ISOLATION AND CHARACTERIZATION OF AURORA-A KINASE INTERACTING PROTEIN (AKIP), A NOVEL NEGATIVE REGULATOR FOR AURORA-A KINASE

LIM SHEN KIAT

(BSc [Hons], National University of Singapore, Singapore)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2006

Acknowledgements

I would like to thank my direct supervisor, Dr. Ganesan Gopalan, Senior Scientist of Division of Cellular and Molecular Research (CMR), National Cancer Centre (NCC) for his patient guidance and constant encouragement throughout my PhD study. I am also grateful to him for critically reading and correcting my thesis.

I would also like to thank my co-supervisors, Prof Hui Kam Man, Director of CMR Division, NCC and Prof Uttam Surana, Principal Investigator of Institute of Molecular and Cellular Biology (IMCB) for their close monitoring on the progress of my PhD project and offering of constructive and helpful suggestions during the yearly committee meeting.

Million thanks to both Dr.Ganesan Gopalan and Prof Hui Kam Man for their grant support for my PhD project throughout.

My sincere appreciation goes to all the current and ex- colleagues in my lab and Prof. Hui's lab, NCC for their friendship, encouragement and technical assistance throughout my PhD study.

I would like to specially thank my beloved wife, Shing Tsui Tsui for her dedicated love and sacrifice as well as her endless moral support and encouragement. Last but not least, I thank my family and friends for their continuous support and encouragement as well.

A million million thanks to all of you again for making everything possible for me.

Lim Shen Kiat
March 2006

Table of Contents

List of Tables

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 6

Chapter 7

Abbrevations

aa	amino acid
A Box	Aurora box
AD	Activation domain
ADH	Alcohol dehydrogenase
AIK1	Human Aurora-A Kinase
AKIP	Aurora-A Interacting Protein
ALLM	N-acetyl-Leu-Leu-methional
ALLN	N-acetyl-Leu-Leu-norleucinal
AML	acute myelogenous leukemia
Amp	Ampicillin
APC/C	anaphase-promoting complex/cyclosome
ATP	Adenosine triphosphate
AURKA	Aurora-A Kinase
AZ	antizyme
AZI	Antizyme Inhibitor
bp	base pair
BSA	bovine serum albumin
CDK	cyclin dependent kinase
cDNA	Complementary DNA
Chfr	Checkpoint protein with FHA and Ring domain
CHO	Chinese Hamster Ovary
CHX	Cycloheximide
CIN	Chromosome Instability
conc	concentration
CO-IP	Co-immunoprecipitation
DAD	D box activating domain
DB domain	DNA binding domain
D box	Destruction box
DMSO	Dimethyl Sulfoxide
Dox	Doxycycline
dNTP	deoxynucleotide triphosphate
EST	expressed sequence tag
Ex	exposure
FBS	fetal bovine serum
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
Gal	Galactose
GAP	GTPase activating protein
Glu	Glucose

HCC	Hepatocellular carcinoma
His	Histidine
Hr	hour
HURP	Hepatoma Upregulated Protein
IAK1	mouse Aurora-A kinase
Ile	Isoleucine
Imp	Importin
IP	Immunoprecipitation
kDa	kilo Dalton
Leu	Leucine
LiAc	Lithium Acetate
M phase	Mitosis phase
mAb	monoclonal antibody
MEN	Mitotic Exit Network
MG132	Carbobenzoxy-L-leucyl-L-leucinal
min	minute
mRNA	messenger RNA
MTOC	microtubule organizing centre
N terminus	amino terminus
NEK	NimA-related kinase
NLS	Nuclear localization signal
nt	nucleotide
ODC	ornithine decarboxylase
ORF	open reading frame
Orn	ornithine
pAb	Polyclonal antibody
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
Phe	Phenylalanine
PI	Propidium Iodide
PKA	protein kinase A
PLK	polo-like kinase
pmol	Pico mole
PP1	protein phosphatase 1
Pu/Put	putrescine
RACE	Rapid amplification of cDNA ends
RB	Retinoblastoma

RT	room temperature
SD	synthetic dropout
SDS	Sodium dodecyl sulphate
sec	second
Ser	Serine
SH3	Src Homology 3
siRNA	small interfering RNA
Spd	spermidine
Spm	spermine
SSD	sensor and substrate discrimination
SUMO	small ubiquitin-like modifier
TBS	Tris-buffered saline
TCR	T cell recptor
Thr	Threonine
TR	N-terminal truncated
Trp	Tryptophan
Ub/Ubiq	Ubiquitin
wt	wild type

Summary

Aurora kinases have evolved as a new family of centrosome- and microtubule-associated serine/threonine kinases that regulate multiple processes in mitosis, such as centrosome duplication and maturation, chromosome condensation, bipolar spindle assembly and dynamics, cytokinesis and checkpoint control. One of its members, Aurora-A kinase is a potential oncogene. Overexpression of Aurora-A kinase causes centrosome amplification and defective chromosome segregation, leading to aneuploidy and tumorigenesis in various cancer cell types.

