that affect the ability of kinetochores to attach to spindle microtubules were found to be detected by the spindle assembly checkpoint (Rudner and Murray, 1996). Low doses of microtubule-polymerization inhibitors and taxol, a microtubule stabilizer, also activated the checkpoint although in both of these cases normal bipolar attachment of chromosomes occurred and the spindle appeared normal (Jordan et al., 1993; Jordan et al., 1996) suggesting that tension may directly control the activity of the checkpoint, or it may stabilize the kinetochore-microtubule attachments, with the checkpoint responding to unoccupied microtubule-binding sites on the kinetochore.

This checkpoint is not, however, an integral part of the mechanism that promotes bi-orientation because mutations of checkpoint proteins such as Mad2 have little effect on the fidelity of bi-orientation during mitosis in the budding yeast (Li and Murray, 1991). The attachment of kinetochores to only a single microtubule, the finding that centromeres are connected to SPBs (spindle pole bodies) even during G1 phase (Winey and O'Toole, 2001) and the asymmetric distribution of old and new spindle pole bodies in the budding yeast (Segal and Bloom, 2001) has made its bi-orientation mechanism particularly easy to study. Ipl1-Sli15 complex, the yeast ortholog of the Aurora B-INCENP, was predicted to

promote bi-orientation. In the complete absence of DNA replication, most unduplicated centromeres cosegregate with old SPBs in *ipl1* and *sli15* mutants while no such bias was found in wild-type cells. The simplest interpretation of this observation was that old SPB-kinetochore connections inherited from G1 persisted throughout the cell cycle in the absence of Ipl1 activity. Ipl1-Sli15 presumably promotes the turnover of these connections, possibly because they do not generate tension; and as a result, unreplicated kinetochores connect with equal probability to microtubules from old and new SPBs (Tanaka et al., 2002). It has been suggested but never fully proven that Ipl1/Aurora B is also required to activate the spindle assembly checkpoint when chromosomes fail to come under tension (Biggins and Murray, 2001; Hauf et al., 2003).

In order to secure the accurate segregation of sister chromatids, the spindle assembly checkpoint acts to block the activation of Anaphase Promoting Complex/Cyclosome (APC/C) until all the chromosomes are correctly bioriented at the metaphase plate. Once activated, Anaphase Promoting Complex enables the activation of a cysteine protease called Esp1 by targeting its inhibitor for degradation (Nasmyth, 1999; Peters, 2002). In turn, Esp1 triggers the segregation of chromatids by cleaving the Scc1 subunit of cohesin complexes that hold the sister chromatids together.

Generation and maintenance of the spindle assembly checkpoint appears to be one of the most complicated events in the mitotic cell cycle. In the absence

of bipolar attachment, these spindle assembly checkpoint proteins which include Mad1, Mad2, Mad3/BubR1, Bub1, Bub3 and Mps1, collaborate to produce an inhibitory signal which diffuses away to block the activity of APC/C throughout the cell. Unattached kinetochores are thought to serve as catalytic sites for the production of this inhibitory signal. Consistent with this idea, all the known checkpoint proteins have been shown to localize to the kinetochores in mitosis. More importantly, Mad1 and Mad2 only localize to the unattached kinetochores (Chen et al., 1998; Hoffman et al., 2001).

Extensive efforts for identifying the nature of this inhibitory signal have led to the purification of MCC (Mitotic Checkpoint Complex) and its sub-complexes such as Mad2-Cdc20 and BubR1-Bub3-Cdc20, all of which are able to inhibit the activation of APC/C in vitro for mitotic substrates (Fang, 2002; Fang et al., 1998a; Hardwick et al., 2000; Sudakin et al., 2001; Tang et al., 2001). MCC contains BubR1, Bub3, Mad2 and Cdc20 which is a so-called APC/C activator required at the metaphase to anaphase transition. MCC stands to be an attractive candidate for a globally acting inhibitory signal because of its much more potent APC/C inhibitory activity than its sub-complexes. Certain evidence suggests that Mad2 and BubR1 function in distinct branches of the checkpoint signaling as elements sensing attachment and tension respectively (Skoufias et al., 2001; Waters et al., 1998; Zhou et al., 2002) although attachment and tension are intimately interrelated. This leads to the possibility that Mad2-containing

complexes may act as a diffusible inhibitor of APC/C in response to loss of microtubule attachment while BubR1-Bub3-Cdc20 may act as a local inhibitor at the kinetochores in response to a lack of tension across kinetochores. Consistent with the latter, BubR1 associates with a plus end-directed kinetochore motor protein called CENP-E whose interaction with microtubules could alter BubR1 activity in response to changes in kinetochore tension (Chan et al., 1999). In addition, Bub1 directly phosphorylates Cdc20 and plays a catalytic role in checkpoint-dependent inhibition of APC/C (Tang et al., 2004).

The mechanism by which MCC (mitotic checkpoint complex) is formed in vivo is not known. However, the formation of Mad2-Cdc20 complex is thought to be central in this process. Throughout the cell cycle Mad1 and Mad2 form a stable complex, whereas a substantial fraction of Mad2 exists as free molecules (Chung and Chen, 2002). The free Mad2 is not available for Cdc20 binding as it most probably retains a conformation which hinders its binding to Cdc20. Because the in vivo formation of Mad2-Cdc20 complex is absolutely dependent on the interaction between Mad1 and Mad2 (Hwang et al., 1998), it is thought that Mad1 binding promotes a conformational change in Mad2 so that Mad2 becomes competent for binding to Cdc20. As Mad1 and Cdc20 share a conserved ten-residue Mad2-binding motif, binding of Mad1 to Mad2 induces a major conformational change in the Mad2 carboxyl-terminal similar to that observed in Mad2-Cdc20 peptide (Luo et al., 2000; Luo et al., 2002; Sironi et al., 2002).

Because Mad2 binds poorly to full-length Cdc20 in vitro, this conformational change is thought to facilitate the binding of Mad2 to Cdc20 at the kinetochores if Mad2 dissociated from Mad1 retains transiently its conformation. However, as revealed by the crystal structure of Mad1-Mad2 complex, the Mad2 C-terminal tail locks in Mad1 and the release of Mad2 is likely to require the unfolding of its C-terminal tail. To envision how Mad1-Mad2 complexes might facilitate the formation of Mad2-Cdc20 complexes, it is necessary to recall that Mad2 forms complexes with itself (Sironi et al., 2001) as well as with Mad1 or Cdc20 and there are two populations of Mad2 at the kinetochores, one turning over rapidly and one relatively stable (Howell et al., 2004; Shah et al., 2004). Because the amount of Mad1 at the kinetochores is much less than the amount of Cdc20 in the cell, Mad1 could bind one Mad2 subunit and thereby influence the probability that a second Mad2 subunit adopts a conformation compatible with Cdc20 binding. A series of biochemical experiments involving the analysis of Mad1-Mad2 complexes by gel filtration has provided key insights into the nature of Mad2 dimers. The results implied that Mad2 dimers form only between Mad2 subunits that are held in different conformations; that is, they form between a Mad2 subunit whose C-terminal tail topologically encloses a Mad1 or Cdc20 peptide and a Mad2 subunit whose C-terminal tail is in a state ready to receive Cdc20 (De Antoni et al., 2005). This model appears consistent with most if not all the facts, but it is far from proven.

Conclusion

Many of the questions posed prior to the revolutionary discovery of molecular biology remain central to modern students of mitosis. These questions are: how does the mitotic spindle form and how do the chromosomes attach to it? What forces are responsible for physically moving the chromosomes and for separating the daughter cells? What other control networks act during mitosis besides the spindle assembly checkpoint? How is the chromosome segregation coupled to the mitotic exit? We now know the identity and the properties of many of the molecules involved in mitosis and we have at hand powerful techniques for identifying the missing components. However, it will require time to understand how spatially and temporally these components are organized to act during mitosis. This becomes evident when the spindle assembly checkpoint is considered as a case where we have a well defined biological problem. Although almost all of its molecular components are identified, spindle checkpoint still remains a poorly understood phenomenon. This is due to our lack of information about the mechanistic and regulatory interplay between the partners of this complicated system.

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Appendix

Names of the genes and mutants are always italicized. For the budding yeast, names of wild-type genes are uppercase and names of recessive mutations are lowercase. For the fission yeast, all gene designations, whether for wild type or mutant forms are lowercase. There is no reliable convention for the names of genes in vertebrates. Gene products are designated in the same way for all organisms: by using the same abbreviations as for the gene, but with only the first letter capitalized and without italics.

CHAPTER TWO

Aurora B is a substrate of the Anaphase Promoting Complex

Introduction

The serine/threonine Aurora B kinase is implicated in a variety of cellular processes such as chromosome condensation, sister chromatid cohesion, mitotic spindle assembly and the regulation of kinetochore-microtubule attachments and cytokinesis. Aurora B is a so called chromosomal passenger protein which localizes between sister-chromatids together with two other passenger proteins, INCENP and Survivin, early in prophase (Cooke et al., 1987; Schumacher et al., 1998; Speliotes et al., 2000). While on chromosome arms, Aurora B contributes to H3 phosphorylation (Adams et al., 2001; Crosio et al., 2002; Giet and Glover, 2001; Goto et al., 2002; Hsu et al., 2000) which was long thought to correlate with chromosome condensation in diverse eukaryotes (Wei et al., 1999). In addition, Aurora B regulates the association of Barren condensin protein and ISWI, a chromatin remodeling ATPase, with chromosomes and controls the distribution of Condensins I and II on the chromosomes (Giet and Glover, 2001; MacCallum et al., 2002; Ono et al., 2004). In unperturbed mitosis, residual amount of cohesin maintains sister chromatid cohesion throughout metaphase and

the majority of cohesin is removed from chromosome arms during prophase by the action of Plk1 and partly by Aurora B (Gimenez-Abian et al., 2004; Losada et al., 2002; Sumara et al., 2002). As cells proceed into metaphase, Aurora B, which is distributed along the chromosome arms, becomes localized to the centromeres (Adams et al., 2000; Ainsztein et al., 1998; Bolton et al., 2002; Delacour-Larose et al., 2004; Uren et al., 2000; Wheatley et al., 2001). Association of Aurora B with another chromosomal passenger protein named Borealin/Dasra B is required for the chromatin-induced microtubule stabilization and spindle formation (Gassmann et al., 2004; Sampath et al., 2004).

A question of central importance in mitosis is how cells ensure that sister kinetochores attach to microtubules with opposite orientations. In order to maintain genomic stability, cells have to avoid attachment of sister kinetochores to microtubules emerging from the same pole (synthelic attachments) and microtubules with opposing orientations from attaching to the binding sites on the same kinetochore (merotelic attachments) (Petronczki et al., 2003). In vertebrates, Aurora B phosphorylates and regulates the localization of MCAK (mitotic-centromere-associated kinesin) and its microtubule depolymerizing activity (Andrews et al., 2004; Lan et al., 2004). Regulation of MCAK activity by Aurora B-dependent phosphorylation is required for correcting synthelic attachments and merotelic attachments (Kline-Smith et al., 2004). Inhibiting the function of Aurora B causes cells to enter anaphase in the presence of numerous mono-

oriented chromosomes, which have one or both of the sister kinetochores attached to a single spindle pole (Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003). Furthermore, in the absence of Aurora B function, kinetochore localization of the spindle checkpoint protein BubR1 is greatly diminished and the spindle checkpoint is compromised in the presence of taxol, a spindle toxin which activates the checkpoint by stabilizing the microtubules (Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003).

After Cyclin B degradation, Aurora B is released from the kinetochores and becomes localized to the central spindle (Murata-Hori et al., 2002; Parry et al., 2003). Relocalization of Aurora B is dependent on MKlp2 (Gruneberg et al., 2004) which may exert dual control over Aurora B localization because it is also a binding partner for the phosphatase Cdc14A, whose budding yeast homolog directs Aurora B to spindles by dephosphorylating INCENP (Pereira and Schiebel, 2003). Central spindle assembly requires the centralspindlin complex composed of MKlp1 kinesin-like protein and a Rho family GAP, MgcRacGAP (Mishima et al., 2004). Aurora B phosphorylates both of these proteins and their phosphorylation is required for the completion of cytokinesis (Guse et al., 2005; Minoshima et al., 2003). As the spindle disassembles, in the budding yeast, Aurora B follows the plus end of the depolymerizing spindle microtubules and moves towards the spindle poles (Buvelot et al., 2003).

The activity of Aurora B is tightly regulated during the cell cycle through its association with INCENP and Survivin, phosphorylation and eventually with the proteasome-targeted degradation (Bishop and Schumacher, 2002; Bolton et al., 2002; Chen et al., 2003; Honda et al., 2003; Shu et al., 2003; Wheatley et al., 2004; Yasui et al., 2004). Autophosphorylation of T232 is indispensable for its activity and is stimulated through interaction with INCENP (Yasui et al., 2004). Phosphorylation of two adjacent serine residues on the carboxyl terminus of INCENP by Aurora B stimulates the kinase activity of this complex (Bishop and Schumacher, 2002; Honda et al., 2003). And for the full activation of Aurora B, Survivin binding is also required (Chen et al., 2003). The complex is presumably disrupted when Survivin is phosphorylated at T117 by Aurora B (Wheatley et al., 2004) and the protein levels of Aurora B, INCENP and Survivin start to be down regulated and show similar changes during the rest of the cell cycle (Honda et al., 2003).

The ubiquitin-proteasome pathway is the major system in the eukaryotic cell for the selective degradation of proteins (Hershko and Ciechanover, 1998). The events of late mitosis, from sister chromatid separation to exit from mitosis, are governed by APC/C (anaphase promoting complex/ cyclosome), a cell cycle-regulated ubiquitin ligase that assembles multiubiquitin chains on regulatory proteins and thereby targets them for degradation (Peters, 2002). APC/C activation requires the association of substrate specificity factors Cdc20 or Cdh1

(Fang et al., 1998; Kramer et al., 1998). During the metaphase to anaphase transition, APC/C^{Cdc20} mediates the ubiquitination and degradation of Securin and Cyclin B which are the only essential targets of APC/C in the budding yeast (Thornton and Toczyski, 2003). The activation of APC/C^{Cdh1} in turn depends on the degradation of Cyclin B and the initiation of the mitotic exit network (Bembenek and Yu, 2001; Jaspersen et al., 1999; Jaspersen et al., 1998; Visintin et al., 1998; Yeong et al., 2000; Zachariae et al., 1998). Once these events have occurred, the specificity factor Cdh1 works with APC/C to target Cdc20 (Pfleger and Kirschner, 2000) and other proteins such as Clb2, Ase1, Cdc6, SnoN, Cin8p, Aurora A, Cdc25A, ribonucleotide reductase R2, Plk1, Skp2, Cks1 and itself for degradation during exit from mitosis and G1 (Bashir et al., 2004; Castro et al., 2002a; Chabes et al., 2003; Donzelli et al., 2002; Hildebrandt and Hoyt, 2001; Lindon and Pines, 2004; Listovsky et al., 2004; Littlepage and Ruderman, 2002; Petersen et al., 2000; Schwab et al., 1997; Taguchi et al., 2002; Visintin et al., 1997; Wan et al., 2001; Wei et al., 2004).

Recognition of substrates by APC/C-specificity factor complexes and their subsequent ubiquitination requires short sequence elements. The best characterized of these are the D box (R-X-X-L, X= any amino acid) and the KEN box (K-E-N) which are found in several APC/C substrates (King et al., 1996; Pfleger and Kirschner, 2000). Additional sequence elements, such as a “GxEN motif” and a so called “A box”, have been identified later (Castro et al., 2003;

Littlepage and Ruderman, 2002). It is poorly understood how these sequence elements contribute to recognition and ubiquitination by APC/C-specificity factor complexes in different instances although they seem to contribute to a cooperative process of assembly between APC/C, specificity factor and substrate (Burton et al., 2005; Carroll et al., 2005; Kraft et al., 2005; Passmore et al., 2003; Yamano et al., 2004). It is widely assumed that substrates can only associate with APC/C in the presence of Cdc20 or Cdh1 (Passmore et al., 2003). Cdc20 and Cdh1 can bind to APC/C substrates directly (Burton and Solomon, 2001; Hilioti et al., 2001; Ohtoshi et al., 2000; Pfleger et al., 2001; Schwab et al., 2001; Sorensen et al., 2001) although only some of these interactions were found to depend on D or KEN boxes (Sorensen et al., 2001). The hypothesis that Cdc20 and Cdh1 function as substrate adapters has been challenged by the observation that tandem repeats of N-terminal fragment of the fission yeast Cyclin B associates in a D box-dependent manner with APC/C but not with Cdc20 in mitotic *Xenopus* egg extracts (Yamano et al., 2004). Among the twelve known subunits of APC/C, Doc1/Apc10 appears to be a good candidate for binding substrates because the structure of Doc1/Apc10 assumes a ligand binding function (Au et al., 2002; Wendt et al., 2001) and it contributes to the recruitment of D-box containing substrates (Carroll et al., 2005; Passmore et al., 2003). And there is also little evidence so far that the observed binding of APC/C substrates to Cdc20 and Cdh1 is actually required for the substrate ubiquitination (Sorensen et al., 2001).

Unlike its homolog Aurora A, which is a well-established substrate for APC/C^{Cdh1} (Crane et al., 2004), Aurora B was shown not to be targeted for degradation by APC/C^{Cdh1} in *Xenopus* extracts although it possessed the same degradation motifs like its homolog (Castro et al., 2002b). Here, we show that Aurora B is degraded during exit from mitosis in a Cdh1 dependent manner and the KEN box of Aurora B is required for its APC/C^{Cdh1} mediated ubiquitination and therefore, its degradation. Using in vitro ubiquitination assays, we provide direct evidence for the ubiquitination of Aurora B by APC/C^{Cdh1} and show that the mutation of its KEN box abolishes the formation of the ubiquitinated intermediates. In addition, KEN box-mutated form of Aurora B is stabilized in interphase extracts supplemented with Cdh1 and interestingly, epitope tagging at either end makes Aurora B unsusceptible to degradation. Aurora B overexpressed in HeLa cells can be efficiently degraded when Cdh1 is ectopically expressed and addition of a cell-permeable proteasome inhibitor, MG132, reverses the effect of ectopically expressed Cdh1. Moreover, the mutant form of Cdh1, which lacks the "C box" and the "IR" motif responsible for binding to APC/C but not to the substrate, is incapable of inducing the degradation of overexpressed Aurora B and KEN-box mutated form of Aurora B is stabilized in the presence of overexpressed Cdh1. Finally, we show that siRNA silencing of Cdh1 stabilizes Aurora B during exit from mitosis. In conclusion, Aurora B is a Cdh1 dependent substrate of the anaphase promoting complex and degraded during exit from mitosis. Recently,

Nguyen et al. (Nguyen et al., 2005) identified KEN box and A box as important determinants of Aurora B degradation in vivo and perplexingly, Stewart et al. (Stewart and Fang, 2005) identified a conserved D-box on the carboxyl terminus of Aurora B as the required motif for ubiquitination by APC/C^{Cdh1} and its degradation.

Experimental Procedures

Cell culture, synchronization procedures and cell cycle analysis

HeLa S3 (ATCC CCL-2.2) cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 IU/ml and 100 μg/ml, respectively) in 37^{o}C and 5% CO_2 incubator. To synchronize cells at the prometaphase stage, asynchronous cells were treated with 100 ng/ml nocodazole (Sigma) for 18 hours. To release cells from drug-induced mitotic arrest, cells were washed twice with phosphate-buffered saline (PBS), were seeded into fresh medium for further incubation and were harvested at set time points. For flow cytometry analysis, cells harvested at various time points were washed once with ice-cold PBS, and suspended in ice-cold PBS and fixed in nine volumes of 70% ethanol at least for 32 hours at 4^{o}C, washed with ice-cold PBS, and then treated with propidium iodide staining solution (0.2 mg/ml RNase A (Sigma), 0.02 mg/ ml Propidium iodide (Sigma), 0.1% Triton-X in PBS) for 30 minutes. At least 10,000 cells were scored using FACScan flow cytometer and cell cycle distribution was analyzed with CellQuest software (Becton Dickinson).

Protein expression and purification

For the production of human Cdh1 and Cdc20 proteins, recombinant baculoviruses encoding these proteins fused at the N-termini with the six-His tag were constructed using the Bac-to-Bac system (Invitrogen). Sf9 cells were used

for virus amplification and protein expression. 50 hours after infection,cells were collected by centrifugation and protein extracts were prepared in a buffer containing 50mM NaH_2PO_4, pH 8.0, 450 mM NaCl, 10 mM imidazole, 1% NP-40, 10 mM β-Mercaptoethanol, 10 μg/ml each of chymostatin, leupeptin, and pepstain. After keeping the lysates on a rotating wheel for 20 min at 4^{o}C and sonication, lysates were cleared by centrifuging at 18000 rpm for 30 min at 4^{o}C. The precleared lysates were then incubated with Ni-NTA beads (Qiagen) and washed with the lysis buffer. After washing with four column volumes of 20 mM and one column volume of 40 mM imidazole containing wash buffers (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl), proteins were eluted with a step gradient of imidazole (50mM-100mM-250mM) in Tris buffer (50 mM Tris, pH 8.0, 100 mM NaCl). The buffer containing the proteins was later changed to XB (500 mM KCl, 0.1 mM $CaCl_2$ 1 mM $MgCl_2$, 10 mM Hepes, pH 7.7, 50 mM Sucrose) using PD-10 columns (Amersham Biosciences).