Our objective is to identify the negative regulator(s) for mammalian Aurora-A kinase. Exploiting the lethal phenotype associated with overexpression of Aurora-A kinase in yeast, we performed a dosage suppressor screen in yeast and successfully isolated a novel negative regulator of Aurora-A kinase, named as AKIP (Aurora-A Kinase Interacting Protein). AKIP is an ubiquitously expressed nuclear protein that interacts specifically with human Aurora-A *in vivo*. AKIP targets Aurora-A for protein destabilization in a proteasome-dependent manner. AKIP-Aurora-A interaction is essential for the AKIP-mediated Aurora-A degradation.

Aurora-A kinase normally undergoes cell cycle-dependent turnover through the Cdh1-mediated APC/C-ubiquitin-proteasome pathway. In an attempt to investigate the mechanism of AKIP-mediated Aurora-A degradation, AKIP was found to potentiate the proteasome-dependent

degradation of Aurora-A by an alternative mechanism that is independent of ubiquitination. This implies Aurora-A kinase can be delivered to the proteasome for degradation via two distinct ubiquitin-dependent and ubiquitin-independent pathways. AKIP inhibits Aurora-A ubiquitination, through its interaction with the potential ubiquitination region of Aurora-A.

Interestingly, AKIP-mediated Aurora-A degradation is functionally linked to a family of protein, called antizyme (AZ), which plays the proteasomal targeting role and mediates the Ub-independent degradation of some proteins. Antizyme can directly down-regulate Aurora-A protein stability, which is dependent on antizyme:Aurora-A interaction. Interestingly, defective antizyme:Aurora-A interaction or inhibition of antizyme function impairs AKIP-mediated Aurora-A degradation, implying AKIP and antizyme function on the same or parallel pathways in the ubiquitin-independent degradation of Aurora-A. AKIP indeed acts upstream of antizyme by enhancing binding of antizyme to Aurora-A, thereby targeting Aurora-A for proteasomal degradation.

SECTION 1

Introduction and Literature Review

(Introduction and Literature Review)

Chapter 1

Aurora Kinase Family and Roles of Aurora-A Kinase in Tumorigenesis

---

1.1 Mitosis

1.1.1 Overview of Eukaryotic Cell Cycle-Mitosis

Mitosis, though it is the shortest phase of the cell cycle, is highly structurally dynamic and plays a critical role in segregating the newly synthesized chromosomes symmetrically and accurately into the two daughter cells. By end of S phase, the centrosome duplication and DNA replication are accomplished. When the cells first enter into prophase, the chromatin condenses and the nuclear envelope breaks down. At the end of prophase, the mature centrosome pair separates and migrates to the opposite poles of the nucleus to serve as two microtubule-organizing centres (MTOCs). Prometaphase follows where the microtubules nucleate from the MTOCs, forming the bipolar spindle. Subsequent to progression into metaphase, the kinetochores capture the plus ends of microtubules and this facilitates the chromosomal bi-orientation and alignment at the metaphase plate in the center of mitotic spindle. In the meantime, there is a continuous activation of mitotic checkpoint to monitor the microtubule attachment to kinetochores and tension. Upon progressing into anaphase, the chromatids start to segregate to the opposite spindle poles and this process is facilitated by the gliding of polar-oriented microtubules. ATPase driven motors such as dynein, kinesins and kinesin-related proteins and their dynamic temporal and spatial coordination play an essential role during the process. During the telophase, nuclear division occurs. Actin and myosin also redistribute to form an actin ring, called post-mitotic bridge in the midzone region between the poles. Contraction of the actin ring initiates the destruction of the post-mitotic bridge and

cytokinesis [1-2]. An overview of eukaryotic cell cycle, in particularly M phase is shown in Figure 1-1.

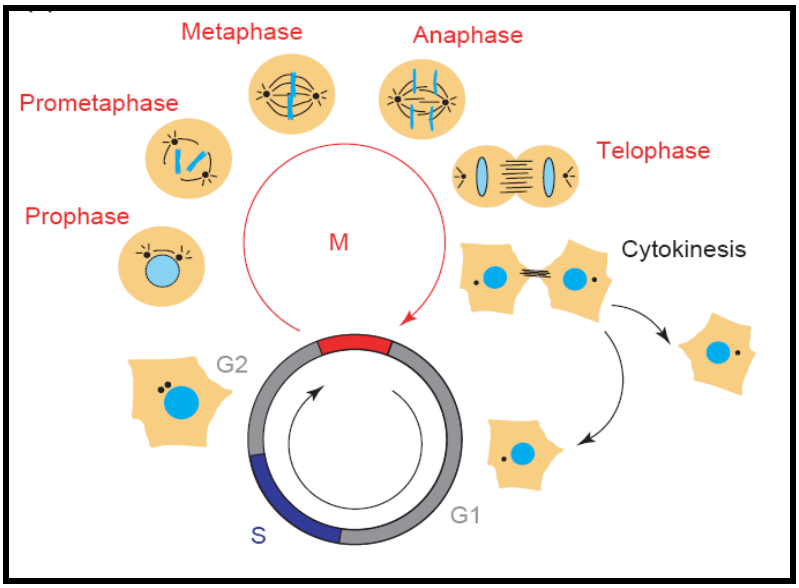


Figure 1-1: Overview of Eukaryotic Cell Cycle
(Figure adapted from ref [3])

1.1.2. Regulation by Mitotic Kinases

All these mitotic events are tightly governed by three regulatory mechanisms: protein localization, proteolysis and phosphorylation. Several protein kinases and their opposing phosphatases had been identified [2]. The best-studied kinases for the cell cycle progression are the cyclin-dependent kinases (CDKs) [4], which complex with cyclins and regulate various processes in mitosis, from DNA replication till mitotic entry and exit. Besides, the polo-like kinases (PLKs) [5] regulate the centrosome maturation, CDK1 activation and inactivation, and cytokinesis. In addition, the NimA-related kinases (NEKs) [6] regulate the centrosome cycle. Moreover, the kinetochore-localized Bub1 [7] kinase regulates the anaphase checkpoint signaling. Table 1-1 summarizes all the above kinases implicated in

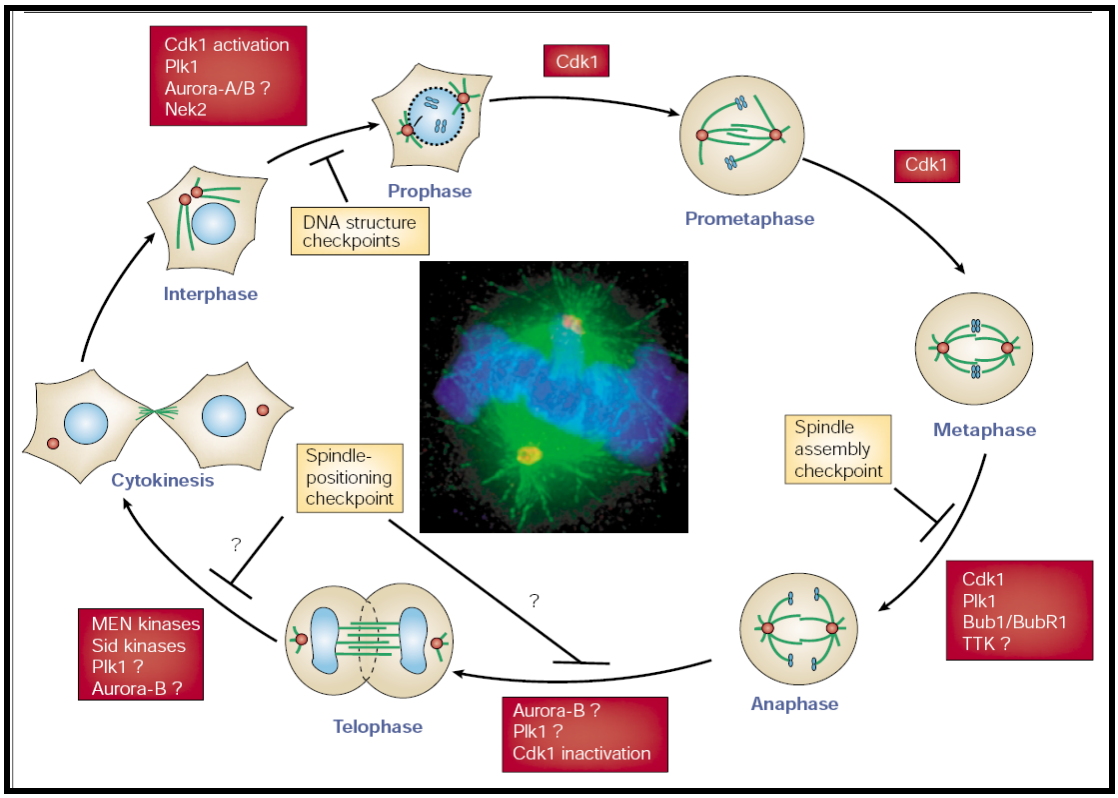
mitotic progression and checkpoints. Figure 1-2 displays where the major checkpoints exert quality control over mitotic progression and where mitotic kinases are thought to act.