APC/C ubiquitination assays

To purify interphase APC/C, the anti-CDC27 beads were incubated with 10 volumes of interphase *Xenopus* egg extract for 2 hr at 4^{o}C and washed five times with high salt XB buffer (500 mM KCl, 0.1 mM $CaCl_2$ 1 mM $MgCl_2$, 10 mM Hepes, pH 7.7, 50 mM Sucrose, 0.5% NP-40) and twice with XB (500 mM KCl, 0.1 mM $CaCl_2$ 1 mM $MgCl_2$, 10 mM Hepes, pH 7.7, 50 mM Sucrose). The interphase APC/C beads were then incubated for 1 hour at room temperature with

human Cdc20 or Cdh1. After incubation, APC/C beads were washed twice with XB and assayed for Aurora B ubiquitination activity. Ubiquitination reaction mixture contains an energy regenerating system (7.5 mM phosphocreatine, 1 mM ATP, 1 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.7), 1.25 mg/ml bovine ubiquitin (Sigma), and 1.5 μl of ^{35}S-labeled-substrate, 200 μg/ml human E1, 50 μg/ml UBCx, and 3 μl of the APC/C beads. The reactions were incubated at room temperature for 1 hour, quenched with 6xSDS-sample buffer, boiled and one half of each sample was separated on 12% SDS-polyacrylamide gels. Gels were fixed in a fixing solution (50% methanol, 40% H_2O, 10% glacial acetic acid) for 30 min, equilibrated in drying buffer (7% methanol, 7% glacial acetic acid, 1% glycerol) for 5 min, dried using a conventional gel drier and analyzed by phosphorimaging.

Degradation assays

^{35}S-labeled-substrate was made by in vitro translation using a rabbit reticulocyte lysate (TNT, Promega) and ^{35}S-methionine. We mixed 5 μl of TNT reticulocyte lysate with 30 μl of interphase *Xenopus* extract at room temperature supplemented with 0.5 mg/ml bovine ubiquitin and with an energy regenerating system (7.5 mM phosphocreatine, 1 mM ATP, 1 mM $MgCl_2$, and 1 mM EGTA, pH 7.7). Where indicated, 10 μl of XB buffer or 10 μl of recombinant human Cdh1 to a final concentration of 0.4 nM was added to the mixture. Aliquots of 5 μl were quenched with 6xSDS-sample buffer at the indicated time points, boiled and one

sixth of each sample was separated on 12% SDS-polyacrylamide gels. Gels were fixed in fixing solution for 30 min, equilibrated in drying buffer for 5min, dried using a conventional gel drier and analyzed by phosphorimaging.

Plasmids, transfections and siRNA

Aurora B was PCR amplified from the human fetal thymus cDNA library (Clontech) with primers introduced 5' Fse I and 3'Asc I sites and cloned into the Fse I/Asc I sites of the FA modified pCS2 expression vector. Aurora B N-terminal and carboxyl–terminal deletion mutants were made by PCR amplification of the desired regions and cloned into the Fse I/Asc I sites of the FA modified pCS2 vector. We used the following primers (sense, 5'CGGCCAACCATGGCCCAGG CGGCGGCCTCCTACCCCTGGCCCTAC3'; antisense, 5'GTAGGGCCAGGG GTAGGAGGCCGCCGCCTGGGCCATGGTTGGCCG3') to mutate ^{4}KEN^{6} to ^{4}AAA^{6} using QuickChange site-directed mutagenesis kit (Stratagene). To mutate the third D box (^{315}RLPL^{318}) to (^{315}ALPA^{318}), we used the following primers (sense, 5'CTCAGGCATAACCCCTCGGAAGCGCTGCCCGCGGCCCAGGTC TCAGCCCACCCT3'; antisense, 5'AGGGTGGGCTGAGACCTGGGCCGCGG GCAGCGCTTCCGAGGGGTTATGCCTGAG3').

HA-tagged Cdh1 plasmid (pCS2-HA-Cdh1) was generated by subcloning human Cdh1 from the pCS2-Cdh1 vector. QuickChange Site-directed mutagenesis kit was used to generate the C box deletion (^{46}DRFIPSR^{52}) using the following primers (sense, 5'CGCCCAGCAAGCACGGAGCCGGAGCCAACTG

GAG3'; antisense, 5'CTCCAGTTGGCTCCGGCTCCGTGCTTGCTGGGCG3'). For the truncation of the C-terminal IR motif (^{492}FTRIR^{496}), C box deleted Cdh1 was PCR amplified with a primer that contains a stop codon at amino acid position 492 and cloned into the Fse I/Asc I sites of the pCS2-HA vector.

For transient transfections, the day before transfection 1.2 x 10^5 HeLa Tet-on (Clontech) cells per well were seeded in a 6-well plate and cells were grown to 40-50% confluency. Cells were transfected with 50 ng of each of the plasmids using the Effectene reagent (Qiagen) according to the manufacturer's protocol. Where indicated, cells were treated with MG132 (Calbiochem) to a final concentration of 10 μM 8 hours prior to harvesting. Twenty-four hours after transfection, cells were harvested by trypsinization, washed with PBS and resuspended in PBS containing 0.1% NP-40 and 10 μg/ml each of chymostatin, leupeptin, and pepstain. Protein concentration in total cell lysates was determined using Bio-Rad protein assay and equal amount of each lysate was separated on SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes using the Bio-Rad semi-dry electrophoretic transfer unit and were analyzed by western blotting.

siRNA oligonucleotides targeting Cdh1 were chemically synthesized at an in-house facility, and correspond to the nucleotides 372-390 of human Cdh1 coding sequence. The day before transfection, 2x10^5 HeLa Tet-on cells per 60 mM dish were seeded and cells were grown to 40% confluency. The annealing of

the siRNAs and subsequent transfection of the RNA duplexes into cells were performed using the Oligofectamine reagent (Invitrogen) exactly as described by the manufacturer. 12 hours after transfection, 100 mM thymidine (Sigma) in PBS was added to a final concentration of 2 mM in order to synchronize the cells at the G1/S boundary. 18 hours later, cells were released into fresh medium and cells were harvested at set time points. Asynchronous cells were harvested 48 hours after transfection with the siRNA.

Antibodies and ECL detection

The following antibodies were used in this study: mouse monoclonal anti-rat Aurora B (α-Aim1) antibody from BD Biosciences, rabbit polyclonal anti-human Aurora B (BL1074) antibody from Bethyl Laboratories, mouse monoclonal anti-human Cdh1 (Cdh1(Ab-2)) antibody from Oncogene, mouse monoclonal anti-human Cyclin B1 (Cyclin B1(D-11)) antibody from SantaCruz, rabbit polyclonal anti-GFP (A-11122) antibody from Molecular Probes, mouse monoclonal anti-HA (12CA5) antibody from Roche, custom made rabbit polyclonal anti-APC2 antibody (1:1,000-fold dilution). All of the commercially available antibodies were used at the dilutions recommended by the manufacturers. Depending on the primary antibody choice, the ECL™ anti-rabbit IgG horseradhish peroxidase linked whole antibody or the ECL™ anti-mouse IgG horseradhish peroxidase linked whole antibody was used as the secondary antibody for the ECL (Amersham) detection.

Results

Aurora B levels are down-regulated during exit from mitosis

Since the identification of its drosophila and rat homologs (Bischoff et al., 1998; Terada et al., 1998), it is known that the protein level of Aurora B is down regulated during exit from mitosis. We and others observed that mammalian Aurora B is degraded during exit from mitosis like its homologs (Honda et al., 2003; Lindon and Pines, 2004). Analysis of extracts prepared from HeLa S3 cells synchronized throughout the mitotic exit by release from nocodazole-induced arrest showed that its degradation followed a slower kinetics than Cyclin B1 degradation (Fig. 1A). Because the activation of APC/C^{Cdh1} follows the APC/C^{Cdc20} targeted degradation of Cyclin B1 (Baumer et al., 2000; Yeong et al., 2000), we reasoned that Aurora B could be degraded in a Cdh1 dependent manner like its family member Aurora A (Castro et al., 2002a; Littlepage and Ruderman, 2002; Taguchi et al., 2002).

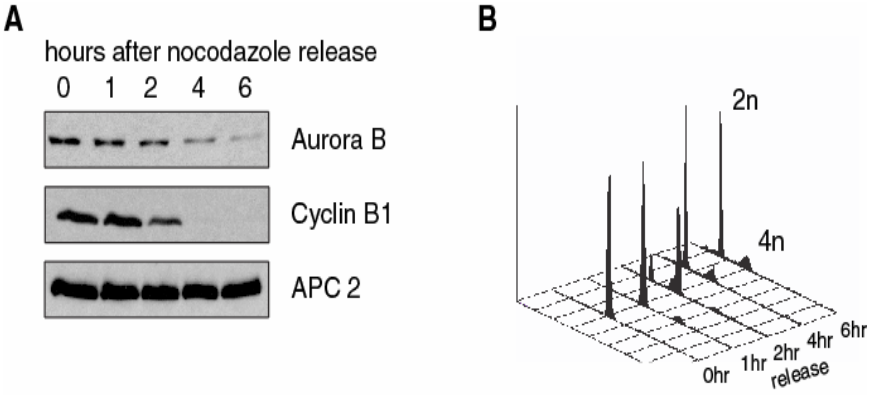

Figure 1 Aurora B level is down-regulated during exit from mitosis. (**A**) Western blot analysis of total cell lysates. Asynchronously growing Hela S3 cells were treated with nocodazole to a final concentration of 100 ng/ml for 18 hours in order to synchronize cells at the prometaphase stage. Cells were released into fresh medium and harvested at set time points. Equal amount of each cell lysate harvested at set time points were analyzed by western blotting with antibodies against APC2, Cyclin B1 and Aurora B (α-Aim1). APC2 level was used as a loading control. (**B**) FACS analysis of cells released from nocodazole-induced mitotic arrest.

Aurora B is ubiquitinated by APC/C^{Cdh1} in vitro

In order to test our hypothesis, we first wanted to see whether the ubiquitination of Aurora B could be mediated by APC/C. When the APC/C-dependent ubiquitination reaction was reconstituted in vitro using the immunoprecipitated *Xenopus* interphase APC/C and human components, Aurora B was found to be efficiently ubiquitinated in the presence of Cdh1 while Cdc20 didn't stimulate the ubiquitination of Aurora B (Fig. 2A). APC/C^{Cdh1} recognizes its substrates using two motifs known as "KEN" box and "D" box (Pfleger and Kirschner, 2000).

Aurora B contains a putative KEN box and four putative D boxes (Fig. 2C). We sought to know among these five putative motifs which one is required for its ubiquitination by APC/C^{Cdh1}. We ruled out the first and the second D boxes because they lie in close proximity to the kinase active site (Sessa et al., 2005) and were unlikely to be exposed to be accessible to recognition by APC/C^{Cdh1}. We initially generated N-terminal and C-terminal deletions of Aurora B which targeted to remove the KEN box and the last two D boxes respectively. Deletion of the first 33 amino acids or deletion of the last 51 amino acids but not that of the last 21 amino acids was sufficient to block the ubiquitination of Aurora B (Fig. 2A). Since within these regions a KEN box and a D box lie, we turned our attention on these putative motifs and using site directed mutagenesis, we generated KEN to AAA and D box substitution mutants (Fig. 2C). Among these two mutant forms, only the KEN mutant of Aurora B failed to be ubiquitinated by APC/C^{Cdh1}, while the D box mutant although less efficiently was still multi-ubiquitinated by APC/C^{Cdh1} (Fig. 2B).

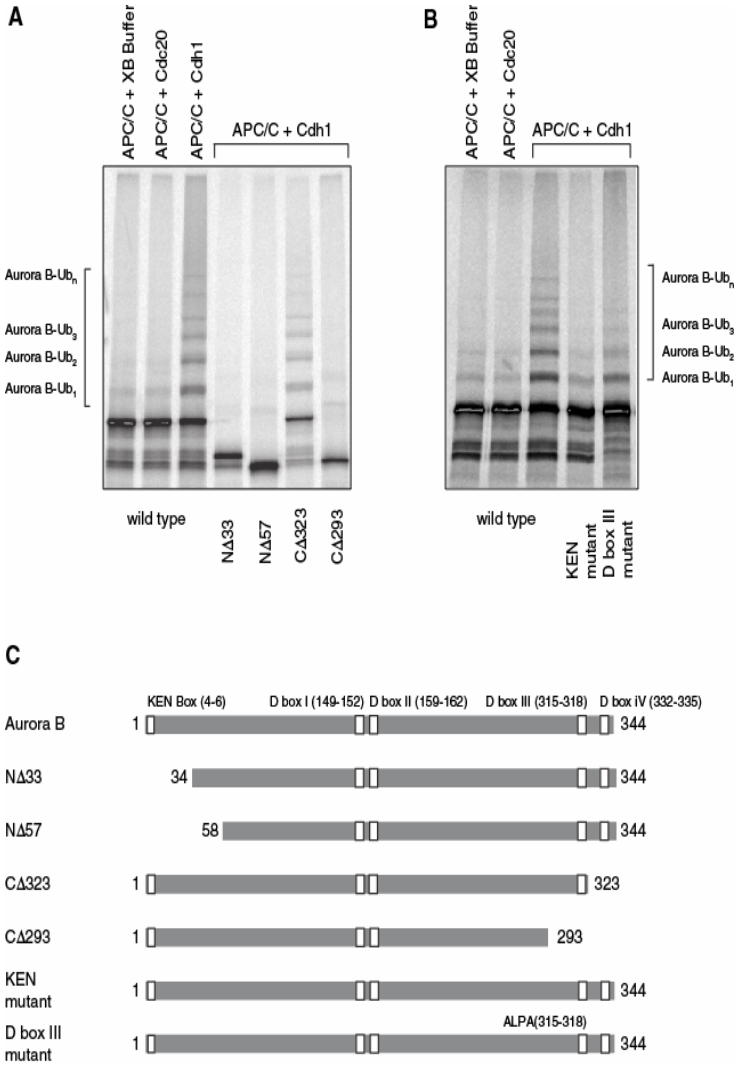

Figure 2 KEN box dependent ubiquitination of Aurora B by APC/C^{Cdh1} in vitro. (**A**) *Xenopus* interphase APC/C when stimulated with Cdh1 exhibited the ubiquitination of wild type Aurora B. The deletion mutants of Aurora B (NΔ33, NΔ57, CΔ293) were not ubiquitinated by APC/C^{Cdh1}. (**B**) For further characterization of the deletion mutants, the ^{4}KEN^{6} box on Aurora B was mutated to ^{4}AAA^{6} and the third D box (^{315}RLPL^{318}) on Aurora B was mutated to ^{315}ALPL^{318}. These mutants (the KEN mutant and the D box III mutant) were tested for APC/C^{Cdh1} ubiquitination activity. Mutation of the KEN box inhibited multi-ubiquitination of Aurora B by APC/C^{Cdh1}. (**C**) Schematic representation of the degradation motifs (the KEN box and the D boxes) present on Aurora B and schematic representation of the mutant forms of Aurora B used in this study.

The KEN mutant of Aurora B is stabilized in *Xenopus* interphase extracts supplemented with Cdh1

Xenopus interphase extracts offer a convenient way of testing whether Cdh1 is sufficient to trigger the destruction of APC/C substrates, since these extracts lack endogenous Cdh1 (Lorca et al., 1998) and the addition of recombinant Cdh1 induces the degradation of known Cdh1 substrates (Pfleger and Kirschner, 2000). Presumably, in these extracts APC/C is inactive because of the absence of a bound activator. Addition of recombinant Cdh1 protein to *Xenopus* interphase extracts induced the degradation of Aurora B although the kinetics of degradation was much slower compared to that of Aurora A (Fig. 3A). The difference in degradation kinetics could be attributed to the different motifs used by APC/C^{Cdh1} for recognizing these substrates since the deletion of an N-terminal A box, phosphorylation of a serine residue within the A box or the deletion of the most C-terminal D box (Castro et al., 2002a; Littlepage and Ruderman, 2002) make Aurora A unsusceptible to degradation in *Xenopus* interphase extracts or to an unknown regulatory mechanism which makes Aurora A a better substrate. And in accordance for its failure to be ubiquitinated, the mutation of the KEN box blocked Cdh1-dependent degradation of Aurora B in these extracts (Fig. 3B), confirming that KEN box is an important determinant of Aurora B degradation.

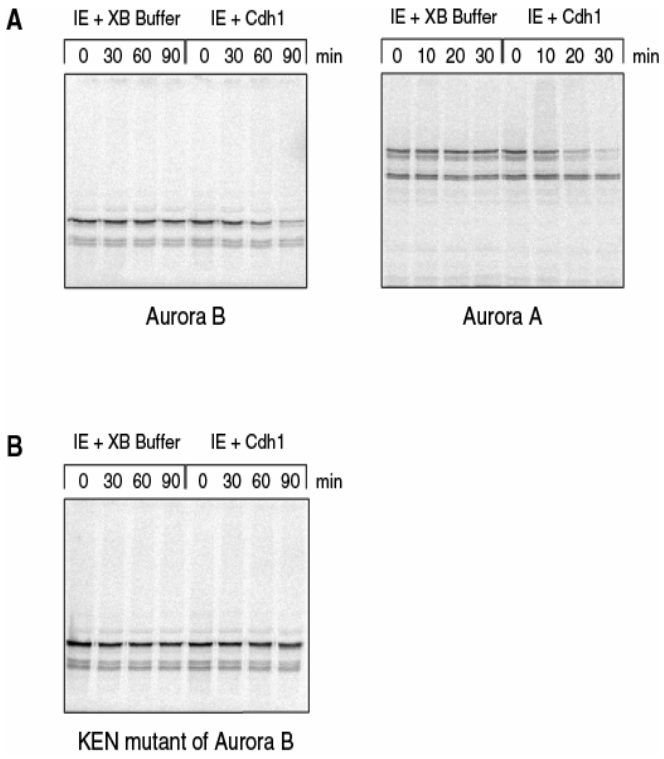

Figure 3 Cdh1 stimulated degradation of Aurora B in *Xenopus* interphase extracts is dependent on a functional KEN box. (**A**) Aurora B and Aurora A are both efficient substrates of APC/C in *Xenopus* interphase extracts when stimulated by Cdh1. The degradation of Aurora B follows a slower kinetics than the degradation of Aurora A. (**B**) The KEN box mutated form of Aurora B is stabilized in *Xenopus* interphase extracts supplemented with Cdh1.

Epitope tags interfere with the degradation and ubiquitination of Aurora B

Because we planned to study Aurora B degradation in vivo, we constructed epitope tagged versions of Aurora B to distinguish it from the endogenous copies; and before moving to in vivo studies, we wanted to see how epitope-tagged Aurora B behaves in *Xenopus* extracts supplemented with Cdh1 and in vitro ubiquitination assays. During the course of this work, we met with previously unsuspected problems in these commonly used assays. We found that epitope tagging of Aurora B at either end makes it unsusceptible to degradation in the presence of Cdh1 (Fig. 4A) and the efficiency of ubiquitination is reduced at a great extent (Fig. 4B). A similar observation was also made by Crane et al. (Crane et al., 2004) when GFP tagged Aurora A was used in the degradation assays although the effect was less pronounced.

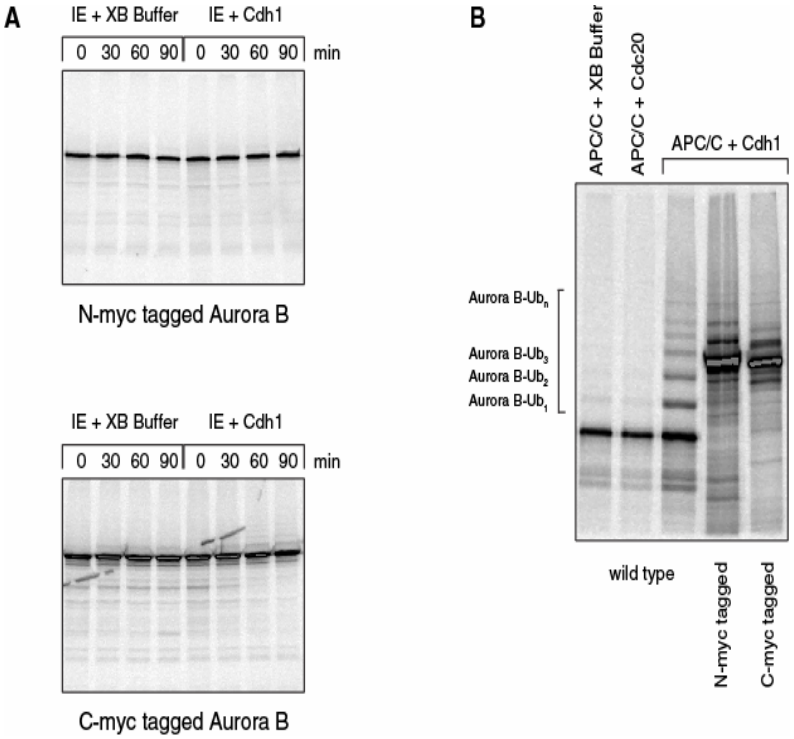

Figure 4 Epitope tags interfere with the degradation and ubiquitination of Aurora B. (**A**) Epitope tagging at either end stabilized Aurora B in *Xenopus* interphase extracts supplemented with Cdh1. (**B**) Epitope tagging at either end led to a diminished level of Aurora B ubiquitination by APC/C^{Cdh1}.

Aurora B is an efficient substrate for Cdh1 in vivo; not its KEN box mutant

Since we had shown that Aurora B is targeted for degradation in vitro in the presence of Cdh1, we investigated the requirement of Cdh1 for Aurora B degradation in vivo (Fig. 5A). Aurora B coexpressed with human Cdh1 in HeLa Tet-On cells produced a significant decrease in the steady–state levels of Aurora B twenty-four hours after post-transfection and in the presence of MG132, a proteasome inhibitor, the down-regulatory effect of Cdh1 on Aurora B level was blocked. We also showed that a mutant form of human Cdh1 which lacks the "C box" (Schwab et al., 2001) and the "IR" motif (Vodermaier et al., 2003) required for binding to APC/C but not to its substrate was unable to induce the down regulation of Aurora B level (Fig. 5A). These results collectively indicated that Cdh1 can induce the proteasome-targeted degradation of Aurora B through APC/C mediated ubiquitination of Aurora B. To test the in vivo requirement of the KEN box for Aurora B degradation, we transfected cells with wild type or the KEN box mutated form of Aurora B in the presence or absence of exogenously expressed Cdh1. Initially, we had puzzling results since the antibody (α-Aim1) we used didn't recognize the KEN mutant form of Aurora B (Fig. 5B). A possible explanation would be that KEN to AAA mutation destroyed the epitope recognized by this antibody, since this antibody is raised against an immunogen lying in the amino-terminus of AIM-1 (rat homolog of human Aurora B). We looked for another antibody and used BL1074 (Bethyl Lab.) which is raised

against the C-terminus of human Aurora B. Using this antibody, we were able to show that the KEN mutant of Aurora B was stabilized in the presence of overexpressed Cdh1, confirming the in vivo role of the KEN box as an important determinant for the degradation of Aurora B (Fig. 5B).

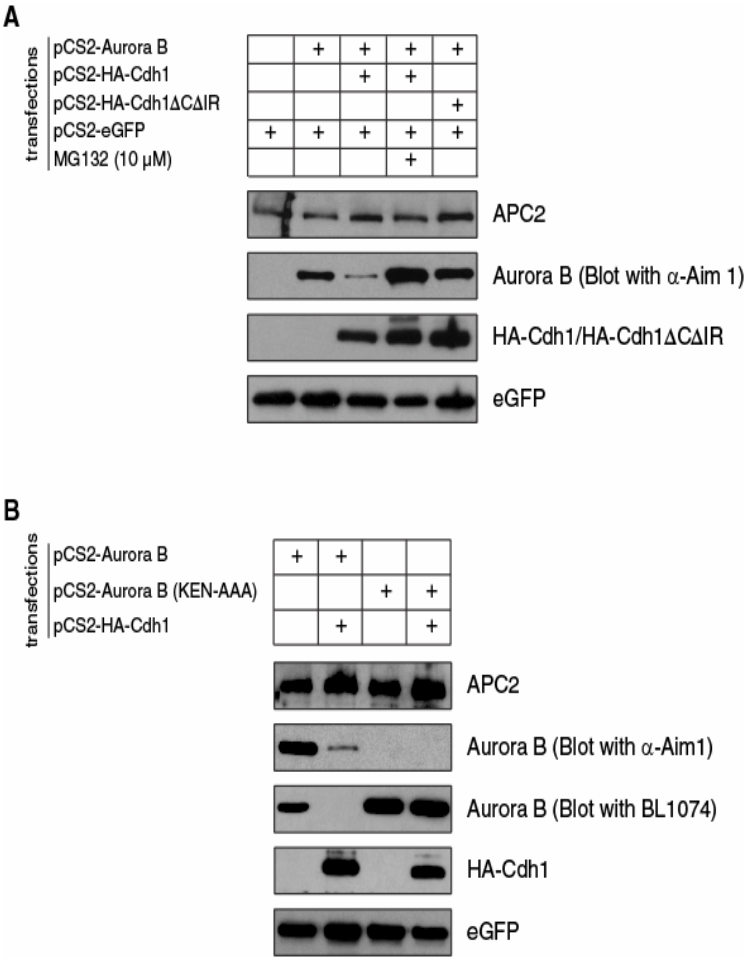

Figure 5 Aurora B degradation in vivo can be stimulated by Cdh1 and is dependent on a functional KEN box. (**A**) When coexpressed in HeLa Tet-on cells, Cdh1 stimulated the proteasome-targeted degradation of overexpressed Aurora B. HeLa Tet-on cells were transfected with the indicated plasmids and were grown for 24 hours before harvesting the cells. MG132 was added 8 hours prior to harvesting. eGFP was used as an internal control to compare the protein level of overexpressed genes. Equal amount of each total cell lysate was separated on SDS-polyacrylamide gels and analyzed by western blotting with the antibodies against APC2, Aurora B (α-Aim1), HA epitope and eGFP. APC2 was used as a loading control. (**B**) The KEN mutant of Aurora B can not act as a substrate of APC/C^{Cdh1} and KEN to AAA mutation disrupts the epitope recognized by α-Aim1; therefore, another antibody (BL1074) was used to detect the KEN mutant of Aurora B.

RNAi silencing of Cdh1 stabilizes endogenous Aurora B during exit from mitosis

We finally tested the regulatory role of Cdh1 on Aurora B levels in vivo through the depletion of Cdh1 by siRNA. HeLa Tet-on cells were treated with Cdh1 or mock siRNA; cells were synchronized at the G1/S boundary by thymidine block and released into the S phase. In mock treated cells, Aurora B level was down regulated between 11 and 18 hours after the release; whereas in those cells depleted of Cdh1 by siRNA, Aurora B remained at high levels during the same time course (Fig. 6). In contrast, the turnover of Cyclin B1 was slightly affected and retarded, probably because the inhibition of Cdh1 allows Cdc20 to persist and APC/C^{Cdc20} can substitute for the APC/C^{Cdh1} targeted pool of Cyclin B1 (Fig. 6). This observation also lets us to draw the reciprocal conclusion that Cdc20 is unable to target Aurora B for degradation.

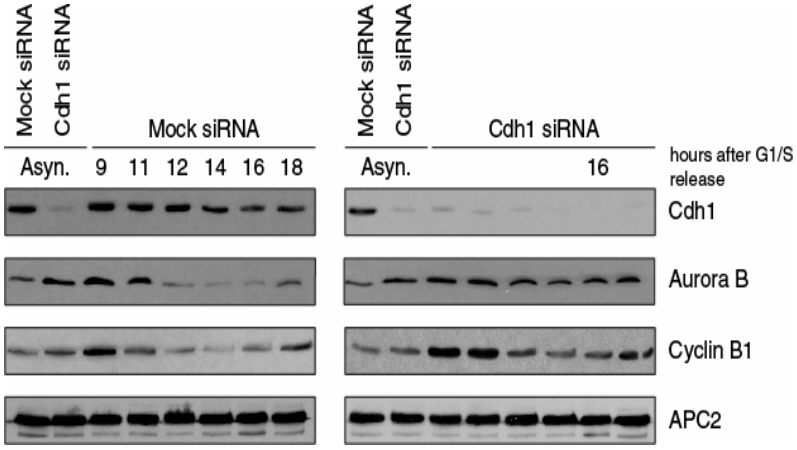

Figure 6 Silencing of Cdh1 stabilizes Aurora B during exit from mitosis. HeLa Tet-on cells transfected with Cdh1 or mock siRNAs were treated with 2 mM thymidine 6 hours after transfection. Cells were grown in the presence of thymidine for an additional 18 hours before releasing into S phase. Cells were collected at set time points and equal amount of each total cell lysate were separated on SDS-polyacrylamide gels and analyzed by western blotting with antibodies against Cdh1, Aurora B (α-Aim1), Cyclin B1 and APC2. APC2 level was used as a loading control.

Discussion

Aurora B is a serine/threonine kinase which plays critical roles required for the proper execution of mitosis. Its activity is tightly regulated with its association with two other chromosomal passenger proteins, INCENP and Survivin, through phosphorylation and the proteasome-targeted degradation (Bishop and Schumacher, 2002; Bolton et al., 2002; Chen et al., 2003; Honda et al., 2003; Shu et al., 2003; Wheatley et al., 2004; Yasui et al., 2004). In the present study, we showed that APC/C^{Cdh1} is able to ubiquitinate Aurora B in vitro and its ubiquitination requires an intact KEN box. We also confirmed the functionality of the KEN box using in vitro degradation assays. Unexpectedly, we found that in these commonly used ubiquitination and degradation assays, epitope tagging of the substrate may interfere with the outcome of experiments. In this certain instance, epitope tagging of Aurora B at either end greatly reduced its ubiquitination and conferred stability to Aurora B in *Xenopus* extracts supplemented with Cdh1. Overexpression studies in HeLa cells showed that Cdh1 is able to induce the down-regulation of Aurora B. Furthermore, overexpression of the mutant Cdh1 which lacks the "C box" (Schwab et al., 2001) and the "IR"motif (Vodermaier et al., 2003) required for binding to APC/C was not effective in down-regulating Aurora B. And treatment with a proteasome inhibitor, MG132, was able to rescue Cdh1 dependent degradation of Aurora B in HeLa cells. These results collectively indicated that Cdh1 is sufficient for the

targeting of Aurora B via the ubiquitin-proteasome pathway. We also showed that the KEN motif, which is found in several Cdh1 substrates, conferred resistance to Cdh1 dependent degradation in HeLa cells when mutated on Aurora B. We finally showed that HeLa cells synchronized at the G1/S boundary and released into S phase maintained elevated levels of Aurora B during exit from mitosis when Cdh1 is silenced by siRNA. In these cells, although slightly delayed, Cyclin B1 levels were still down-regulated. Experiments in yeast and flies have led to the suggestion that APC/C^{Cdc20} complexes target regulators of early mitosis for destruction, whereas APC/C^{Cdh1}complexes target proteins for destruction later in exit from mitosis and in G1 (Sigrist and Lehner, 1997; Visintin et al., 1997). Degradation of Cyclin B1 occurs in two phases (Baumer et al., 2000; Yeong et al., 2000). The first phase of Cyclin B1 degradation, which is restricted to the spindle and chromosomes, commences at the metaphase to anaphase transition and depends on Cdc20. The second phase of Cyclin B1 destruction that occurs in the cytoplasm during telophase requires the activation of Cdh1 (Clute and Pines, 1999; Huang and Raff, 1999; Raff et al., 2002). The first phase of Cyclin B1 degradation is a prerequisite for the second and this is implicated by the inhibition of Cdh1 association with APC/C due to its phosphorylation by Cdk1/Cyclin B1 and Cdc14 mediated reversal of this phosphorylation (Jaspersen et al., 1999; Zachariae et al., 1998). Because the silencing of Cdh1 slightly retarded the degradation of Cyclin B1, we feel safe to conclude that Aurora B degradation is

not dependent on Cdc20 and instead requires the association of Cdh1 with APC/C.

In earlier studies, where the subject of investigation was Aurora A, another member of the same family, the KEN box was proved to be nonfunctional. Instead, the most C-terminal D box was identified as the recognition motif required for the degradation of Aurora A (Castro et al., 2002a; Littlepage and Ruderman, 2002; Taguchi et al., 2002). Castro et al., who previously demonstrated that *Xenopus* Aurora A is degraded at late mitosis by APC/C^{Cdh1} in a D-box dependent manner (Castro et al., 2002a), showed that Aurora B is not targeted for degradation by this ubiquitin ligase using similar assays (Castro et al., 2002b). Moreover, it was shown that the N-terminus of Aurora A, which can direct its own degradation in the absence of any conventional D boxes (Littlepage and Ruderman, 2002), confers degradation capacity on the C-terminus of Aurora B and GFP (Castro et al., 2002b). Interestingly, mutation of the most C-terminal D box on Aurora B blocked the degradation capacity conferred by the N-terminus of Aurora A on the C-terminus of Aurora B (Castro et al., 2002b). It is possible to speculate that the A box that lies in the N-terminus of Aurora A is required to activate silent D boxes but this proposition is not sufficient enough to explain all of the previous observations made. In this regard, it would be interesting to know how the distinction is made by Cdh1 on substrates structurally very similar (Sessa et al., 2005). While this

study was in preparation, another work published by Nguyen et al. (Nguyen et al., 2005) showed that Aurora B is ubiquitinated in vivo and overexpression of either Cdc20 or Cdh1 induced the degradation of Aurora B and the formation of high molecular weight Aurora B intermediates in vivo. Few substrates such as Hsl1p, human Securin and Xkid were earlier shown to be degraded by both APC/C^{Cdc20} and APC^{Cdh1} although the significance of these observations remains to be established (Burton and Solomon, 2000; Burton and Solomon, 2001; Castro et al., 2003; Zur and Brandeis, 2001). At least in the case of human Securin, its degradation by APC/C^{Cdh1} seems to be artificial since the timing of Securin degradation is set by the spindle checkpoint (Hagting et al., 2002). The authors also identified two motifs; a KEN box and an A box, as the required determinants for Aurora B degradation. In this study, we can not provide any evidence for the role of the A box while we are in agreement with their published results. We believe that the emerging picture indicates that a complex regulation is involved in yet a simple process, targeting of Aurora kinases by APC/C^{Cdh1}. The picture becomes even more complicated with the added results of Stewart et al. (Stewart and Fang, 2005) who identified the third D box as the motif required for the targeted degradation of Aurora B and its ubiquitination by APC/C^{Cdh1}. In our hands, we observed a decreased ubiquitination of Aurora B when the third D box was mutated. However, we didn't observe an increased stability of this mutant in *Xenopus* interphase extracts supplemented with Cdh1 and moreover this mutant

form of Aurora B was unstable even in the absence of Cdh1 (Appendix-Fig. 1); therefore, we are reluctant to make conclusions.

Several groups have found that APC/C substrates can bind to Cdc20 and Cdh1 (Burton and Solomon, 2001; Hilioti et al., 2001; Schwab et al., 2001) and the ability of overexpressed Cdc20 and Cdh1 to destabilize specific substrates in vivo corresponds to their ability to bind these substrates (Schwab et al., 2001). However, it is not clear whether these so called recognition motifs are actually responsible for the binding of the substrate to Cdc20 or Cdh1, or whether APC/C subunits are actually responsible for the binding. While it is still under debate how APC/C bound to Cdc20 or Cdh1 recognizes the substrates, it is inconceivable to understand the distinction in mode of recognition. It would be interesting to use Aurora A and Aurora B as model substrates in structural studies to gain further insight about how D box, KEN box and A box contribute to the ubiquitination of Aurora kinases by APC/C^{Cdh1} and how the presence of each motif influences the presence of others.

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Appendix

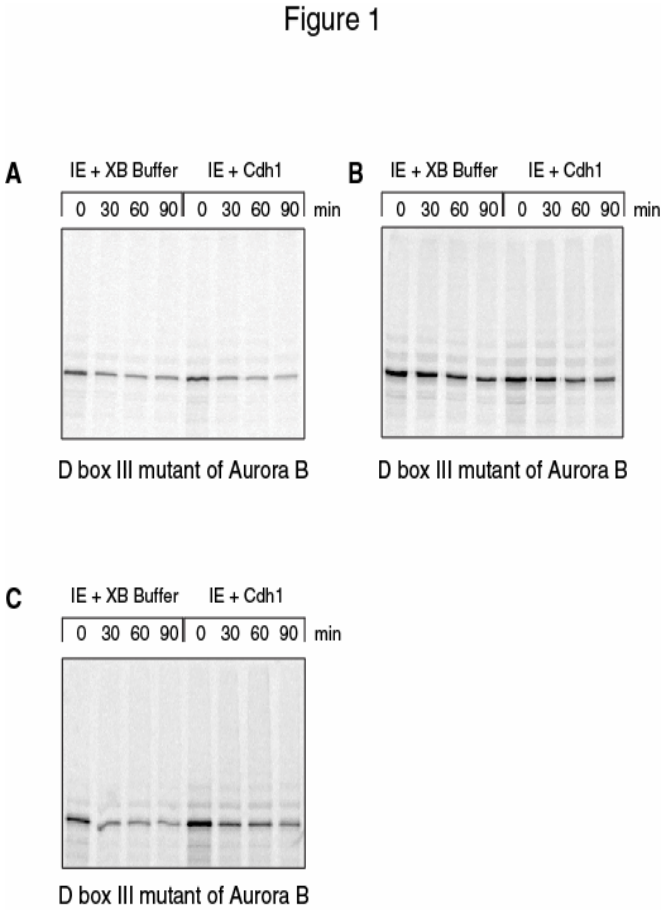

Figure 1 The D box III mutant of Aurora B is unstable in Xenopus interphase extracts. Three different experimental setups which show the observed destability of the D box III mutant of Aurora B in *Xenopus* interphase extracts in the presence or absence of Cdh1.

CHAPTER THREE

Screening for Aurora B kinase substrates using an in vitro expression cloning system

Introduction

Phosphorylation is the main device a cell uses to control the function of its proteins. The responsible enzymes, protein kinases, regulate a myriad of processes from cell division to neuronal communication in memory. Kinases impart their signal by transferring the γ-phosphate group from ATP to the hydroxyl group of a recipient serine, threonine or tyrosine residue on a protein. The choice of target is decided by the catalytic domain of the kinase, which usually recognizes a docking domain on the substrate and a specific, short sequence of amino acids (the consensus motif) that surround the residue to be phosphorylated. Each kinase can have several substrates and be itself a substrate for other kinases.

To start to delineate the complex network of pathways involving kinases, researchers want to match every kinase in a cell to specific phosphorylation sites on target proteins. To this end, several methods have been developed to identify the potential protein kinase substrates. Approaches to identifying candidate

substrates of protein kinases have historically included genetic screens, the isolation of kinase-binding proteins, and biochemical purification. Genetic screens and subsequent epistasis analysis in model organisms such as yeast, worms and flies have been very successful in placing substrates downstream of specific protein kinases within the signaling pathways. In the budding yeast, screens for mating-defective mutants which are resistant to growth inhibition by α factor have led to the elucidation of a protein kinase cascade leading from cell surface pheromone receptors to the downstream transcription factor Ste12, which binds at a nucleotide sequence located in the upstream regions of many α factor-inducible genes (Herskowitz, 1995).

Many of the protein kinase substrates have been identified through yeast two-hybrid screens, with the kinase of interest as "bait" (Yang et al., 1992). Since in most cases the interaction between a protein kinase and its substrate is transient, and upon substrate phosphorylation the association is disrupted, allowing a single kinase to catalyze the phosphorylation of multiple substrates, these interactions are difficult to trap and identify. However, in some cases, kinases bind their substrates by interactions outside the catalytic pocket, and such associations can often be detected in two-hybrid screens. Although two-hybrid screens to identify kinase substrates have been used with some success, problems with a high rate of false positives and with distinguishing between kinase-binding partners and bona fide in vivo substrates limit the usefulness of this approach. As

with all approaches, confirmation of the candidate substrates by secondary assays is a must.

Historically, the positions of phosphorylation sites on important cellular proteins were often determined prior to the identification of the responsible kinase. To identify kinases, proteins in cell lysates were separated by column chromatography and protein fractions were assayed for kinase activity toward a substrate of interest. Similar techniques have been used with limited success for the identification of proteins within lysate fractions that can be phosphorylated in vitro by specific protein kinases, as scored by the incorporation of ^{32}P on two-dimensional gel electrophoresis. Obvious problems arise from such an approach, including the presence of kinases within the lysates phosphorylating themselves and other proteins, leading to high levels of background and false-positives. Some of these problems have been circumvented with the development of a method termed as KESTREL (kinase substrate tracking and elucidation) which aims to reduce the background phosphorylation in cell extracts by introducing simple modifications to standard protocols and to increase the probability of identifying potential substrates by comparing the specificities of protein kinases that are closely related in structure (Knebel et al., 2001).

Over the years, several other methods have been developed for the identification of in vitro protein kinase substrates. Although useful for the identification of candidate substrates for a given kinase, in vitro kinase assays can

often be misleading and provide too many candidate substrates to be further validated by more rigorous in vivo approaches. The use of high concentrations of the purified kinase is partially responsible for the loss of specificity and the removal of kinase from the cell often results in loss of its physiological regulatory mechanisms.