Table 1-1: Mitotic Kinases (Table adapted from ref [1])

	Mammalian members	Founding members	Comments
The Cdk family	Cdk1 (Cdc2)	Cdc2p (Sp)/Cdc28p (Sc)	Mammalian Cdk1 functions in association with both A- and B-type cyclins
The Polo family	Plk1	Polo (Dm)	The vertebrate Polo families comprise additional members (see BOX 2)
The Aurora family	Aurora-A Aurora-B Aurora-C	Aurora (Dm)/Ipl1p (Sc)	The Aurora nomenclature is explained in TABLE 2
The NIMA family	Nek2	NIMA (An)	The vertebrate Nek families comprise additional members (see BOX 3). Whether NIMA and Nek2 represent bona fide functional homologues is not known at present
Mitotic checkpoint	Bub1 BubR1 TTK/Esk	Bub1p (Sc) Mps1p (Sc)	 TTK and Esk are the names given to putative human and mouse homologues, respectively, of budding yeast Mps1p
MEN/SIN kinases	?	Multiple yeast kinases (Sc and Sp) (see FIG. 5)	Several metazoan kinases (Ndr/LATS family members) are structurally related to a yeast SIN/MEN kinase (budding yeast Dbf2p/Mob1p and fission yeast Sid2p/Mob1p), but no functional homologies have yet been shown

*This Table is not meant to be exhaustive, and kinases with widely pleiotropic functions, such as MAP kinases and PKA (the cAMP-dependent kinase), are only mentioned in passing. This should not detract from the fact that both MAP kinases and PKA probably have important roles in the regulation of M phase, at least in some cell types. Furthermore, several of the 'mitotic' kinases listed here are highly expressed in the germ line, implying probable functions also in meiosis.
(Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; An, *Aspergillus nidulans*; Dm, *Drosophila melanogaster*.)

Figure 1-2: Cell Cycle and Kinase Signaling Cascades (Figure adapted from ref[1])



1.2. Aurora Kinases

1.2.1. Members of Aurora Kinase Family

Recently, a new family of conserved mitotic serine/threonine kinase, named as Aurora kinase [1, 8-9] had been identified and played the implicated roles in centrosome separation and maturation, spindle assembly and stability, chromosome condensation, congression and segregation and cytokinesis. Homologues of Aurora kinase had been isolated in various organisms, including yeast, *Caenorhabditis elegans*, Drosophila and vertebrates. Mammalian genome encodes for three members, namely Aurora-A (also known as Aurora-2, AIR-1, AIK1, AIRK1, AYK1, BTAK, Eg2, IAK1, STK15), Aurora-B (also known as Aurora-1, AIM-1, AIK2, AIR-2, AIRK-2, ARK2, IAL-1 and STK12) and Aurora-C (also known as AIK3), while for other metazoans, like *Xenopus laevis*, *Drosophila melanogaster* and *Caenorhabditis elegans*, only Aurora-A and Aurora–B kinases were found, whereas the yeast genomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* encoded only one Aurora-like homolog. Ipl1p from budding yeast *S. cerevisiae* and Aurora from *Drosophila melanogaster* are the founding members of Aurora kinase family. Ipl1p was identified through a genetic screen for mutations that led to increased chromosome missegregation [10]. Table 1-2 summarizes the nomenclature of the Aurora family kinases.

Table 1-2: Nomenclature of Aurora Family Kinases (Table adapted from ref[1])

*The aurora-A, -B, -C nomenclature has been approved by many scientists working in the aurora field. For the sake of clarity, the future use of this unifying nomenclature is recommended.

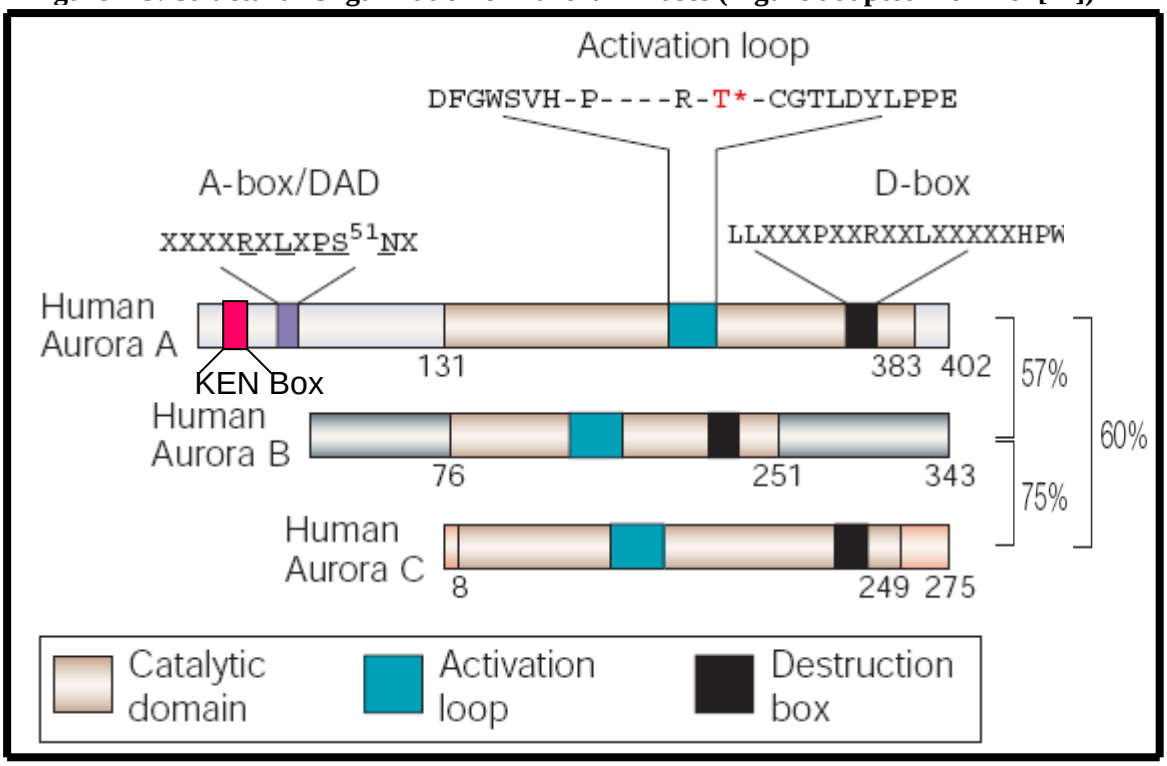
‡Ipl1p is the only aurora family member in budding yeast and cannot be attributed to the any particular subfamily.