One strategy to identify substrates of a kinase is to use phage expression libraries and is based on the fact that cellular proteins immobilized on a membrane filter can be phosphorylated by a soluble kinase with specificity similar to that obtained in conventional liquid-phase phosphorylation (Valtorta et al., 1986). The detection could be performed either by immunoscreening of phage expression libraries with antibodies that specifically recognize a group of phosphoproteins (Westendorf et al., 1994) or by autoradiography if proteins on these filters are subjected to phosphorylation by the purified kinase in the presence of [γ-^{32}ATP] (Fukunaga and Hunter, 1997). The frequent improper folding of cDNA-encoded proteins presents one potential problem and the utility of this approach for identifying genuine substrates of a given kinase will greatly depend on the in vitro specificity of the kinase. Kinases that utilize noncatalytic domains to interact with their substrates are likely to give a higher degree of success. Some protein kinases form stable complexes with their respective substrates in vivo and this fact was exploited for the identification of kinase

substrates by screening phage expression libraries by a binding assay (Zhao et al., 1998).

Oriented peptide library screens have been useful for determining the preferred phosphorylation motif of a given kinase. This approach greatly simplifies the prediction of phosphorylation sites on an independently identified substrate, allows the identification of candidate substrates through bioinformatics and can be effectively used in combination with other substrate identification methods such as immunoblotting or immunoprecipitating with phosphomotif antibodies. In this method, a degenerate library of peptides oriented around an invariant serine, threonine or tyrosine residue is subjected to an in vitro kinase assay and the resulting phosphopeptides which constitutes a small fraction of the peptides within the mixture are isolated on an IMAC (immobilized metal affinity chromatography) column. The mixture of phosphopeptides is quantitatively sequenced by Edman degradation to determine the peptide-substrate specificity of the kinase (Songyang et al., 1994). Recent refinements to this technique have made it better suited to high-throughput screens. In the oriented peptide array library approach, the pools of oriented peptide libraries are arranged as scan arrays, in which each spot corresponds to a fixed amino acid at a particular position flanking the tyrosine, serine or threonine (Hutti et al., 2004).

Knowledge of the minimal requirements for a kinase to phosphorylate a given protein sequence, determined by the previously identified phosphorylation

sites or peptide library methods, can be used to raise phosphomotif antibodies (Zhang et al., 2002). These antibodies are raised against a library of degenerate phosphopeptides with residues required by the specific kinase and a phosphoserine, phosphothreonine, or phosphotyrosine locked in at the appropriate positions. Phosphomotif antibodies can then be used to perform immunoblots on two-dimensional gels or to immunoprecipitate phosphorylated proteins in order to identify candidate substrates by mass spectroscopy methods. Phosphomotif antibodies generated against peptides will often not be able to bind and immunoprecipitate proteins in their native folded state. Therefore, efficient immunoprecipitation with these antibodies may require prior denaturation of proteins within the cell lysates. These approaches require an effective and highly specific means of activating and/or inhibiting the kinase whose substrates are being analyzed. The specific inhibition of a kinase can be achieved by antibody injection, silencing with small-interfering RNAs, or by pharmacological inhibition. Since inhibitors that can strictly distinguish between specific kinases are rare, generating functionally silent active-site mutations to sensitize a target kinase to inhibition by a small molecule can be used as an alternative (Bishop et al., 2001).

The most promising method for identifying kinase substrates has been the recently developed chemical-genetic method which aims to generate mutant alleles of a kinase that can utilize specific ATP analogs, in addition to ATP

(Ubersax et al., 2003). The mutation is introduced into the structurally conserved ATP-binding pocket in a region where the N^6 amine of ATP usually sits and this mutation allows the use of an ATP analog with a large hydrophobic moiety positioned on the N^6 amine. This approach eliminates all background problems when searching for substrates by in vitro kinase assays on fractions from cell lysates and in principle can be applicable to the in vivo labeling of substrates when the mutant kinase is expressed.

In the current study, we used an in vitro expression cloning system which can be an alternative to all of the previously mentioned methods for identifying the candidate substrates of a given kinase (Stukenberg et al., 1997). In this approach, pools of clones from a human adult brain cDNA library or alternatively from a normalized *Xenopus* cDNA library are first transcribed and translated in vitro to generate pools of the radiolabeled proteins. These protein pools are then incubated with a recombinant kinase and proteins that become phosphorylated after incubation with the kinase are identified by observing for a decreased mobility upon gel electrophoresis. We aimed to find substrates for Aurora B kinase because of its involvement in the error-correcting machinery that destabilizes incorrect microtubule-kinetochore attachments. In the absence of Ipl1 (the budding yeast homolog of Aurora B), synthelic attachments in which both kinetochores on a pair of sister chromatids bind to microtubules emanating from a single spindle pole are not corrected and persist (Petronczki et al., 2003). The

best-understood connection of Aurora B in this process comes from the studies in the budding yeast (Tanaka et al., 2002). Two properties of the yeast kinetochores and spindle pole bodies provided an opportunity to investigate the role of Ipl1. In the budding yeast, whose kinetochores possess a single microtubule binding site, centromeres are connected to spindle pole bodies even during G1 and buds inherit the old SPB. In cells deficient to replicate their chromosomes but nevertheless duplicate their spindle pole bodies, in the absence of Ipl1 function 85% of the time sister chromatids are segregated to the old spindle pole. Because the biased segregation of chromosomes to the bud in *ipl1* mutants was eliminated by the transient disruption of microtubules, it was proposed that Ipl1 promotes the turnover of mono-oriented (synthelic) kinetochore-microtubule interactions. The kinetochore in the budding yeast is organized into distinct subcomplexes that have been placed into inner, central, and outer domains based on two criteria (Biggins and Walczak, 2003): (1) in vitro centromere or microtubule binding activities and (2) in vivo dependency relationships for centromere association. The inner kinetochore CBF3 complex binds to the centromeric DNA and is required for the centromere association of all other kinetochore proteins. The central kinetochore includes the conserved Ndc80 complex implicated in the stabilization of kinetochore-microtubule attachments and the large Ctf19 complex, which can be subdivided into at least three subcomplexes. The outer kinetochore contains the Dam1 complex, which is brought to the kinetochores during microtubule capture.

Through a series of experiments, in vivo and in vitro, Ipl1 phosphorylation sites were found on three components of the Dam1 complex (Cheeseman et al., 2002). Eliminating the mapped phosphorylation sites in Spc34, Ask1 didn't result in detectable defects, while the phosphorylation site mutants of Dam1 phenocopied the inactivation of Ipl1 in terms of the chromosome missegregation. In addition, cells with alterations designed to mimic the constitutive phosphorylation of Dam1 showed evidence of lagging chromosomes and were able to partially suppress the defects of Ipl1 mutants. Since lagging chromosomes are often indicative of weak kinetochore-microtubule attachments, these findings lend support to the possibility that Ipl1 eliminates synthelic attachments by weakening the kinetochore-microtubule attachments through the phosphorylation of Dam1. In support of this view, Dam1-Ndc80 interaction was found to be weakened by mutations mimicking phosphorylation at Ipl1 sites (Shang et al., 2003). The relevant substrates for Aurora B in higher organisms remain to be identified although the correction mechanisms can be quite different. Since in vertebrates there are multiple microtubule binding sites on one kinetochore, it is possible that kinetochores never get completely detached to correct synthelic attachments; but instead one of the kinetochores may acquire microtubules from the opposite pole, becoming merotelic, and such a transient merotelic attachment would allow the kinetochores face toward opposite poles and eventually facilitate biorientation through the attachment of additional microtubules from the opposite pole. In

mammalian cells, when Aurora B has been inactivated by treatment with a chemical inhibitor and then reactivated by washing away the inhibitor, chromosomes keeping both kinetochores attached to the microtubules first move to the pole and later gain bipolar attachments in a process that appears to involve the disassembly of one kinetochore fiber (Lampson et al., 2004). In vertebrates, the localization and the activity of the motor protein MCAK (mitotic centromere-associated kinesin), which acts as a microtubule depolymerase, was found to be regulated through its phosphorylation by Aurora B and persistent disruption of the regulatory mechanism acting on MCAK, either by the expression of phosphomimicking or nonphosphorylatable mutants of MCAK, increased the frequency of synthelic attachments (Andrews et al., 2004; Kline-Smith et al., 2004; Lan et al., 2004; Ohi et al., 2004). Therefore, Aurora B may facilitate the biorientation of chromosomes by directing MCAK to depolymerize the incorrectly attached microtubules. In this study, we failed to identify candidate substrates which may be relevant to the involvement of Aurora B in the error-correcting machinery that destabilizes incorrect microtubule-kinetochore attachments. Using this approach, we have isolated cDNAs encoding 19 proteins that are phosphorylated by recombinant Aurora B in vitro. Nine of these cDNAs were recovered from the *Xenopus* cDNA library while the rest were recovered from the human adult brain cDNA library. We mapped the in vitro phosphorylation sites on two candidate substrates, human beta 5-tubulin and

human MCM7, using site-directed mutagenesis. We found that the consensus motif (K/R)X(S/T)(I/L/V) deduced from the phosphorylation sites previously identified in the budding yeast (Cheeseman et al., 2002) can be useful in predicting the phosphorylation sites on an independently identified substrate.

Experimental Procedures

Plasmids, cloning and site-directed mutagenesis

The full-length coding sequence for human Aurora B was PCR amplified with the following primers (forward, 5'ACGCGGATCCGATGGCCCAGAAGGAGAAC TC3'; reverse, 5'CCGGAATTCTCAGGCGACAGATTGAAGGGC3') using the pCS2-N-myc-Aurora B as a template and cloned into the Bam HI/Eco RI sites of pHis-Parallel1. The kinase-dead version of Aurora B was subsequently generated by using QuickChange site-directed mutagenesis kit (Stratagene) to mutate ^{106}K to ^{106}R with the following primers (sense, 5'TTTCATCGTGGCGCTCAGGGTCC TCTTCAAGTCCC3'; antisense, 5'GGGACTTGAAGAGGACCCTGAGCGCC ACGATGAAA3'). The full-length coding sequence for human beta 5-tubulin (NCBI accession number: BC020946) was PCR amplified with the following primers (forward, 5'TGCTGGCCGGCCCATGAGGGAAATCGTGCACATCC3' ; reverse, 5'ATTGGCGCGCCTTAGGCCTCCTCTTCGGCCTC3') using the human adult brain cDNA library clone Plate B-B8(G4) as a template and cloned into the Fse I/Asc I sites of the FA modified pCS2 expression vector. The full-length coding sequence for human MCM7 transcript variant 1 (NCBI accession number: NM 005916) and transcript variant 2 (NCBI accession number: NM182776) were PCR amplified from a human fetal thymus cDNA library (Clontech) using the following primers respectively (transcript variant 1 forward,

5'TGCTGGCCGGCCGATGGCACTGAAGGACTACGCG3'; transcript variant 2 forward, 5'TGCTGGCCGGCCGATGGTGGTGGCCACTTACACT3'; reverse, 5'ATTGGCGCGCCTCAGACAAAAGTGATCCGTGTCC3') and cloned into the Fse I/Asc I sites of the FA modified pCS2 expression vector. The putative Aurora B phosphorylation sites on human beta 5-tubulin ($RI^{48}SV$), MCM7 transcript variant 1 ($RD^{422}SV$, $RC^{483}SI$, $RR^{500}SL$, $KD^{643}SL$) and MCM7 transcript variant 2 ($RD^{246}SV$, $RC^{307}SI$, $RR^{324}SL$, $KD^{467}SL$) were individually mutated to alanine using QuickChange site-directed mutagenesis kit (Stratagene).

Protein expression and purification

pHis-Parallel1 bacterial expression plasmid harboring the coding sequence for human Aurora B or the kinase dead mutant of Aurora B (K106R) were transformed into Rosetta(DE3) cells and the transformants were selected on LB (amp^+chl^+) plates. Individual clones were inoculated into 20 ml of LB (amp^+chl^+) medium and were grown overnight at 37°C with vigorous shaking. Overnight grown cultures were diluted into 2 lt of LB (amp^+chl^+) medium and were grown at 37°C with vigorous shaking till $O.D_{600}$ reaches around 0.6. The cultures were cooled down to room temperature and IPTG (Roche) was added to a final concentration of 0.5 mM. Cells were grown at room temperature for an additional 6 hours with vigorous shaking.

Cells were harvested by centrifugation at 2000 rpm for 20 min at 4°C using a Beckman J6 rotor and each culture was resuspended in 20 ml of lysis

buffer (50 mM Tris-Cl, pH 7.5, 250 mM NaCl, 5mM $MgCl_2$, 10% glycerol and 10 μg/ml each of chymostatin, leupeptin, and pepstain). Lysozyme was added to a final concentration of 1mg/ml before incubating the cell suspensions on ice for 30 min. After the incubation, the lysates were sonicated on ice for 6 minutes with 30 sec pauses using a medium-tip sonicator at amplitude set to half. 20% Triton-X in 50mM Tris-Cl, pH 7.5, 250 mM NaCl, 5 mM $MgCl_2$ and 10% glycerol was added to a final concentration of 0.1% and the lysates were gently mixed for 10 min to aid the solubilization of proteins. Cell lysates were spinned down at 35000 rpm for 1 hour at 4^oC. After centrifugation, protean sulfate (30 mg/ml) was added to the supernatants at a final concentration of 1 mg/ml and the mixture was gently incubated for 5 min at 4^oC. The supernatants were centrifuged at 20000 rpm for 20 min at 4^oC in order to precipitate the protean sulfate bound to DNA. Each of the cleared supernatants was loaded onto 2ml of Ni-NTA (Qiagen) beads equilibrated with PBS. After washing with 10 column volumes of lysis buffer with 40 mM imidazole, the bound proteins were eluted with 5 column volumes of elution buffer (20 mM Tris-Cl, pH 7.7, 150 mM NaCl, 250 mM imidazole) and were dialyzed against a buffer containing 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, pH 8.0, 1 mM DTT, 1 mM PMSF over a period of 3 hours with two changes.

In vitro kinase assay with recombinant His-Aurora B

5µl of the wild-type (WT) or the catalytically inactive (KR) recombinant His-Aurora B was incubated with 0.5 mg/ml H3 in kinase reaction buffer (50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM ATP, 1 mM DTT) supplemented with 1µl of [γ-^{32}P]ATP (2µCi/µl) for 30 min at 30^{o}C. Reactions were alternatively supplemented with 5 µl of the purified recombinant IN-box segment of INCENP$^{799\text{-}919}$ sufficient enough to saturate recombinant Aurora B in solution. Kinase reactions were terminated by adding 6xSDS-sample buffer. Samples were resolved on a 12% SDS-polyacrylamide gel. Gel was fixed in a fixing solution (50% methanol, 40% H_2O, 10% glacial acetic acid) for 30 min, equilibrated in drying buffer (7% methanol, 7% glacial acetic acid, 1% glycerol) for 5 min, dried using a conventional gel drier and analyzed by phosphorimaging.

In vitro expression cloning screen

The human adult brain cDNA library (Promega) which is subdivided into 96 well plates containing 50-100 clones per well, was previously in vitro transcribed and translated in the presence of [^{35}S]-methionine (Amersham) using Gold TnT SP6 Express System (Promega) in our lab to generate functional proteins according to the manufacturer's protocol. 2 µl of each *Xenopus* cDNA library pool was in vitro transcribed and translated in the presence of 1µl of [^{35}S]-methionine (Amersham) using 12 µl of TnT SP6 Quick Coupled Transcription/Translation System. The [^{35}S]-methionine-labeled protein pools (2.5 µl for each) were incubated with 15µl

of either the wild-type (WT) or the catalytically inactive (KR) recombinant His-Aurora B and 2.5µl of 8x kinase reaction buffer (400 mM Tris-Cl, pH 7.5, 80 mM $MgCl_2$, 2 mM ATP, 8 mM DTT, 40 mM NaF, 40 mM β-glycerophosphate, 0.4 mM Sodium vanadate) in 96 well plates at 30^oC for 90 minutes while shaking at 200 rpm in an incubator. The reactions were stopped by adding 6xSDS-sample buffer. Samples were separated on 20-cm 12% SDS-polyacrylamide gels in order to increase the possibility of resolving the phosphorylated and non-phosphorylated forms. Gels were fixed in a fixing solution (50% methanol, 40% H_2O, 10% glacial acetic acid) for 30 min, equilibrated in drying buffer (7% methanol, 7% glacial acetic acid, 1% glycerol) for 5 min, dried using a conventional gel drier and analyzed by phosphorimaging.

Secondary screen, isolating positive cDNAs

1µl of each cDNA pool on the master plate, which were identified to harbor the positive translation products during the initial screen, was diluted 100 times and 1 µl of each diluted cDNA pool was transformed into DH5α cells and the cells were plated on LB ampicillin plates at the appropriate dilutions to achieve 100-200 colonies per plate. 96 colonies were picked up for each cDNA pool and each clone was transferred into an individual well of a 96 well growth block containing 2 ml of LB (amp^+) per well. Cultures were grown overnight at 37^oC with appropriate agitation. 800 µl of each culture was used to pool the clones grown in the 96 well growth block into 12 columns (numbered as 1 to12) and 8 rows

(labeled as A to H). Pooled cultures were collected by centrifugation at 4000 rpm for 5 minutes and the plasmid DNAs in each pool were purified using Qiagen's miniprep kit according to the manufacturer's protocol. 96 well growth blocks were stored at 4^{o}C until the secondary screen was finally over. 1 µl of each pool of purified cDNAs (0.25 µg/µl) was in vitro transcribed and translated in the presence of 1µl of [^{35}S]-methionine (Amersham) using 10 µl of TnT SP6 Quick Coupled Transcription/Translation System. The [^{35}S]-methionine-labeled protein pools (2.5 µl) were incubated with 15µl of either the wild-type (WT) or the catalytically inactive (KR) recombinant His-Aurora B and 2.5µl of 8x kinase reaction buffer (400 mM Tris-Cl, pH 7.5, 80 mM $MgCl_2$, 2 mM ATP, 8 mM DTT, 40 mM NaF, 40 mM β-glycerophosphate, 0.4 mM Sodium vanadate) in 96 well plates at 30^{o}C for 90 minutes while shaking at 200 rpm in an incubator. The reactions were stopped by adding 6xSDS-sample buffer. Samples were separated on 20-cm 12% SDS-polyacrylamide gels in order to increase the possibility of resolving the phosphorylated and nonphosphorylated forms. Gels were fixed in a fixing solution (50% methanol, 40% H_2O, 10% glacial acetic acid) for 30 min, equilibrated in drying buffer (7% methanol, 7% glacial acetic acid, 1% glycerol) for 5 min, dried using a conventional gel drier and analyzed by phosphorimaging. If the same electrophoretic mobility shift pattern of a single translation product was observed within both a row and a column, this translation product was identified as positive and, the well containing that positive clone on the 96 well

growth block was identified as the well corresponding to the intersection of that row and column. After the secondary screen, individual positive clones on the 96 well growth block were inoculated into 5 ml of LB (amp^+) and were grown at 37^{o}C overnight with vigorous shaking. Plasmids harboring positive cDNA clones were recovered by Qiagen's miniprep kit and each of the clones was tested in kinase reactions as described above. The cDNA clones identified as positive after the final kinase reactions were sequenced.

Mapping in vitro phosphorylation sites on candidate substrates

1 µl of each pCS2 plasmid (0.25 µg/µl) encoding for the wild type and the mutant form of human beta 5-tubulin and that of human MCM7 transcript variants were in vitro transcribed and translated in the presence of 1µl of [^{35}S]-methionine (Amersham) using 10 µl of TnT SP6 Quick Coupled Transcription/Translation System. The [^{35}S]-methionine-labeled proteins (2.5 µl) were incubated with 15µl of either the wild-type (WT) or the catalytically inactive (KR) recombinant His-Aurora B and 2.5µl of 8x kinase reaction buffer (400 mM Tris-Cl, pH 7.5, 80 mM $MgCl_2$, 2 mM ATP, 8 mM DTT, 40 mM NaF, 40 mM β-glycerophosphate, 0.4 mM Sodium vanadate) in 96 well plates at 30^{o}C for 90 minutes while shaking at 200 rpm in an incubator. The reactions were stopped by adding 6xSDS-sample buffer. Samples were separated on a 20-cm 12% SDS-polyacrylamide gel in order to increase the possibility of resolving the phosphorylated and nonphosphorylated forms. Gel was fixed in a fixing solution (50% methanol, 40% H_2O, 10% glacial

acetic acid) for 30 min, equilibrated in drying buffer (7% methanol, 7% glacial acetic acid, 1% glycerol) for 5 min, dried using a conventional gel drier and analyzed by phosphorimaging.