Nomenclature guide*					
Mammals	**Other names**	***Xenopus***	***C. elegans***	***Drosophila***	***S. cerevisiae***
Aurora-A	Aurora-2, HsAIRK1, ARK1, Aik, BTAK, STK-15 (human); ARK1, Ayk1, IAK1 (mouse)	Eg2	AIR-1	aurora	Ipl1p‡
Aurora-B	Aurora-1, HsAIRK2, ARK2, Aik2, AIM-1, STK-12 (human); ARK2, STK-1 (mouse); AIM-1 (rat)	AIRK2	AIR-2	IAL	
Aurora-C	Aurora-3, HsAIRK3, AIE2, Aik3, STK-13 (human); AIE1 (mouse)				

1.2.2. Domain Organization of Aurora Kinases

The three Aurora kinases (309-403 a.a) share the similar domain organization, with their catalytic kinase domain flanked by very short C-terminal tail (15-20 a.a.) and N-terminal domain of variable length (39-129 a.a.). The N-terminal domain is highly variable in sequence and length between Aurora members and this confers selectivity and specificity for protein-protein interaction, whereas the catalytic kinase domain is highly conserved (67-76% identity), even across different organisms. The most conserved motif is the activation loop, which contains a highly conserved threonine residue (Thr288). Though all three Aurora kinases are similar in structure, they display different expression patterns, subcellular localizations and timing of activation [8, 11-12]. Figure 1-3 shows the domain organization of the Aurora kinases.

Figure 1-3: Structural Organization of Aurora Kinases (Figure adapted from ref [11])



1.2.3. Aurora Kinases Expression, Subcellular Localization and Functions in Mitosis

Aurora-A kinase is ubiquitously expressed, low in most tissues, yet high in tissues with high mitotic and meiotic index, such as thymus, fetal liver and testis. Aurora-A mRNA and protein expression levels as well as its kinase activity are cell cycle regulated, low in G1/S phase, peaking in G2/M and then dropping upon mitotic exit into the next G1. Aurora-A kinase displays dynamic subcellular localization, localized initially to the duplicated centrosomes at the end of S phase, translocating to mitotic spindle from prophase through telophase. Activation of centrosomal Aurora-A at late G2 phase is essential for centrosome maturation and mitotic entry. Its further activation and translocation are required for centrosome separation, leading to subsequent bipolar spindle formation and chromosomal alignment.

Upon completing cytokinesis, Aurora-A kinase has to be rapidly degraded and inactivated. In summary, Aurora-A kinase plays a critical mitotic role in centrosome separation and maturation, microtubule nucleation and bipolar spindle assembly [8-9, 11-16].

Aurora-B kinase is also highly expressed in tissues with a high mitotic index. Its mRNA and protein expression levels are also cell cycle regulated, peaking at G2/M phase and its kinase activity reaches the maximal from metaphase till end of mitosis. Aurora-B is identified as one of the components for the "chromosomal passenger protein" complex (Aurora-B-INCENP-survivin-borealin), which plays an important role in coordinating the chromosomal functions and cytoskeletal functions. Therefore Aurora-B kinase displays highly dynamic localization change in mitosis. Aurora-B associates along the chromosome arms during prophase, and is later concentrated at inner centromeres (kinetochore) in metaphase. At the anaphase onset, it translocates to the spindle midzone and cell cortex, the site for cleavage furrow formation. Aurora-B, thus, has multiple roles in mitosis, which include chromosome condensation, cohesion, bi-orientation, cytokinesis and spindle assembly checkpoint [8-9, 11-16].