Results

Testing the kinase activity of recombinant Aurora B

Aurora B is a serine/threonine kinase that associates in vivo with the proteins INCENP and Survivin. Together, these proteins first localize along the length of chromosomes during prophase and during prometaphase, become concentrated at the inner centromere region where they have been implicated in the error-correction mechanism facilitating the biorientation of sister chromatids tethered by cohesin (Carmena and Earnshaw, 2003). The kinase activity of Aurora B is stimulated by INCENP and the C-terminal region of INCENP is sufficient for the activation of Aurora B. Phosphorylation of the carboxyl terminus of INCENP by Aurora B stimulates the kinase activity of Aurora B and a nonphosphorylatable mutant of INCENP is a poor activator of Aurora B, demonstrating that the phosphorylation of INCENP is important for the activation of Aurora B (Honda et al., 2003). Our own experience also showed that when Aurora B was purified from Sf9 cells, INCENP stimulated the kinase activity of Aurora B while Survivin was incapable of activating the kinase activity of Aurora B against histone H3 (data not shown). Histone H3 was used as a substrate for testing the kinase activity of Aurora B because histone H3 was earlier shown to be phosphorylated by Aurora B at two different positions, Ser10 and Ser28, both in vivo and in vitro (Goto et al., 1999; Sugiyama et al., 2002). Since we needed a large amount of

recombinant Aurora B for our screen, we used a bacterial expression system to express the wild-type and the catalytically inactive His-tagged-Aurora B. The recombinant proteins were purified using Ni-NTA chromatography and dialyzed against a Tris containing buffer to remove the imidazole. The catalytically inactive mutant was poorly expressed but nevertheless was sufficient to serve as control in our kinase assays (Fig. 1A). Since the removal of imidazole from the solution caused His-tagged-Aurora B to form insoluble aggregates, we wanted to know whether this preparation of recombinant Aurora B is still catalytically active. In order to test the catalytic activity of our prep, we examined whether or not Aurora B phosphorylates the full-length histone H3 in vitro. Figure 1B shows that histone H3 is phosphorylated by recombinant Aurora B. We also tested whether the carboxyl-terminal domain of INCENP$^{799\text{-}919}$ would be an efficient substrate for recombinant Aurora B or whether it would stimulate the kinase activity of Aurora B; however, in this system the carboxyl-terminal domain of INCENP$^{799\text{-}919}$ failed to be a substrate for Aurora B and to stimulate its kinase activity (Fig. 1B). Essentially, this indicated that recombinant Aurora B failed to bind to the carboxyl-terminal domain of INCENP (IN-box segment), which is normally responsible for binding to Aurora B (Bishop and Schumacher, 2002). Actually, using in vitro pull down assays, we extended this observation and showed that recombinant IN-box segment can bind to radiolabeled Aurora B and A with equal efficiency (data not shown). Since the phosphorylation of INCENP

on this segment requires a different consensus motif than normally observed in Aurora B substrates (Honda et al., 2003), it is possible that a misfolded form of Aurora B would fail to phosphorylate the carboxyl-terminal of INCENP.

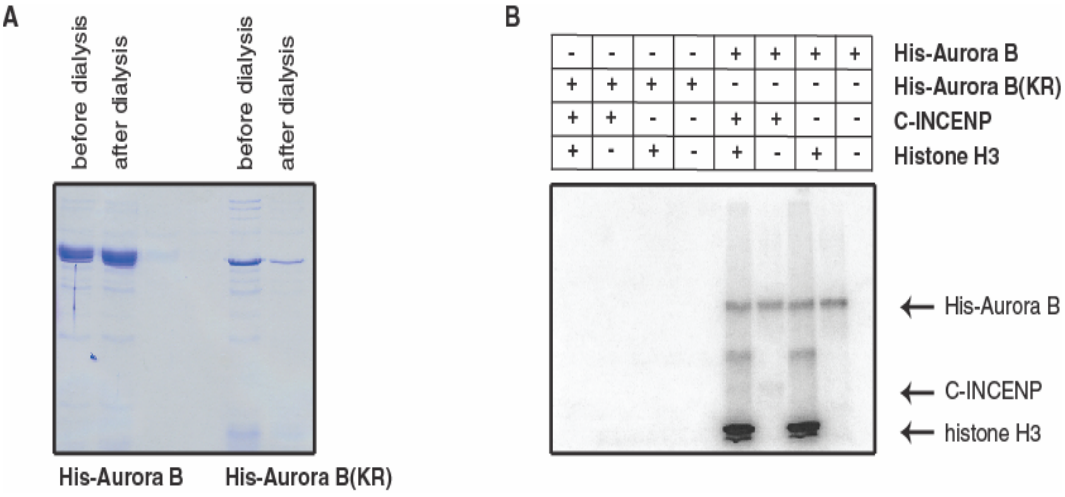

Figure 1 Recombinant Aurora B purified from bacteria phosphorylates H3. (**A**) 15 µl of recombinant Aurora B (the wild type/His-Aurora B or the catalytically inactive/His-Aurora B(KR)) preparations before and after dialysis were resolved on 12% SDS-polyacrylamide gel and analyzed by Commasie blue staining. (**B**) 5 µl of recombinant Aurora B (the wild-type or the catalytically inactive) was assayed for histone H3 kinase activity in the presence or absence of equal amount of the carboxyl terminal domain of INCENP (799-919) using [γ-^{32}P]ATP.

In vitro expression cloning screen for Aurora B substrates

Even though our recombinant Aurora B was insoluble and incapable of phosphorylating INCENP, efficient phosphorylation of histone H3 encouraged us to screen for in vitro Aurora B substrates using this preparation. In order to identify the candidate substrates of Aurora B, we used an in vitro expression

cloning system. In this system, small pools of the human adult brain cDNA library were transcribed and translated in vitro in the presence of [^{35}S]-methionine. The [^{35}S]-methionine-labeled protein pools were incubated with either His-tagged Aurora B or the catalytically inactive His-tagged Aurora B(KR) and analyzed by SDS-PAGE followed by autoradiography. We initially screened a single plate (plate E) of the human adult brain cDNA library where each plate contains 96 small pools of 50-100 cDNA clones. Almost 20% of the 96 small cDNA pools tested exhibited mobility shift changes in His-tagged Aurora B treated protein pools but not in His-tagged Aurora B(KR) treated protein pools (Fig. 2).

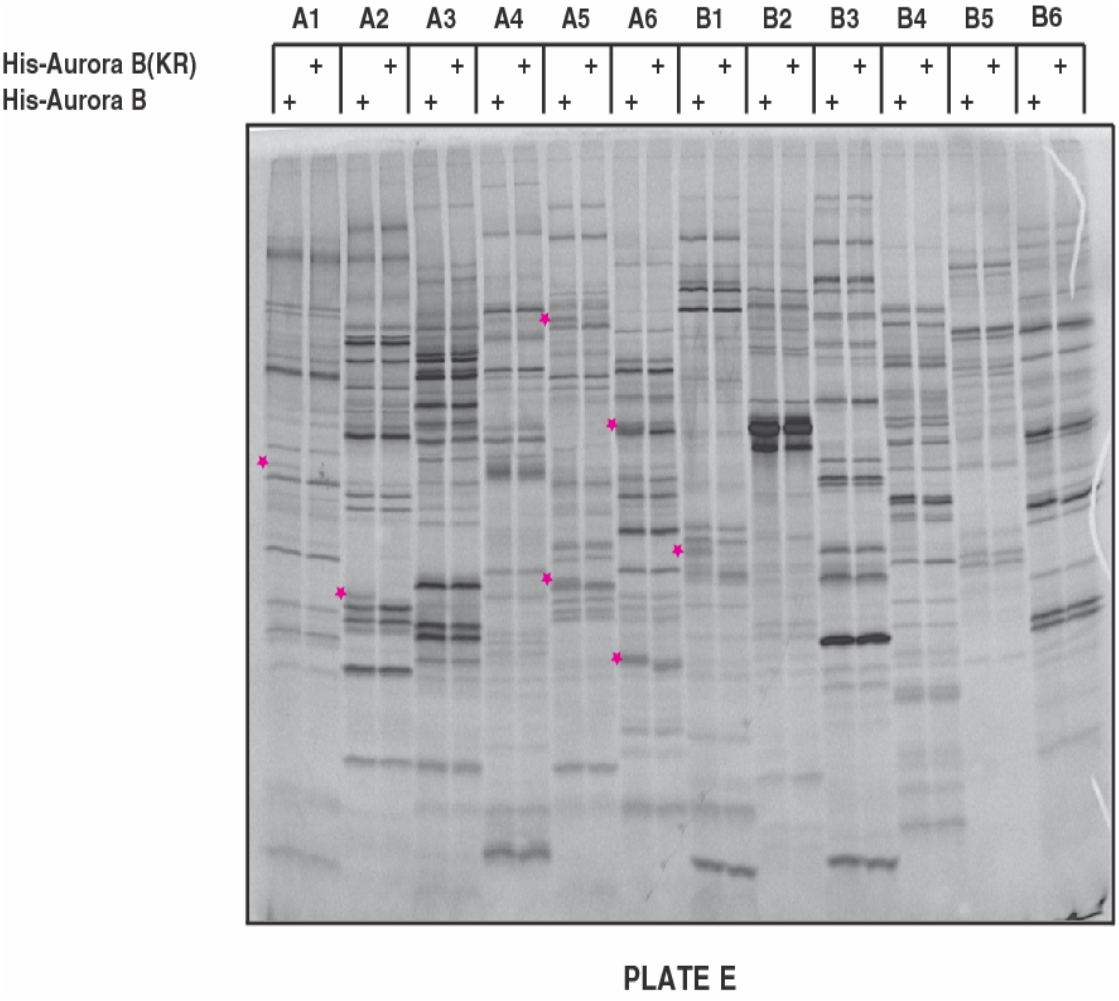

Figure 2 A sample of bands identified as positive during the primary screen on Plate E. The [^{35}S]-methionine-labeled protein pools were incubated with the wild-type or the catalytically inactive (KR) recombinant His-tagged-Aurora B, respectively. Potential substrates present are illustrated by the electrophoretic mobility shift (as shown by purple stars) in His -Aurora B but not in His-Aurora B(KR) treated lanes on SDS-PAGE.

We randomly selected seven of the small cDNA pools on plate E that showed decreased mobility and checked whether the same bands in these pools would be phosphorylated by other mitotic kinases such as Plk1 and Cdc2/Cyclin B. In none of the pools tested, Plk1 or Cdc2/Cyclin B stimulated the mobility shifts observed with Aurora B and the mobility of the bands was similar in Plk1 and Cdc2/Cyclin B treated pools. Although there is an observed specificity of phosphorylation with Aurora B in these pools (Fig. 3), we can not conclusively say that this is true since we lack the evidence for the activity of Plk1 and Cdc2/Cyclin B in these pools. The primary reason for the lack of activity could be due to the amount of Plk1 and Cdc2/Cyclin B used since these proteins were produced in Sf9 cells and were available in low quantities. It would be essential to show that at least some of the bands in these pools showed decreased mobility when treated with Plk1 and Cdc2/Cyclin B or these kinases show activity towards a given substrate.

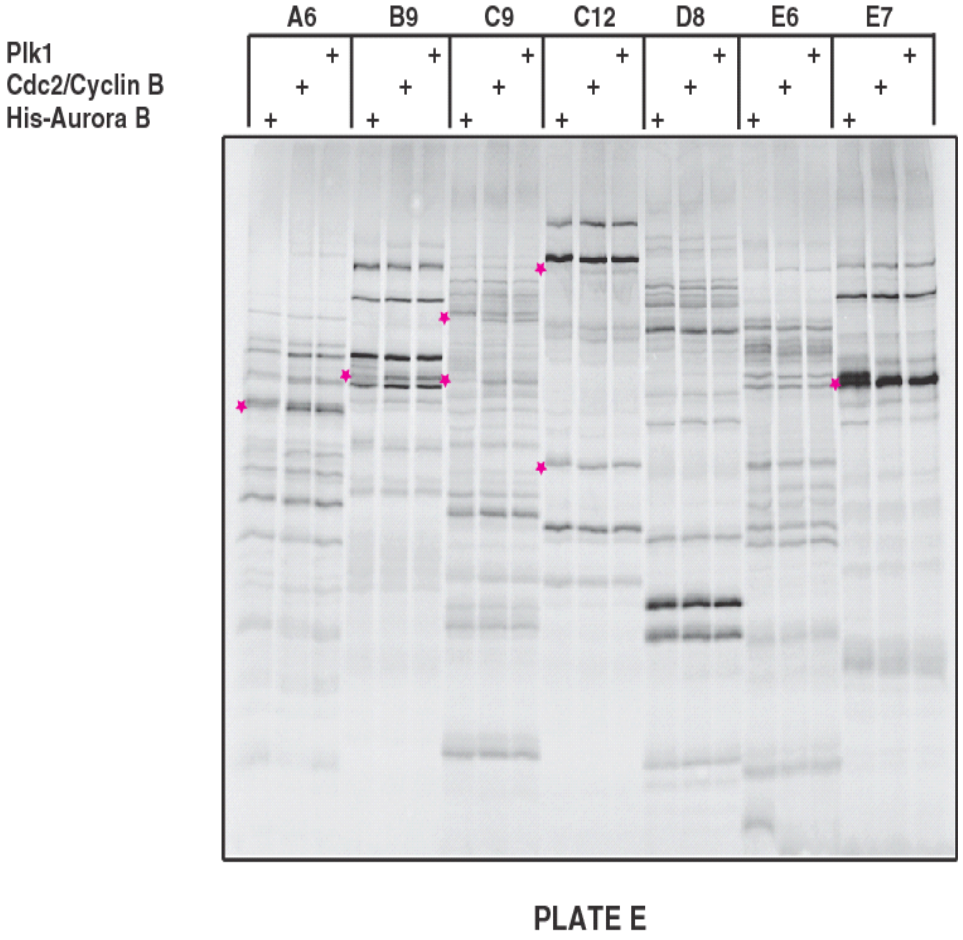

Figure 3 Candidate bands are not phosphorylated by recombinant Plk1 or Cdc2/Cyclin B. Seven of the cDNA pools that showed bands with decreased mobility after treatment with recombinant Aurora B (as shown by purple stars) were alternatively treated with 3µl of Sf9 produced Plk1 or Cdc2/Cyclin B.

We screened three more plates (plate B, C and D) of the human adult brain cDNA library for mobility shifts upon incubation with His-tagged-Aurora B. After the primary screen, around 50 pools were found to have translation products that

showed decreased mobility when treated with recombinant Aurora B. We isolated the plasmids encoding for the cDNAs from 9 of those pools identified as positive (Plate B-A6, B-B6, B-B8, B-C5, B-D6, E-A6, E-B9, E-C9 and E-E7) and carried out the secondary screen as described in "Experimental Procedures". As shown in Figure 4, when the secondary screen was carried out for a given cDNA pool (Plate B-A6), the same bands were found to be phosphorylated in multiple rows and columns. In most of the cases, these translation products were found to be different than the ones suspected to be phosphorylated in the primary screen when the size of the bands is used as a criterion. In this certain case, two translation products were found to be phosphorylated. One of the translation products was identified in two rows (A and D) and in two columns (5 and 10) while the other translation product was identified in two rows (E and H) and in three columns (1, 7 and 9), which means that cDNAs encoding for the translation products can originate from any one of the wells corresponding to the intersection of positive rows and columns (A5, A10, D5, D10 or E1, E7, E9, G1, G7, G9). In order to eliminate the ambiguity, we fixed one of the rows and followed columns (D5, D10 and E1, E7) for purifying the plasmids encoding for each translation product. After the secondary screen, isolation of the plasmids and the final screen was carried over 7 pools (Plate B-A6, B-B8, B-C5, B-D6, E-A6, E-B9 and E-E7) out of 9. In some cases, some of the bands found to be phosphorylated in the secondary screen were eliminated in order to reduce the number of candidate

clones and were not subjected to further characterization. A similar ambiguity about the origin of plasmids was also associated with four other pools (Plate B-C5, E-A6, E-B9 and E-E7).

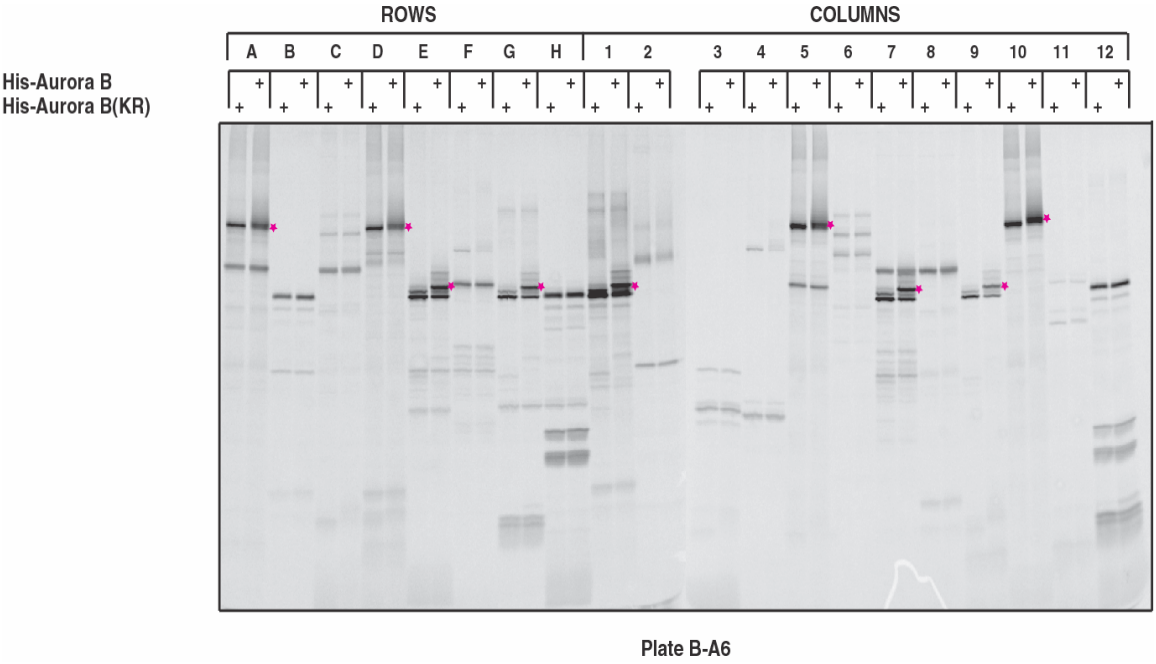

Figure 4 Secondary screening of the plasmids derived from cDNA pool A6 on plate B. After culturing individual clones on a 96 well growth block, cultures on a single row or column were pooled before carrying out the plasmid isolation. Plasmids derived from each row or column were used to produce [^{35}S]-methionine-labeled protein pools. Radiolabeled protein pools were incubated with the wild-type or the catalytically inactive (KR) recombinant His-tagged-Aurora B, respectively. Potential substrates present are illustrated by the electrophoretic mobility shift (as shown by purple stars) in His -Aurora B but not in His-Aurora B(KR) treated lanes on SDS-PAGE.

After the isolation of plasmids, each clone was in vitro transcribed and translated in the presence of [^{35}S]-methionine, and the radiolabeled products were incubated with the wild type and the catalytically inactive recombinant Aurora B and the reactions were resolved on SDS-PAGE and analyzed by autoradiography. After this final screen, twelve plasmids were sent to sequencing and they were found to encode for transcription factor 4 (Plate B-A6(D10)), ubiquitin-like protein SB132 (Plate B-A6(E1)), phospholipase C-like 2 (Plate B-B8(D7)), beta 5-tubulin (Plate B-B8(G4)), HDAC2 (Plate B-C5(A3)), adenocarcinoma antigen (ART1/P17) (Plate B-C5(A6)), beta 5-tubulin (Plate B-D6(C7)), beta 5-tubulin (Plate E-A6(E9)), NADH dehydrogenase β subcomplex 9 (Plate E-A6(G11)), amyloid beta (A4) precursor protein (Plate E-B9(A11)), beta 4-tubulin (Plate E-B9(D11)), clusterin (Plate E-E7(D3)) (Fig. 5).