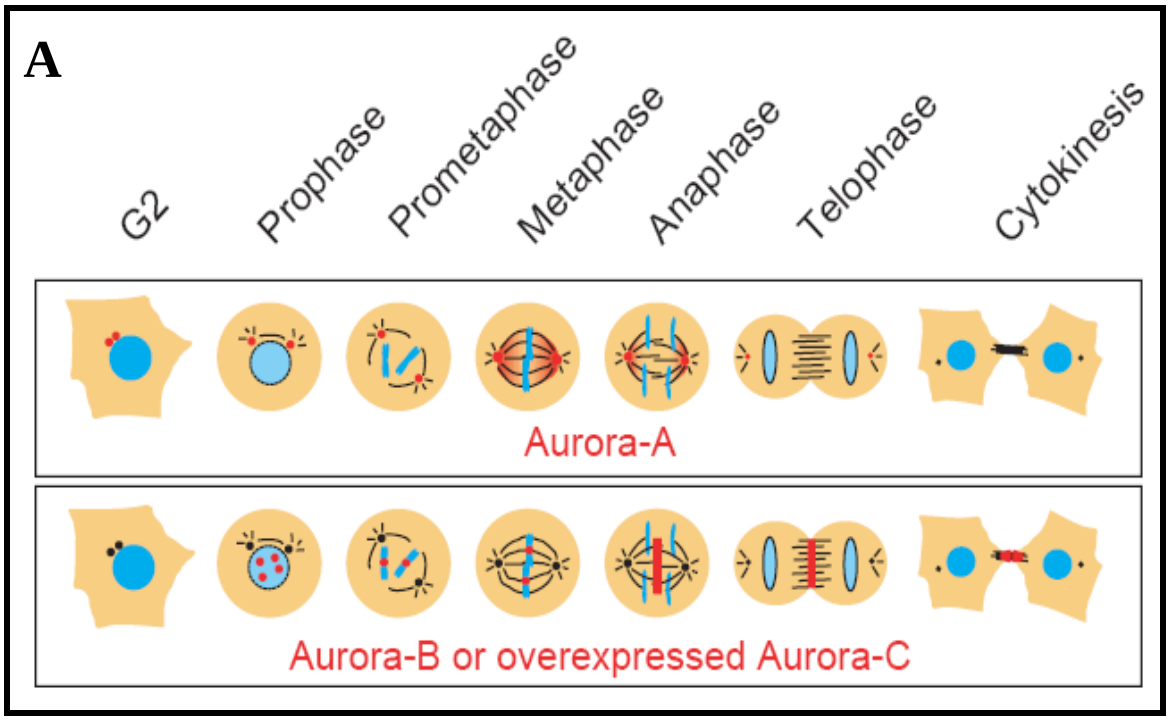
Aurora-C, though found prominently in testis, is also detected in other cell types and is overexpressed in certain cancer cell lines. Just like Aurora-A and –B, its mRNA and protein expression levels are cell cycle-dependent, peaking at G2/M. Like Aurora-B, Aurora-C is also a chromosomal passenger protein, localizing initially to the centromeres and then to the

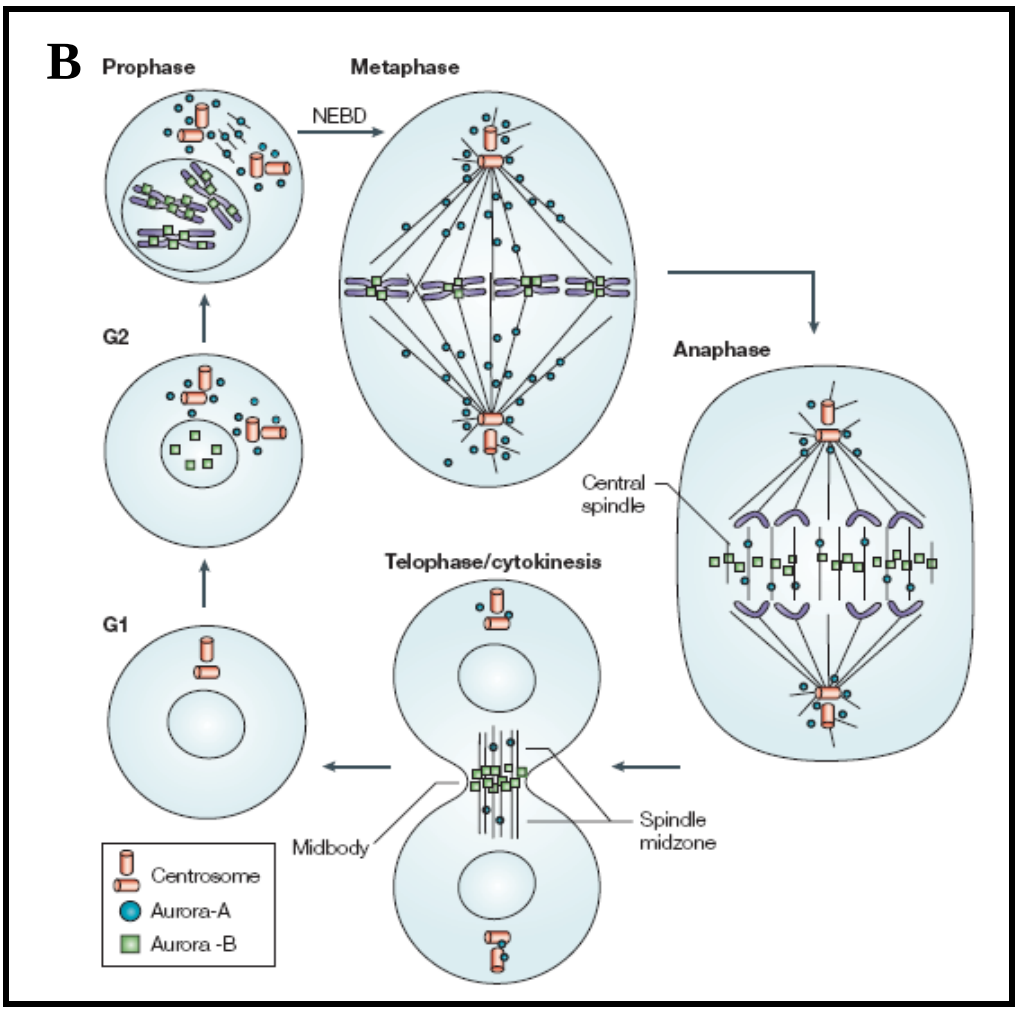
spindle midzone. Hence, the function of Aurora-C kinase overlaps with and complements the function of Aurora-B kinase in mitosis [8-9, 11-16].