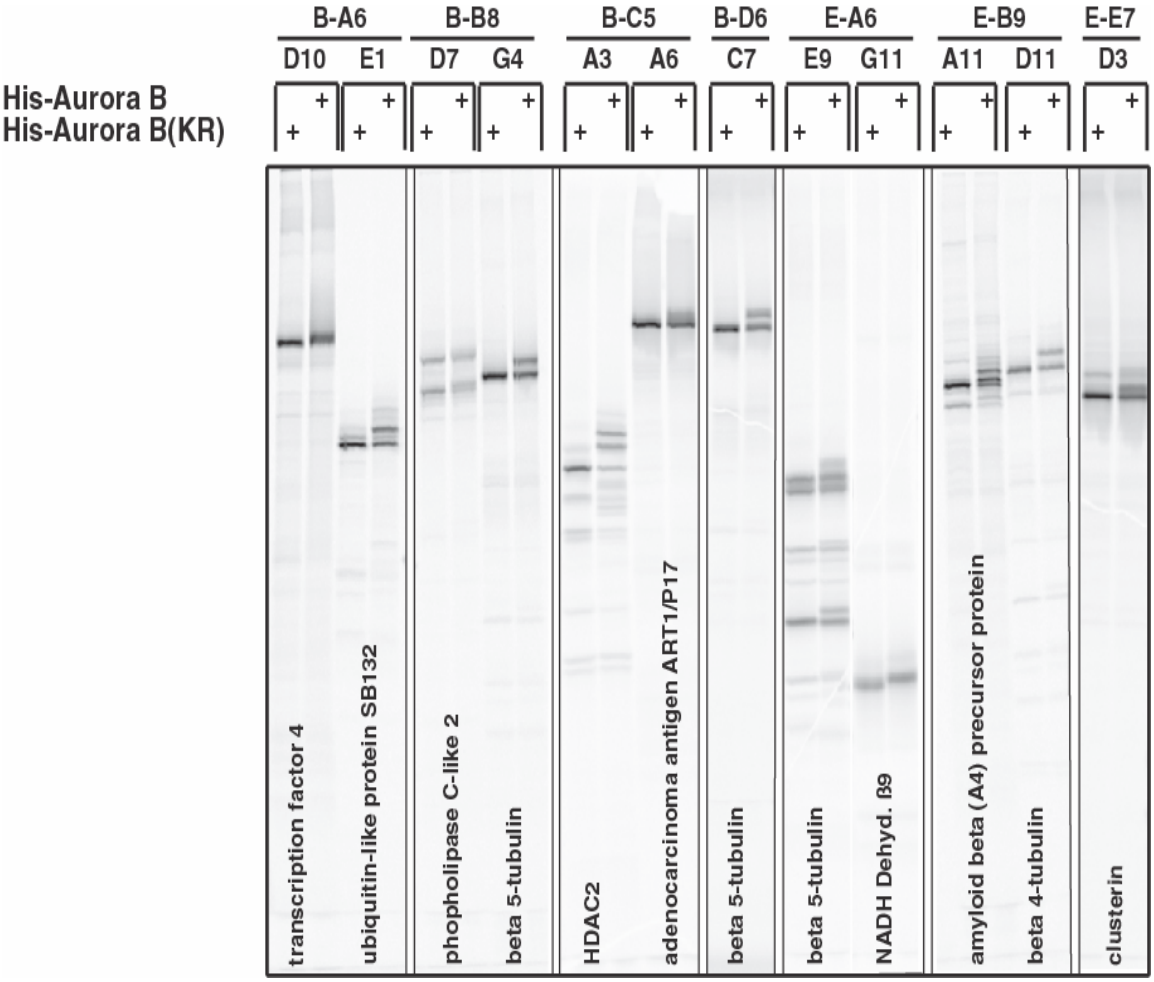

Figure 5 Positive translation products identified in human adult brain cDNA library. Twelve translation products were found to be phosphorylated in the human adult brain cDNA library by recombinant Aurora B during the final screen. The identity of each cDNA coding for an individual translation product was verified by DNA sequencing.

In addition to the human adult brain cDNA library, we screened a normalized *Xenopus* cDNA library for identifying Aurora B kinase substrates. There were two meaningful reasons to use this library for further screening. We

suspected that the candidate substrates we identified were recovered because of their likely abundance in human adult brain tissue and the use of a normalized library would potentially increase the diversity of the substrates identified. More importantly, because human adult brain tissue is composed of quiescent cells which are incapable of undergoing cell division, proteins involved in cell cycle regulation were unlikely to be expressed in brain tissue. In most cases, the abundance of such proteins fluctuate during somatic cell cycle and are subject to tight control through transcriptional and posttranslational mechanisms. Therefore, it would be crucial to use a library derived from a tissue or an organism where cells are dividing and yet the transcriptional control over cell cycle is relieved. In *Xenopus*, after fertilization, cell division proceeds without growth at a rapid rate in a nearly synchronous manner for a total of 11 to 12 cleavages. During the period of rapid cleavage, there is no observable RNA transcription. After 12 cleavages, the embryo undergoes a concerted developmental transition affecting all cells, called the midblastula transition (MBT) at which time transcription is initiated; the cell cycle slows down, G1 and G2 phases appear, cell division becomes asynchronous and cells display motile activity (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). The normalized *Xenopus* cDNA library we used in this study was derived from pre-MBT staged eggs and therefore was expected to be more suitable for identifying the downstream targets of Aurora B

whose RNA level and protein level peak during G2 and return to low levels as the cells re-enter G1 during a normal somatic cell cycle (Terada et al., 1998).

We transcribed and translated in vitro 2 plates of the normalized *Xenopus* cDNA library in the presence of [^{35}S]-methionine and performed the primary screen as described for the human adult brain cDNA library. After the primary screen, we found that the *Xenopus* library is poorly transcribed or translated and only one fourth of the total cDNA pool was found to give radiolabeled translation products; actually, none of the cDNA pools on Plate 2 were translated. Nevertheless, we identified several bands that showed decreased mobility when the protein pools are incubated with His-tagged recombinant Aurora B. We recovered the plasmids from 6 cDNA pools (Plate 1-C2, 1-C6, 1-D2, 1-D3, 1-F2, 1-F9) and performed secondary screen in the usual manner. After the final screen, out of the 6 cDNA pools, we found eleven clones to code for candidate substrates and the identities of these potential substrates were analyzed by DNA sequencing. Two of these cDNA clones were found to code for E.Coli related proteins (Plate 1-C2(B1), Plate 1-D3(E11)). The others were found to code for MGC83814 (Plate 1-C6(A8)), MGC89203-similar to glutamate dehydrogenase (Plate 1-C6(C6)), egg-specific protein (p100) (Plate 1-C6(C8)), LOC446970- similar to axotrophin (Plate 1-D2(B3)), lectin (Plate 1-D2(F5)), homologous to pre-mRNA processing factor 31 (Plate 1-D3(C1)), MCM7 (Plate 1-D3(H12)), homologous to ring finger

protein 38 (Plate 1-F2(B4)), homologous to NADH dehydrogenase Fe-S protein 2-NDUFS2 (Plate 1-F9(A3)) (Fig. 6).

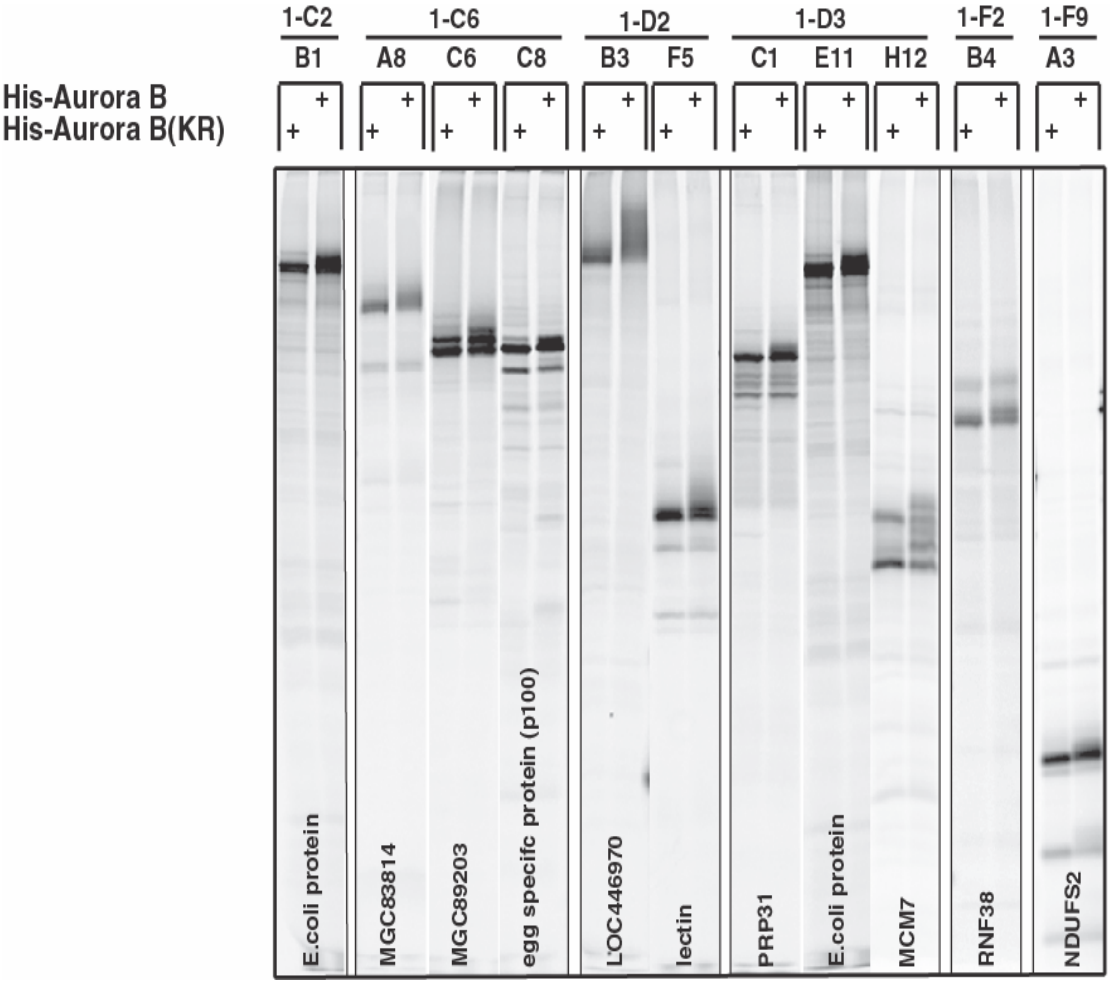

Figure 6 Positive translation products identified in the *Xenopus* cDNA library. Twelve translation products were found to be phosphorylated in the normalized *Xenopus* cDNA library by recombinant Aurora B during the final screen. The identity of each cDNA coding for an individual translation product was verified by DNA sequencing.

Mapping in vitro phosphorylation sites on candidate substrates

Among the 19 proteins identified as candidate substrates, none of them was easily identified as physiologically relevant substrates of Aurora B. We wanted to map in vitro phosphorylation sites on two of these substrates, beta 5-tubulin and MCM7. The human adult brain cDNA library clone Plate B-B8(G4) was used as a template to clone the full-length coding sequence for human beta 5-tubulin into the pCS2 expression vector. Since MCM7 was identified in *Xenopus* cDNA library, we cloned the full-length transcript variants of MCM7 by PCR amplification from a human fetal thymus cDNA library into the pCS2 expression vector. We used the consensus motif (K/R)X(S/T)(I/L/V) deduced from the Ipl1 phosphorylation sites previously identified in the budding yeast to search for putative Aurora B phosphorylation sites on these candidate substrates. We identified one consensus site (RI^{48}SV) on human beta 5-tubulin. This site was not conserved among all isoforms of human beta-tubulin and it was missing in the budding yeast beta-tubulin (Tub2) and the human beta-tubulin, which forms mitotic spindles together with alpha-tubulin. We identified four consensus sites (RD^{422}SV, RC^{483}SI, RR^{500}SL and KD^{643}SL) on human MCM7 isoform1. The first consensus site was conserved among the mouse and rat homologs while missing in the xenopus homolog. All other three sites were well conserved in all species examined. In order to identify the in vitro phosphorylation sites, the serine of each consensus site on human beta 5-tubulin and MCM7 was substituted with alanine

using site-directed mutagenesis to generate nonphosphorylatable mutants. Each mutant was in vitro transcribed and translated in the presence of [^{35}S]-methionine and the radiolabeled translation products were assayed for the disappearance of mobility shift which was normally observed after incubation with recombinant Aurora B. Human beta 5-tubulin was found to be phosphorylated on the consensus site (RI^{48}SV) and the mutation of the first consensus site (RD^{422}SV) on human MCM7 relieved its phosphorylation (Fig. 7).

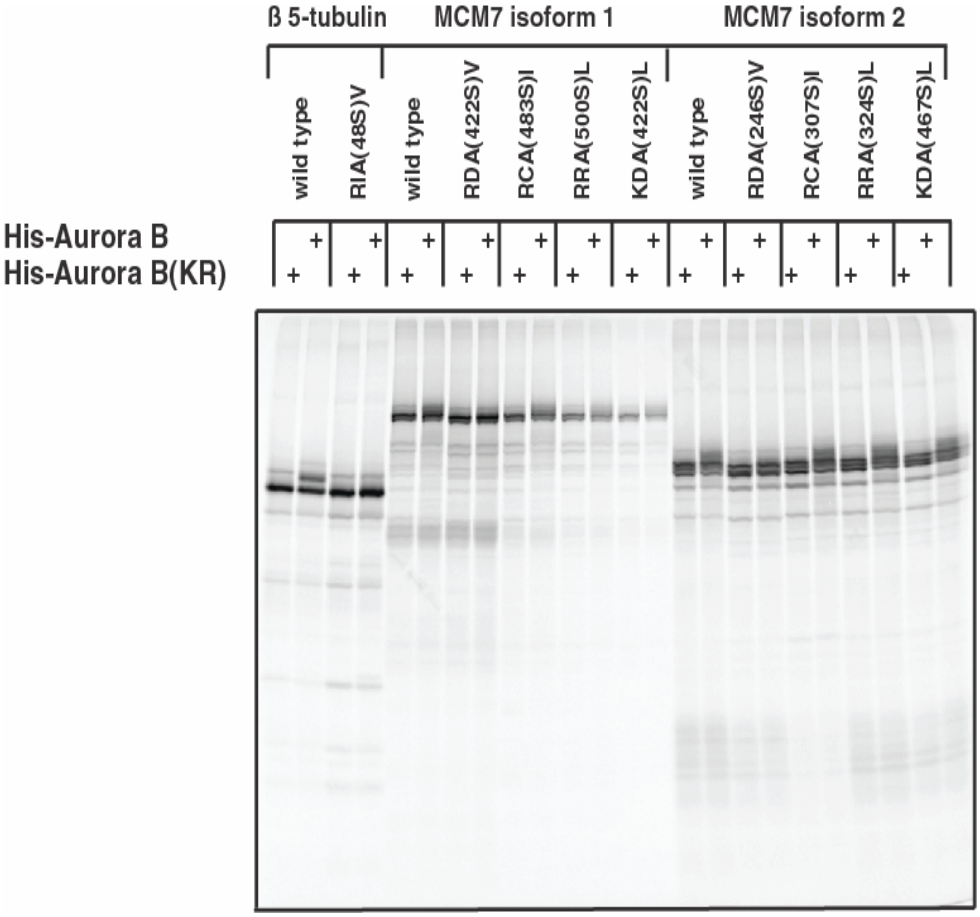

Figure 7 Mapping in vitro phosphorylation sites on candidate substrates. Putative Aurora B phosphorylation site (serine to alanine) mutants of human beta 5-tubulin and human MCM7 isoforms on the putative Aurora B phosphorylation sites were assayed with recombinant Aurora B for their capacity to be phosphorylated.

Discussion

In this study, we screened for Aurora B substrates using an in vitro expression cloning system and the recombinant form of Aurora B. We identified 19 cDNA clones which encode for translation products capable of being phosphorylated by recombinant Aurora B in vitro and we mapped the in vitro phosphorylation sites on two candidate proteins using site-directed mutagenesis. Ten of these cDNA clones were recovered from the human adult brain cDNA library while nine others were recovered from the *Xenopus* cDNA library. None of the identified substrates were found to be common in both libraries. An overall survey of the identified substrates indicates that these clones were picked because of their likely abundance in these libraries. For example, in the human adult brain cDNA library four of the clones were coding for beta tubulin isoforms which may constitute dynamic cellular structures required for neuronal morphogenesis and functioning. Neurons are among the cell types that develop polarized structures. A neuron is composed of a cell body, dendrites, and a long axon along the direction of impulse propagation. The axon lacks protein synthesis machinery, and thus all the proteins required in the axon must be transported down the axon after they are synthesized in the cell body. Most proteins are conveyed in membranous organelles and microtubules which are arranged longitudinally, with the plus end

pointing away from the cell body serving as rails for the transport of organelles (Hirokawa, 1998).

The approach used in this study suffered from several drawbacks and the candidate substrates were most probably misleading. The recombinant kinase used in this study was not available in soluble form and formed aggregates. It was not clear if the aggreagated form would identify physiologically relevant substrates. The protein kinase fold, which is extremely well conserved among the serine/threonine and tyrosine kinases, is separated into two subdomains (Huse and Kuriyan, 2002). ATP is bound in a deep cleft between the two lobes and sits beneath a highly conserved loop. This phosphate binding loop contains a conserved glycine-rich sequence motif and the glycine residues allow the loop to approach the phosphates of ATP very closely. Peptide substrates bind in an extended conformation across the front end of the nucleotide binding pocket, close to the γ-phosphate of ATP. A centrally located loop known as "the activation loop", typically 20-30 residues in length, provides a platform for the peptide substrate. In Aurora B, as in most kinases, this loop is phosphorylated when the kinase is active (Yasui et al., 2004). Phosphorylation of the activation loop stabilizes it in an open and extended conformation that is permissive for substrate binding. Even though recombinant Aurora B efficiently phosphorylated histone H3, poor autophosphorylation, lack of increased activation in the presence of the C-terminal domain of INCENP clearly indicated that only the catalytic core

is intact in this recombinant form; therefore, only those substrates that can serve as isolated peptides are expected to be phosphorylated by this form of Aurora B. Because the in vitro expression cloning system allowed us to produce the translation products using reticulocyte lysate, the correct folding of the translation products may not occur in this system because reticulocytes are specialized forms of cells with reduced cellular activity. Therefore, the proteins produced by the reticulocyte system were more likely to serve as peptide substrates and in fact all of the substrates identified except four had at least one site that exactly matched the Ipl1 consensus motif (K/R)X(S/T)(I/L/V) and two of the candidate substrates, human beta 5-tubulin and human MCM7 were found to be phosphorylated on a single consensus site by recombinant Aurora B. Another important drawback was the source and quality of the cDNA libraries. The human adult brain cDNA library was not a good choice for screening cell-cycle related substrates of Aurora B; however, we were restricted in our choice since this was the only library available in this format. Moreover the translated pools were already available for use since the library was previously used for screening sumoylated substrates. The normalized *Xenopus* cDNA library derived from pre-MBT staged eggs was a better choice; however, the quality of this library was poor as evidenced by the isolation of E.coli related clones and the inefficient translation of the cDNA pools. And finally, this approach provided too many candidate substrates to be further validated by more rigorous in vivo approaches.

Among the 19 candidate substrates, the most plausible substrate was MCM7. Mcm2-7 proteins form a functional hexameric complex that comprises an important part of the "pre-replication complex" found at replication origins during G1 phase. On exit from metaphase, the replication licensing system becomes activated, so that each origin becomes loaded with Mcm2-7. The replication licensing system remains active throughout most of G1 but inactivated as cells approach S phase. This means that no further Mcm2-7 can be loaded onto origins in S phase, G2 and early mitosis. Only licensed origins containing Mcm2-7 can initiate a pair of replication forks. When initiation occurs at an origin, the bound Mcm2-7 is displaced so that the origin can not fire again (Blow and Hodgson, 2002). The coincidence of the timing of Mcm complex recruitment and the inactivation of Aurora B activity brought the idea that Aurora B could maintain Mcm complex in its unbound state by phosphorylating Mcm7. Also, one piece of evidence showed that replication licensing is coupled to mitosis. During mitosis, the smallest subunit of human ORC, Orc6, localizes to kinetochores and silencing Orc6 resulted in the formation of multinucleated cells (Prasanth et al., 2002). However, the in vitro phosphorylation site we mapped on human MCM7 was not present on the *Xenopus* homolog identified in the the screen.

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CHAPTER FOUR

Employment of the Hsp90-Cdc37 chaperone system as a tool for coexpressing the Aurora B-INCENP complex in bacteria

Introduction

The first sight of Hsp90 and Cdc37 came during the initial analysis of v-Src protein kinase activity, which was found to phosphorylate tyrosine when assayed in an immunoprecipitate (Hunter and Sefton, 1980). Two phosphoproteins of ~80-kDa and 50-kDa were found to coimmunoprecipitate with v-Src from RSV-transformed cells. The 50-kDa phosphoprotein was the first cellular phosphotyrosine containing protein to be identified. The ~80-kDa and 50-kDa proteins were encountered in a number of other situations, particularly in association with protein kinases involved in signal-transduction pathways. The 80-kDa protein was soon identified as a member of the Hsp90 heat shock protein family (Xu and Lindquist, 1993) and the identification of the 50-kDa protein as Cdc37 came unexpectedly from studying the protein kinases that regulate the cell cycle.

Although a simple two-step mechanism involving cyclin binding and phosphorylation by Cdk activating kinase (CAK) was sufficient to describe the

assembly and activation of Cdk2/Cyclin complexes, there were early indications that Cdk4/6-Cyclin D assembly required additional factors. In quiescent fibroblasts stimulated to enter the cell cycle with growth factors, Cdk4/Cyclin D complexes assembled in mid-G1 as D-type cyclins accumulated. However, in quiescent fibroblasts expressing Cdk4 and Cyclin D3 ectopically, both subunits were present but kinase complexes were not formed (Matsushime et al., 1994). Assembly of the Cdk4/Cyclin D3 complex in this context occurred in mid-G1 and required an uncharacterized mitogen dependent step (Kato et al., 1994; Matsuoka et al., 1994). The fact that it is much more difficult to produce the active recombinant Cdk4/Cyclin D in vitro than it is to produce the active Cdk2/Cyclin or Cdc2/Cyclin complexes also supported the idea that the assembly of Cdk4/Cyclin D complexes required additional complexes.

A step forward came with the identification of vertebrate proteins related to the *CDC37* gene, which was first identified in Saccharomyces cerevisiae through a temperature-sensitive mutation that causes cell-cycle arrest in G1 like that seen during pheromone treatment. Mouse and human Cdc37-related proteins have been cloned by sequence homology with the yeast and drosophila proteins (Dai et al., 1996; Stepanova et al., 1996). The first indication that Cdc37 had a role in regulating Cdk/Cyclin function came when it was found that G1 arrest in the *cdc37-1* mutant is accompanied by a decrease in the Cdc28 activity associated with G1 cyclin Cln2, apparently due to a defect in Cdc28-cyclin binding (Gerber

et al., 1995). Mammalian Cdc37 was found to encode the 50-kDa kinase targeting subunit of the chaperone heat shock protein 90 (Hsp90) previously observed in complexes with Raf-1 and v-Src (Stancato et al., 1993; Whitelaw et al., 1991) and was shown to be sufficient to target Cdk4 to Hsp90 (Stepanova et al., 1996). The Hsp90-Cdc37 complex associated preferentially with the fraction of Cdk4 not bound to D-type cyclins and the pharmacological inhibition of Hsp90 function with geldanamycin reduced the level of Cdk4 and reduced the stability of the newly synthesized Cdk4 as revealed by pulse-chase experiments. These observations suggested that Cdc37 in combination with Hsp90 might play a role in the assembly or activation of Cdk4/Cyclin D complexes. Most probably, Hsp90-Cdc37 was responsible for assisting the folding of newly synthesized Cdk4, leaving it in a conformation able to bind Cyclin D.

The heat shock protein Hsp90 and its cofactors form the molecular chaperone complexes that facilitate the structural maturation of its substrates. Hsp90 seems to be a specialized chaperone, targeting specific substrate proteins involved mainly in signal transduction such as steroid hormone receptors and kinases. Hsp90 does not act to fold nonnative proteins but rather binds to substrate proteins at a late stage of folding prior to activation by binding to a ligand or other proteins (Jakob et al., 1995). The Hsp90-assisted maturation of substrate proteins often leads to an enhanced activity and stability. In the absence of hormone, steroid hormone receptors are found as “aporeceptor complexes” consisting of a

receptor monomer, a dimer of the heat shock protein Hsp90, and accessory proteins such as Hsp56, Hsp70, DnaJ and p23 (Smith and Toft, 1993). Subsequent to ligand binding, the "activated receptor" dissociates from the complex, binds to specific DNA sequences, and modulates the transcription of genes. Proper interaction of the receptors with Hsp90 is essential for efficient ligand binding and response, indicating that the aporeceptor complex represents a "poised" conformation of the receptor, ready to respond to the signal (Bohen and Yamamoto, 1993; Bresnick et al., 1989; Smith, 1993). In the absence of ligand, the complex is in dynamic equilibrium with two unliganded (active and inactive), no Hsp90-bound receptor forms. Hsp70 and other chaperones, in an adenosine triphosphate-dependent mechanism, appear to reassemble Hsp90 onto unliganded receptors; thereby maintaining the pool of aporeceptors that can respond to the ligand.

The interaction of Hsp90 with protein kinases appears to depend on kinase recognition by Cdc37. In vitro, Hsp90-Cdc37 associates specifically with misfolded, largely inactive forms of v-Src and the src-like tyrosine kinase Lck (Hartson et al., 1996; Hartson and Matts, 1994). Many of the Hsp90-Cdc37-protein kinase interactions that have been detected involve mutant forms of the protein kinase. The association with mutant protein kinases could represent a nonphysiological interaction, albeit one that is needed to support the weak activity of the mutant protein kinase. However, it seems more likely that the interaction of

Hsp90-Cdc37 with the mutant proteins is more readily detected because a larger fraction of the population is not properly folded and therefore, becomes a target for the Hsp90-Cdc37 association.

Genetic evidence indicated that Hsp90-Cdc37 chaperone may have a broader substrate specificity than has been revealed by biochemically. Cdc37 displayed genetic interactions with several kinases in the budding yeast, including Mps1 (Schutz et al., 1997), which is required for the spindle pole body duplication and the spindle checkpoint, Kin28 (Valay et al., 1995), which is required for RNA polymerase II function, Fus3 (Fujimura, 1994), a mitogen-activated protein kinase in the pheromone response pathway, and casein kinase II (Kimura et al., 1997).

Functional dissection of Cdc37 identified a protein kinase binding domain at the N terminus (Grammatikakis et al., 1999) while the remainder of the protein was found to interact with Hsp90 (Shao et al., 2001). Cdc37 acts as an adaptor facilitating protein kinase interaction with the Hsp90 (Silverstein et al., 1998). Binding to Cdc37 is highly specific, and otherwise closely related protein kinases differ substantially in their interaction as Cdk4 associates with Cdc37 whereas Cdk2 does not (Dai et al., 1996; Stepanova et al., 1996). Cdc37 is not a passive scaffold but participates in the regulation of Hsp90 chaperone cycle (Siligardi et al., 2002). Cdc37 is able to arrest the ATPase cycle to facilitate client protein loading.

Studies on the subcellular localization of Cdc37 provided further hints about its potential client proteins (Lange et al., 2002). Some Cdc37 was detected on the mitotic apparatus and the loss of Cdc37 function during mitosis led to phenotypes that are very similar to those brought by the inactivation of Aurora B. Aurora B was found to interact with and require the Hsp90-Cdc37 complex for its stability. This finding prompted us to use this chaperone system to assist the folding and assembly of Aurora B-INCENP complexes in bacteria during their overexpression. Aurora B exists in a complex with two other proteins, INCENP and Survivin (Bolton et al., 2002). It emerged that activation of Aurora B by INCENP is a two step-process in which INCENP partially activates Aurora B, whereas the full activation requires phosphorylation of a conserved Thr-Ser-Ser motif near the C terminus of INCENP (Honda et al., 2003). Therefore, it was essential to have Aurora B in complex with INCENP to be able to use in biochemical and structural studies; however, to this date it was only possible to coexpress these two proteins in Sf9 cells which produced very low yields while bacterial expression systems didn't produce satisfactory results. Here, we describe the employment of the Hsp90-Cdc37 chaperone system as a versatile tool for expressing kinases in bacteria using Aurora B-INCENP as an example.

Experimental Procedures

Plasmids and cloning

The full-length coding sequence for INCENP and the C-terminal coding sequences (INCENP$^{628\text{-}919a.a}$, INCENP$^{444\text{-}919a.a}$, INCENP$^{307\text{-}919a.a}$ and INCENP$^{136\text{-}919a.a}$) were subcloned from pET3aTr-His vector into the Bam HI / Hind III sites of pETDuet1. The full-length coding sequence for Aurora B was subcloned from pET3aTr vector into pETDuet1 vector using the Bgl II/Nde I sites. The following primers (Forward, 5'ACGCGGATCCGATGGCACGACGGCCCCAGGGCCCA TTC3'; INC628Forward, 5'ACGCGGATCCGATGGAGGAGGTGGAAGCACG C3'; INC444Forward, 5'ACGCGGATCCACCGCAGAGTGCCAGGAGGAAG3 '; INC307Forward, 5'ACGCGGATCCCCCGATCGCCCCGTCTTCCCCG3'; INC136Forward, 5'ACGCGGATCCCATGGCATTGGCTGCACCTTCTTCA3' and Reverse, 5'ACCCAAGCTTTCAGTGCTTCTTCAGGCTGTAGGC3') were used previously to PCR amplify the full-length INCENP and the C-terminal fragments (INCENP$^{628\text{-}919a.a}$, INCENP$^{444\text{-}919a.a}$, INCENP$^{307\text{-}919a.a}$ and INCENP$^{136\text{-}919a.a}$) respectively and clone into pET3aTr-His vector. The full-length coding sequence for Hsp90 (NCBI accession number: BC023006) was PCR amplified from a human fetal thymus cDNA library (Clontech) using the following primers (Forward, 5'CATGCCATGGGAATGACCAAGGCTGACTTGATCAA3'; Reverse, 5'CGCGGATCCTTAGTCTACTTCTTCCATGCGTG3') and cloned into the Nco I/Bam HI sites of pRSFDuet (Novagen). The full-length coding

sequence for Cdc37 (NCBI accession number: BC000083) was PCR amplified from a human fetal thymus cDNA library (Clontech) using the following primers (Forward, 5'GGAATTCCATATGGTGGACTACAGCGTGTGG3'; Reverse, 5'GTCTCTCGAGTCACACACTGACATCCTTCTCAT3') and cloned into the Nde I/Xho I sites of pRSFDuet.

Protein expression and purification

Rosetta(DE3) cells were cotransformed with pRSFDuet-Hsp90-Cdc37 and pETDuet1 vectors harboring Aurora B and the full-length INCENP or the C-terminal fragments of INCENP ($INCENP^{628\text{-}919a.a}$, $INCENP^{444\text{-}919a.a}$, $INCENP^{307\text{-}919a.a}$ and $INCENP^{136\text{-}919a.a}$) and the cotransformants were selected on LB ($amp+kan^{+}chl^{+}$) plate. Individual clones were picked, inoculated in 10 of ml LB ($amp+kan^{+}chl^{+}$) medium and were grown overnight at $37^{o}C$ with vigorous shaking. Overnight grown cultures were diluted into 1 lt of LB ($amp+kan^{+}chl^{+}$) medium and were grown at $37^{o}C$ with vigorous shaking till $O.D_{600}$ reaches around 0.6. The cultures were cooled down to room temperature and IPTG (Roche) was added to a final concentration of 0.5 mM. Cells were grown at room temperature for an additional 4 hours with vigorous shaking.

Cells were harvested by centrifugation at 2000 rpm for 20 min at $4^{o}C$ using a Beckman J6 rotor and each culture was resuspended in 20 ml of lysis buffer (50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 5mM $MgCl_2$, 10% glycerol, 1 mM ATP and 10 μg/ml each of chymostatin, leupeptin, and pepstain). Lysozyme was

added to a final concentration of 1mg/ml before incubating the cell suspensions on ice for 30 min. DNAseI was added to a final concentration of 10 µg/ml and the lysates were incubated at room temperature for 20 min. After the incubation, cell lysates were spinned down at 36000 rpm for 1 hour at 4^{o}C. After centrifugation, each of the cleared supernatants was loaded onto 1ml of Ni-NTA (Qiagen) beads equilibrated with PBS. After washing with 10 column volumes of lysis buffer with 40 mM imidazole, the bound proteins were eluted with 5 column volumes of elution buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 250 mM imidazole). 10 µl of each eluate was separated on SDS-PAGE gel for analysis and detection with Coomassie blue staining.

The Aurora B- INCENP$^{628\text{-}919a.a}$ fractions were pooled and concentrated to 1 ml using Amicon centrifugal filter unit. For the purification of Aurora B-INCENP$^{628\text{-}919a.a}$ complex using gel filtration, Superdex 200 column was equilibrated with a buffer containing 50 mM Tris-Cl, pH 8.0, 300 mM NaCl, 2 mM DTT.

Western Blot, antibodies and ECL detection

Samples were separated on SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes using the Bio-Rad semi-dry electrophoretic transfer unit and were analyzed by western blotting. The following antibodies were used in this study: mouse monoclonal anti-rat Aurora B (α-Aim1) antibody from BD Biosciences, rabbit polyclonal anti-human C-INCENP

antibody. For the production of rabbit polyclonal anti-human C-INCENP antibody, the C-terminal fragment of INCENP (799-919a.a) was expressed in bacteria, purified to homogeneity using Ni-NTA chromatography and gel filtration and supplied to Zymed at a final concentration of 1mg/ml in PBS for immunizing the rabbits. Final bleeds were used for the purification of the polyclonal antibody on Affi-gel matrix coupled to the immunogen. Depending on the primary antibody choice, the ECL^{TM} anti-rabbit IgG horseradhish peroxidase linked whole antibody or ECL^{TM} anti-mouse IgG horseradhish peroxidase linked whole antibody was used as the secondary antibody for the ECL (Amersham) detection.

Results

Production of Aurora B-INCENP complexes via the coexpression of Hsp90-Cdc37 molecular chaperones

Aurora B exists in a complex with at least two other proteins, inner centromere protein (INCENP) and Survivin (Bolton et al., 2002). Using the recombinant proteins, it was found that Aurora B kinase activity was stimulated by INCENP and the C-terminal region of INCENP was sufficient for the activation of Aurora B (Honda et al., 2003). Our efforts to express recombinant Aurora B in bacteria using different fusion tags such as GST and six-histidine yielded in insoluble protein aggregates. We sought to overcome this problem by coexpressing various C-terminal fragments of INCENP which were earlier shown to be required for binding to and activation of Aurora B (data not shown) (Honda et al., 2003). However, this approach also didn't produce any satisfactory results.

The original *aurora* allele was identified in a screen for *Drosophila melanogaster* mutants that were defective in spindle-pole behaviour, and was named after the aurora, a phenomenon of the night sky in the polar regions (Glover et al., 1995). There are three mammalian Aurora homologs which are similar in sequence, in particular within the carboxyl terminal catalytic domain, in which Aurora A and B share 71% identity. The structural studies were successfully employed on the catalytic domains of Aurora A and Aurora B (Cheetham et al., 2002; Sessa et al., 2005). However, the three Auroras differ in

length and sequence of the amino-terminal domain, which presumably contributes to the very distinct localization and function of these kinases.

Since Aurora B was found to interact with the Hsp90-Cdc37 molecular chaperones, we reasoned that Aurora B could be a folding substrate of Hsp90-Cdc37. The fact that Hsp90-Cdc37 binds to its substrates at a late stage of folding prior to activation by binding to a ligand or other proteins, as in the case of Cdk4/Cyclin D, suggested that the presence of INCENP could positively promote the correct folding of Aurora B inside the cell. Therefore, to be able to isolate functionally competent Aurora B-INCENP complexes, we tried to coexpress these two proteins in bacteria in the presence of Hsp90 and Cdc37. In order to do so, we cloned the coding sequence of Hsp90 and Cdc37 into pRSFDuet vector. Similarly, the coding sequence of Aurora B and the full-length INCENP or one of the C-terminal coding sequences ($INCENP^{628\text{-}919a.a}$, $INCENP^{444\text{-}919a.a}$, $INCENP^{307\text{-}919a.a}$ and $INCENP^{136\text{-}919a.a}$) was cloned into pETDuet1 vector. Hsp90 was cloned in between the Nco I/Bam HI sites of pRSFDuet so that among the four cloned genes, only INCENP would harbor the six-histidine tag. Coexpression involves the transformation of *E. coli* with both of these plasmids which have compatible origins of replication and independent antibiotic selection for maintenance and allows the simultaneous expression of these four proteins. Prior to large scale expression, we should have screened the clones for the optimal induction conditions and the solubility; since this is not the usual practice in our lab, each

cotransformant was cultured for large-scale expression. Cells were lysed in the presence of ATP and histidine-tagged fusion proteins were purified by Ni-NTA column chromatography. The eluates were analyzed by SDS-PAGE and coomassie blue staining (Fig. 1A). In each case, there was a major band identified as INCENP estimated by the apparent molecular weight of the protein. Though not obvious, Aurora B protein was also identified in a stoichiometric ratio with INCENP in each case. The identities of the supposed protein bands were confirmed by western blotting (Fig. 1B). Among the four different INCENP fragments, INCENP$^{628\text{-}919a.a}$ seemed to give the highest yield of expression and the best-fit of Aurora B to INCENP stoichiometric ratio. Therefore, we carried further purification on INCENP$^{628\text{-}919a.a}$ eluates using gel filtration and the Aurora B-INCENP$^{628\text{-}919a.a}$ complex was eluted as a single peak around 60-68 ml of elution volume. These two fractions between 60 and 68 ml were combined together, analyzed by coomassie blue staining (Fig. 2A) and western blot (Fig. 2B), confirming that Aurora B-INCENP$^{628\text{-}919a.a}$ purifies as a complex when coexpressed in bacteria in the presence of the molecular chaperones Hsp90-Cdc37. Though we didn't thoroughly test the effect of Hsp90-Cdc37 on Aurora B-INCENP coexpression in bacteria, the apparent success in at least one case leads to the conclusion that Hsp90-Cdc37 promotes the correct folding of Aurora B in the presence of its binding partner INCENP and allows their copurification.

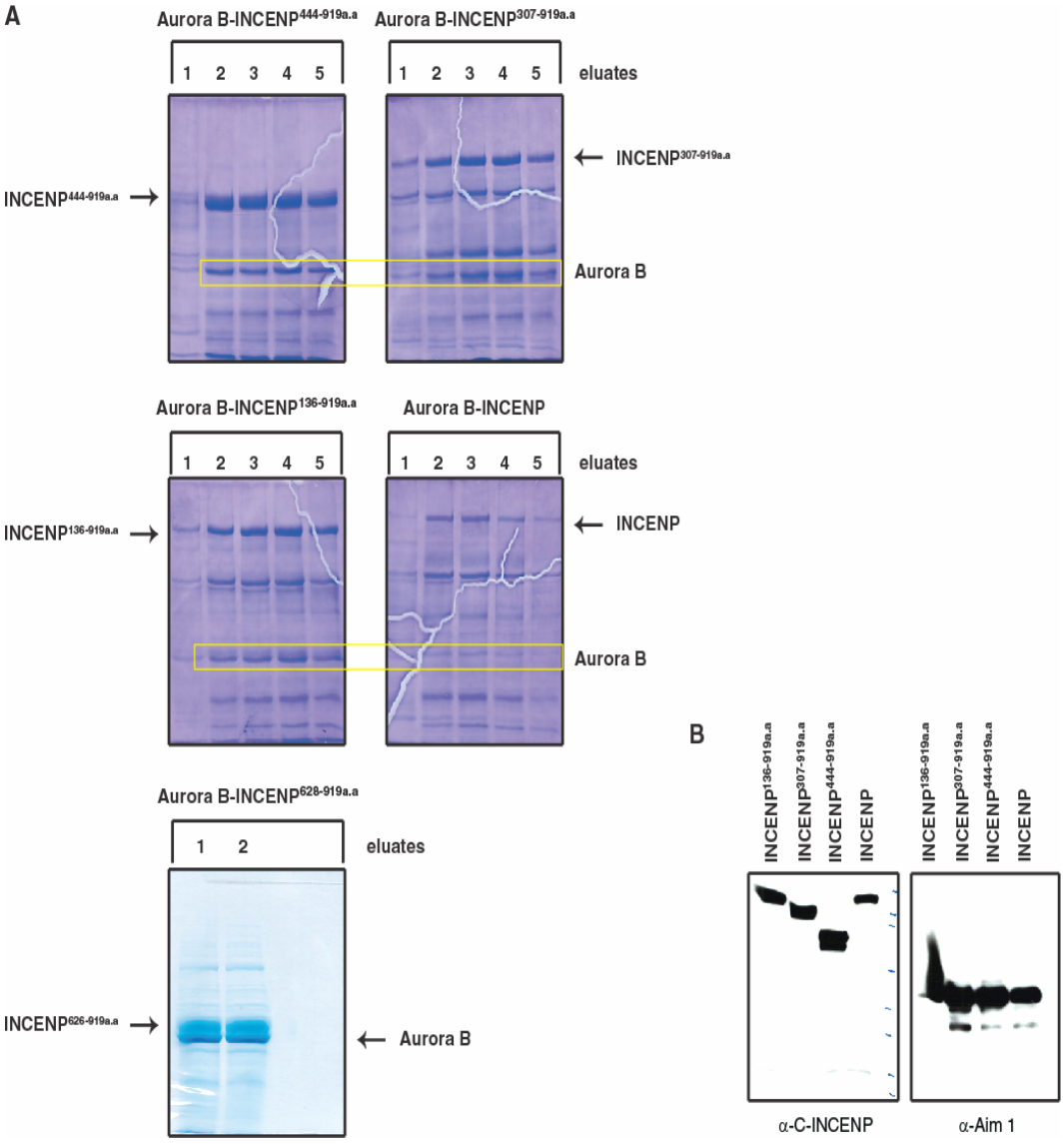

Figure 1 Production of Aurora B-INCENP complexes via the coexpression of Hsp90-Cdc37 molecular chaperones. (**A**) Cultures of *E.coli* Rosetta(DE3) transformed with pRSFDuet-Hsp90-Cdc37 and one of the pETDuet1-INCENP (INCENP$^{628\text{-}919a.a}$, INCENP$^{444\text{-}919a.a}$, INCENP$^{307\text{-}919a.a}$ and INCENP$^{136\text{-}919a.a}$)-Aurora B vectors were induced for 6 hours at room temperature with IPTG at a final concentration of 0.5 mM. Cell lysates were subject to purification using Ni-NTA affinity chromatography. The eluates were resolved on SDS-PAGE gel and analyzed by coomassie blue staining. (**B**) Aurora B and INCENP protein in these eluates were detected by immunoblotting.