Figure 1-4 (A-D) gives an overview of the subcellular distribution of the Aurora kinases and their functional roles throughout mitotic cell cycle.

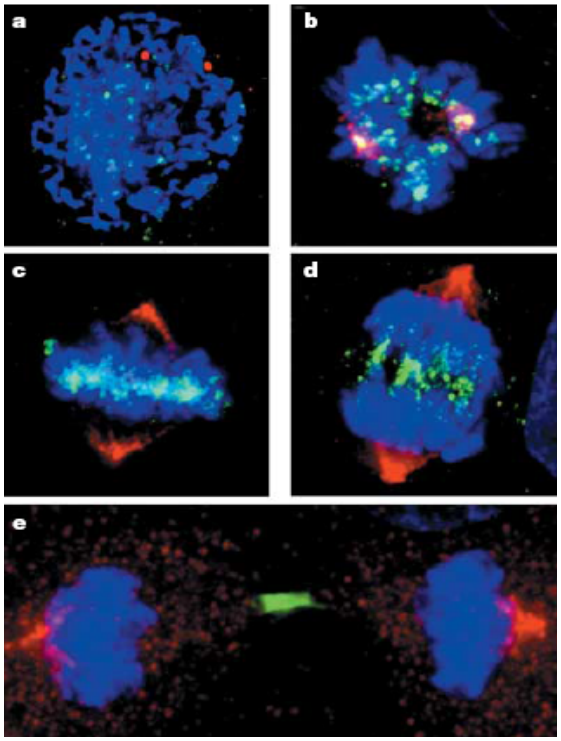
Figure 1-4: Localization of Aurora Kinases During Cell Cycle

(Figure above adapted from ref [3])