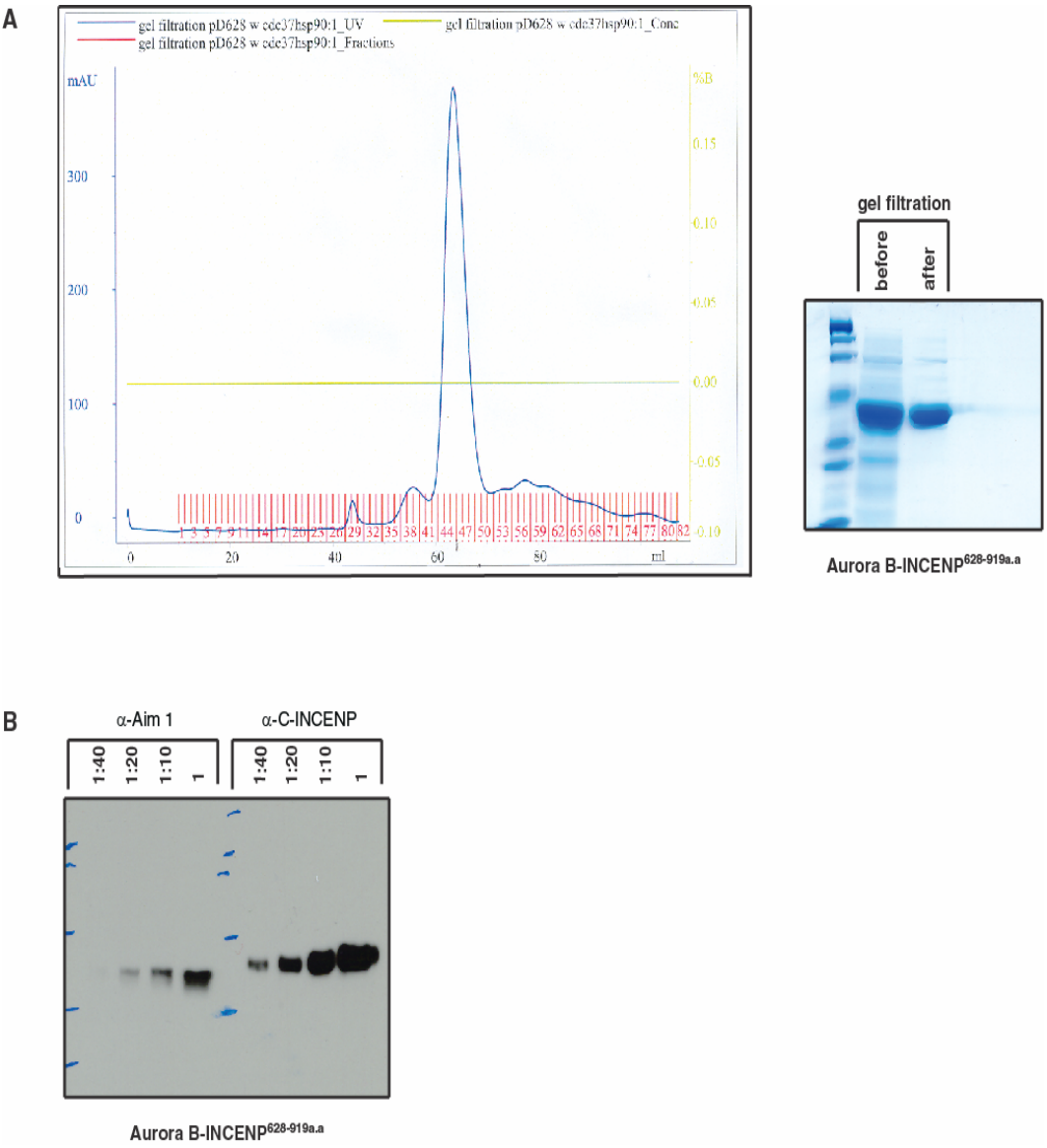

Figure 2 Purification of the Aurora B-INCENP$^{628\text{-}919a.a}$ complex using gel filtration chromatography. (**A**) The Aurora B- INCENP$^{628\text{-}919a.a}$ fractions were pooled and were subjected to gel filtration chromatography. The Aurora B-INCENP$^{628\text{-}919a.a}$ complex was eluted within a single peak between 60 and 68 ml. The protein content of these fractions was compared with the initial fractions using SDS-PAGE analysis. (**B**) 1 µl of the sample used for SDS-PAGE analysis was subjected to dilution and the proteins were detected by immunoblotting.

Discussion

In the crowded milieu of the *E.coli* cytoplasm where transcription and translation are tightly coupled and one chain is released from the ribosome every 35 seconds (Lorimer, 1996), an environment where macromolecule concentration can reach 300-400 mg/ml (Ellis and Minton, 2003), protein folding is an extraordinary challenge. In general, small, single domain host proteins efficiently reach a native conformation owing to their fast folding kinetics, whereas large multidomain and overexpressed recombinant proteins often require the assistance of folding modulators. Folding modulators include molecular chaperones, which favor folding by shielding nonpolar segments that would normally be buried within the core of their substrates from the solvent and the structured hydrophobic domains, and folding catalysts that accelerate the rate-limiting steps, such as the isomerization of peptidyl-prolyl bonds from an abnormal cis to a trans conformation and the formation and reshuffling of disulfide bonds.

The apparent difficulty in expressing functional Aurora B protein in bacteria has reminded us that the correct folding of Aurora B in bacteria may require the action of molecular chaperones. In fact, in one of our previous attempts, we tried to express Aurora B using an *E.coli* expression system overproducing the bacterial chaperones GroES and GroEL (data not shown) (Goenka and Rao, 2001). However, it didn't yield any successful results. The inspiration grasped from the use of bacterial chaperones has motivated us to

search mammalian components that could be relevant to the stability and expression of Aurora B and its binding partner INCENP, which is required for the activation of this kinase. The apparent relationship between Hsp90-Cdc37 molecular chaperones and Aurora B provided us a scheme to work on where we would try to coexpress Aurora B and INCENP in the presence of these molecular chaperones. Our preliminary results have indicated that Aurora B and INCENP can be coexpressed and copurified in this system although the significance of Hsp90-Cdc37 involvement is not determined. However, in our previous trials where we coexpressed the minimal Aurora B binding fragments of INCENP (data not shown) together with Aurora B, we failed to purify soluble Aurora B protein bound to the INCENP fragment. Therefore, the copurification of Aurora B and INCENP$^{628\text{-}919a.a}$ after gel filtration built some confidence about the Hsp90-Cdc37 system as a means of overexpressing the Aurora B-INCENP complex in bacteria.

If this system turns out to be relevant for the coexpression of Aurora B-INCENP complexes and is proven to be actively involved in the correct folding of these proteins in the intracellular environment of bacteria, then the whole system could be adapted to study the regulation of the ATPase cycle of Hsp90 and its regulation by Cdc37 and the client proteins. Our built up knowledge about the regulation of Hsp90 and Cdc37 is derived from the structural information of the Hsp90 and Cdc37 fragments and the functional mapping of the interaction between Hsp90 and Cdc37 and the client proteins (Roe et al., 2004). The

functional relationship determined between Hsp90 and Cdc37 is actually in apparent contradiction with the results we obtained with our expression system. Therefore, it would be useful to reexamine the supposed mechanism. According to what is known, Cdc37, which acts early in the chaperone cycle, can completely inhibit the ATPase activity of Hsp90 (Siligardi et al., 2002). The ability of this recruitment factor to prevent ATP turnover suggests that Cdc37 holds Hsp90 in an "open" conformation in which the putative binding surfaces in the middle segment of the chaperone would be accessible to a client protein kinase bound to the N-terminal domain of Cdc37. Progress from this arrested "loading" phase through the ATPase cycle would absolutely depend on the ejection of Cdc37 C-terminus between the jaws of the Hsp90 clamp to allow it to close. However, Cdc37 could remain persistently associated with the cycling Hsp90 complex via the interaction of its N terminus with the bound client protein kinase. It is not known how the arrested Hsp90-Cdc37 complex converts to a cycling complex and in my opinion would be modulated by the binding of client protein to Cdc37.

This expression system could be improved using *E.coli* cells, which exploits the unique properties of MazF, a bacterial toxin which acts as a sequence-specific endonuclease that exclusively cleaves ssRNAs at ACA sequences (Suzuki et al., 2005). Expression of MazF and a target gene engineered to encode an ACA-less mRNA would result in sustained and high level target expression in the absence of background cellular protein synthesis. This expression system

would dramatically simplify the structural and functional studies of Hsp90-Cdc37 molecular chaperones.

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CHAPTER FIVE

Conclusions and Future Directions

Modern molecular biology is based on a deep understanding of life which involves the complex chemical interactions that take place among the several thousand different molecules found within any cell. Of these, DNA is the master molecule in whose structure is encoded all the information needed to create and direct the chemical machinery of life. Analysis of the flow and regulation of this genetic information among DNA, RNA and protein is the subject of molecular biology. However, the techniques of molecular biology are now being applied to nearly every major field of biology. This change in our understanding of life has launched a dramatic and seemingly sudden biological revolution. However, it is prudent to remember that embedded in the word *revolution* are both *revolve* and *evolution*. These remind us of the long-term, cyclical, and historical undercurrents-however sharp its break from the past appears.

As time goes on, nearly every field of human endeavor is marked by changes. But only among the sciences is there true progress; only there is the record one of continuous advance. And yet, among most branches of science, the process of progress is one of both correction and extension.

The development of molecular biology was in large part driven by the quest to find increasingly purer and more powerful abstractions of essential biological processes. Experimental systems progressed from complex, multicellular organisms (such as pea plants and fruit flies used in the early 1900s) to simple, one-cell organisms (bacteria and viruses, beginning in the 1940s) to purified cellular components (in vitro systems, beginning in the 1960s). While biological systems evidently exhibit complex behaviors, the significance of fixed points in biological studies has been a recurring theme in the literature. I thought this meant that certain processes are well understood and that this knowledge might therefore provide useful lessons for studying system issues in the more complex areas of signal transduction. While a lot is known, I would not characterize it as well understood. Had the problem of language not been so difficult, molecular biology might have made greater headway. Hanahan and Weinberg (Hanahan and Weinberg, 2000) raised the question as to whether progress will consist of "adding further layers of complexity to a scientific literature that is already complex almost beyond measure" or whether the progress will lead to "a science with a conceptual structure and logical coherence that rivals that of chemistry and physics." At the core of the challenge is the need for a new approach, a shift from reductionism to a holistic approach. To make this point clear, I would like to give an example.

In somatic cells, entry into mitosis depends on the successful completion of DNA synthesis. This dependency is established by feedback controls that arrest cell division when damaged or unreplicated DNA is present. Hartwell and Weinert began dissecting the checkpoint that orders DNA replication and mitosis in the budding yeast by examining available radiation-sensitive mutants (Weinert and Hartwell, 1988; Weinert and Hartwell, 1990; Weinert and Hartwell, 1993). The roles of *RAD9* and *RAD17* genes were evaluated by comparing the cell-cycle responses of mutant and wild-type cells to radiation and to a drug that inhibits DNA replication (hydroxyurea); *rad9* and *rad17* mutants failed to arrest after irradiation (X- or UV-), but did arrest when treated with hydroxyurea.

A genetic approach to the study of cell division in the budding yeast, including DNA replication, was also undertaken by Hartwell and coworkers (Culotti and Hartwell, 1971; Hartwell, 1971; Hartwell et al., 1970). The gene products for three of these genes were initially identified (*cdc9*, DNA ligase (Johnston and Nasmyth, 1978); *cdc8,* thymidylate kinase (Jong et al., 1984); *cdc21*, thymidylate synthetase (Bisson and Thorner, 1977)). However, no mutant affecting the central figure in replication, DNA polymerase, was found among the mutants collected, or among other temperature sensitive mutant collections (Dumas et al., 1982; Johnston and Game, 1978; Kuo et al., 1983). Biochemically, three nuclear DNA polymerases have been purified from the budding yeast. They were thought to be distinct polymerases on the basis of genetic and

immunological criteria. An alternative approach to identifying components of the replication apparatus was undertaken. This involved purifying replication proteins, isolating the genes encoding them and constructing the appropriate mutant from the cloned gene.

In addition, the *RAD9* and *RAD17* genes also showed a striking genetic interaction with a class of *CDC* genes that encode the structural genes for DNA replication enzymes, including DNA ligase (*CDC9*); DNA Polymerase I (*CDC17*); DNA Polymerase III (*CDC2*) and *CDC13*. It was found that the cell cycle arrest of *cdc2*, *cdc9*, *cdc13* and *cdc17* after shift to the restrictive temperature required intact *RAD9* and *RAD 17* genes (Weinert and Hartwell, 1993).

The striking interaction of *RAD9* and *RAD17* with *CDC* genes has suggested a genetic strategy to identify additional checkpoint genes (Weinert et al., 1994). Three novel mutants that prevent damaged DNA from arresting the cell cycle, *mec1*, *mec2* and *mec3*, have been identified and also a fourth checkpoint gene, *RAD24* was found by screening the *RAD* mutants. Four of the six mutants showed normal cell-cycle arrest in response to unreplicated rather than damaged DNA, but two, *mec1* and *mec2* (also known as *rad53*), are also defective in their ability to arrest their cell cycle in response to unreplicated DNA. This meant in the budding yeast different sensors detect damaged (G_2-phase pathway) or unreplicated DNA (S-phase pathway) and both feedback controls converged to act

on the same checkpoint. However, the nature of the lesions in DNA that are detected as damaged or unreplicated were not known.

The budding yeast Saccharomyces cerevisiae provides an ideal subject for the studies of division delay. Progression of individual cells through the nuclear cycle can be easily monitored by cell and nuclear morphology. Bud emergence and spindle pole duplication occur approximately at or before the time of DNA synthesis (Nasmyth, 1993). Duplicated spindle pole bodies remain closely attached to each other for a large fraction (>25%) of the cell cycle and the separation of spindle pole bodies does not occur until well after the completion of S phase. Formation of bi-polar spindles between spindle pole bodies facing each other occurs in S phase but is very difficult to observe. After DNA replication, the nucleus migrates from its location in the mother cell to the "neck" between the mother cell and the bud, while the new and old spindle pole bodies separate but remain connected by interdigitating nuclear microtubules, resulting in the formation of a short (~1-2 μm) bipolar spindle that traverses the nucleus (Winey and O'Toole, 2001). Mutants defective in the S-phase pathway were defined as those unable to prevent spindle elongation when DNA replication was blocked by the action of ribonucleotide reductase inhibitor hydroxyurea (Allen et al., 1994). When cells are progressing through mitosis, the spindle and the nuclear envelope elongate through the neck into the bud and one genome moves into the bud while the other remains in the mother cell. Once the genomes move to the distal regions

of the mother cell and bud, the spindle breaks down, the nucleus divides and cytokinesis ensues. Before its breakdown, the final length of the elongated spindle is 6-8 μm.

In the budding yeast, a functional checkpoint response requires the activities of Mec1 and Mec2 (Rad53). The activities of these kinases are required as soon as cells begin DNA replication and an unknown target of the checkpoint pathway prevents the transition to mitosis. Unlike in the fission yeast or the ATR-Chk1 pathway in mammalian cells (Abraham, 2001), inhibition of the premature transition to mitosis in the budding yeast doesn't involve inhibitory tyrosine-phosphorylation of Cdc28 (Amon et al., 1992; Sorger and Murray, 1992). Thus, given the role of Chk1 in Cdk phosphorylation in other systems, it seemed unlikely that S.cerevisiae Chk1 would be required for the checkpoint control. However, when the integrity of the checkpoint in *chk1* mutants was examined in *cdc13* mutants, cells failed to arrest and progressed through multiple cell cycles although *chk1* mutants grown asynchronously were not sensitive to γ- or UV-radiation (Sanchez et al., 1999). Temperature-sensitive *cdc13* mutants accumulate single–stranded DNA at the telomeres and arrest in the G_2 phase of the cell cycle (Garvik et al., 1995).

A new layer of definition about the mechanism of coupling of S phase to mitosis arised with the identification of Pds1, an anaphase inhibitor, which was also shown to be essential for cell cycle arrest in the presence of DNA damage

(G_2 phase pathway), but not inhibition of DNA replication (S phase pathway) (Yamamoto et al., 1996). The initial *pds1-1* allele was identified by its inviability after transient exposure to microtubule inhibitors and its precocious dissociation of sister chromatids in the presence of these microtubule inhibitors. Unlike wild-type cells, γ-irradiation of *pds1* mutant cells or Cdc13 inactivation does not result in the inhibition of mitosis, as evidenced by spindle elongation, sister chromatid separation and cytokinesis. In response to DNA damage, Pds1 is phosphorylated in a Mec1 and Rad9-dependent but Mec2-independent manner (Cohen-Fix and Koshland, 1997) and since in the presence of DNA damage or when DNA replication is inhibited, Mec2 is phosphorylated in a Mec1-dependent manner (Sun et al., 1996), Pds1 and Mec2 were placed on parallel branches of the checkpoint. Similarly, because Chk1 is phosphorylated in a Mec1 and Rad9-dependent but Mec2-independent manner in response to DNA damage, Chk1 and Mec2 were placed on parallel branches of the checkpoint (Sanchez et al., 1999; Walworth and Bernards, 1996). Phosphorylation of Pds1 was later found to be Chk1 dependent in *cdc13* mutants and mutation of Chk1 phosphorylation sites on Pds1 abolished its checkpoint function (Wang et al., 2001).

Pds1 inhibits sister chromatid separation by binding and inhibiting Esp1, a cysteine protease that causes the cleavage of the cohesin Scc1 that holds sister chromatids together (Ciosk et al., 1998). Degradation of Pds1 occurs shortly before anaphase and sets a point in space and time for the liberation of Esp1,

which is a perquisite for anaphase entry. Can we translate the above mentioned observations on the role of Pds1 during the G_2 phase pathway to a fixed point in metaphase to anaphase transition and use to explain the role of Pds1 in checkpoint signaling? Experiments tell us that it can be because mutation of Chk1 phosphorylation sites on Pds1 doesn't interfere with its anaphase inhibitor function and loss of Pds1 phosphorylation correlates with the APC-dependent Pds1 destruction in response to DNA damage (Wang et al., 2001). However, it doesn't explain why mutations on the parallel branch of the checkpoint such as *mec2* fail to arrest under the same conditions (Weinert et al., 1994) and how mutation of Chk1 phosphorylation sites on Pds1 disrupts the coordination of S phase to M phase (Wang et al., 2001). *mec2* mutants should arrest if the parallel branch of the checkpoint is functional and sufficient to block the progression.

In *pds1* mutants, a defective coupling of S phase to M phase can be demonstrated when the replication is partially inhibited with low doses of replication inhibitors by measuring the relative timing of budding, DNA replication, spindle assembly and the onset of anaphase (Clarke et al., 1999). Under these conditions, replication proceeds more slowly, cells perform other aspects of cell cycle progression, budding, and spindle assembly as rapidly as in the absence of HU; cell cycle arrest in G_2 is then necessary to delay anaphase while replication is completed. Although both wild type and *pds1* cells budded and progressed through S phase with similar timing, sister centromere separation

was advanced in *pds1* cells. These examples set with Pds1 implicate that nature's design is not optimization of the behavior over time, but rather optimization of a system's organized complexity. Therefore, understanding in biology would depend on the search for organizing principles rather than on construction of predictive descriptions that outline the evolution of systems. The search for organizing principles requires the accurate description of concepts and focus of investigation. What is meant by DNA damage or what is meant by unreplicated DNA? What is meant by the G_2 phase pathway and S phase pathway? How is coordination achieved in each case? Coordination, which is provided by the checkpoint signaling, is needed if, due to genetic mutations such as *cdc13* mutants or perturbations such as DNA damage or unreplicated DNA, one or more of the subsystems is not sending the information for proper functioning. A discrepancy that has to be restored provides a signal that coordination is needed. What is the nature of the signal in each case? What is the impact of functioning on a low level (such as DNA damage) to functioning on a higher level (such as DNA replication, transcription, translation, protein folding, protein degradation, cell cycle progression)? Which subsystems are ignorant to functioning on a low level? What is the domain of autonomy which is a range of changes on the lower level such can be implicated with low doses of replication inhibitors and the corresponding range of normal behavior on the higher level so that the two levels do not interact in the sense that the changes in functional level are treated as "background noise"

in the higher level? Conceptual understanding would eventually arise from rexaming the fundamentals.

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VITAE

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