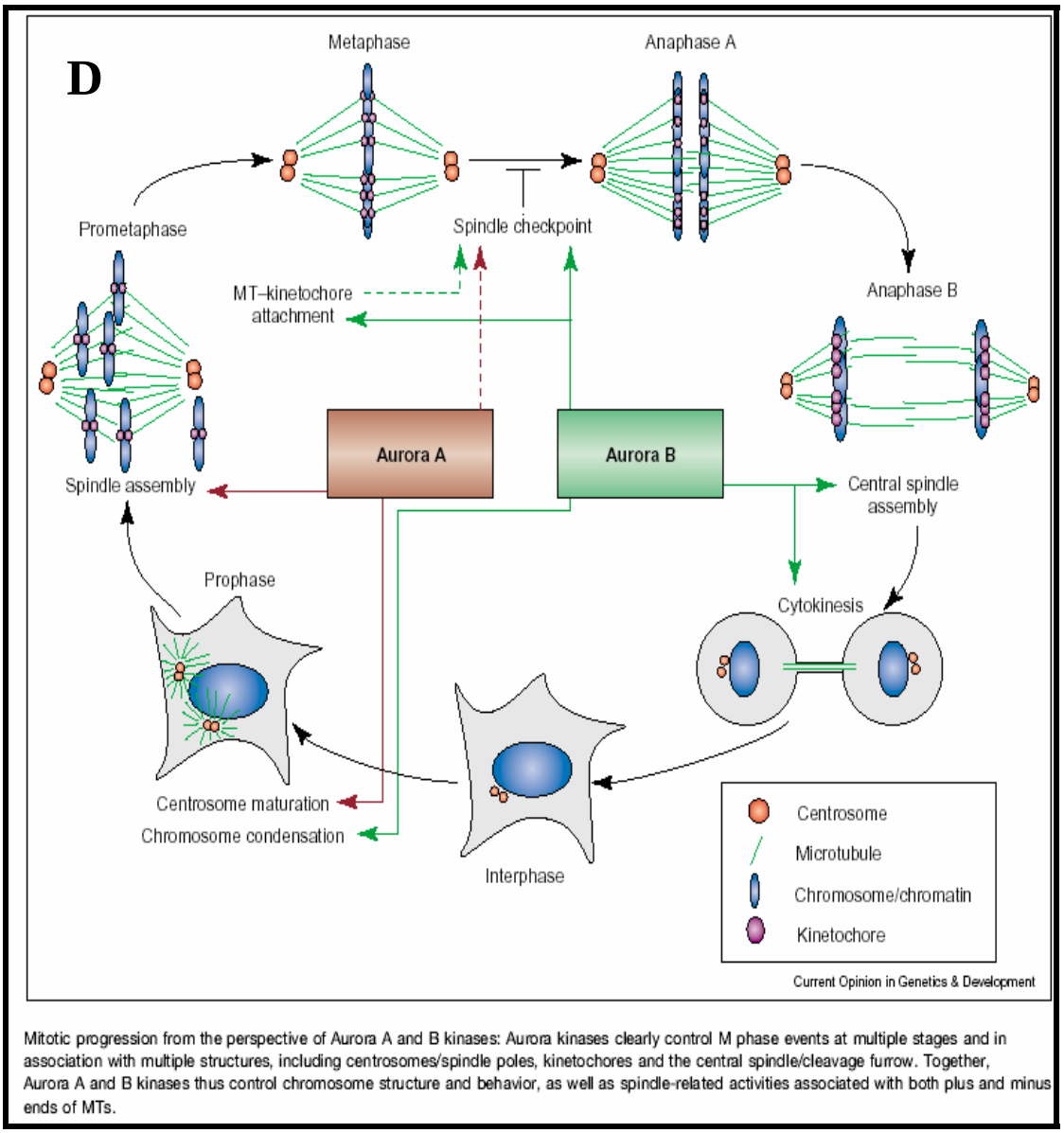


(Figure above adapted from ref [14])



C Subcellular localization of Aurora-A (red) and Aurora-B (green) relative to the chromosomes (blue) during (a) prophase, (b) prometaphase, (c) metaphase, (d) anaphase and (e) telophase. During prophase, Aurora-A localizes to the centrosomes, whereas in later stages of mitosis it is at the spindle poles (c, d and e) and also extends up the spindle. During prometaphase and metaphase (a and b), Aurora-B localizes to the centromeres. After anaphase (d), however, Aurora-B localizes to the spindle midzone, and finally accumulates at the midbody during telophase (e).

(Figure adapted from ref [64])



Mitotic progression from the perspective of Aurora A and B kinases: Aurora kinases clearly control M phase events at multiple stages and in association with multiple structures, including centrosomes/spindle poles, kinetochores and the central spindle/cleavage furrow. Together, Aurora A and B kinases thus control chromosome structure and behavior, as well as spindle-related activities associated with both plus and minus ends of MTs.

(Figure adapted from ref [65])

1.3. Role of Aurora-A Kinase in Tumorigenesis

1.3.1. Association with Multiple Cancers

Aurora-A kinase has been the most strongly implicated in tumorigenesis among all the three members of Aurora kinase family. Aurora-A kinase maps to chromosome 20q13.2-q13.3, a region frequently amplified in many types of cancer. Aurora-A kinase is amplified and/or overexpressed in primary breast (12%), colorectal (52%) and gastric tumours as well as breast, ovarian, colon, prostate, liver, bladder, cervical and gastric cancer cell lines [17-32] (See Table 1-3). High level of 20q13 amplification correlates with poor prognosis. However in some cases, Aurora-A overexpression does not correlate with the gene amplification. For example, only 3% of the hepatocellular carcinomas (HCCs) have Aurora-A amplification although more than 60% of HCCs overexpress Aurora-A mRNA and protein [31]. Other mechanisms like transcriptional activation and defective proteolysis, could lead to this discrepancy.

Table 1-3: Reported Aurora-A Kinase Abnormalities in Human Tumors
(Table adapted from ref [33])

Type of human tumor	Type of Specimen	Findings
Colorectal cancer	Primary	Overexpressed in >50%
Breast cancer	Primary	Amplified in 12% Overexpression in 94%
Gastric cancer	Primary	Amplified in 5% and overexpressed in >50%
Bladder cancer	Primary	Amplification is associated with aneuploidy and tumor aggressiveness
Pancreatic cancer	Primary	Overexpressed in 93% Overexpressed in 53%
Breast, ovarian, colon, prostate, neuroblastoma, cervical, and pancreatic cancers	Cell lines	Both overexpression and amplification noted

1.3.2. Phenotypes Associated with Overexpression of Aurora-A Kinase

Besides these correlative data, a number of studies had shown that overexpression of constitutively active Aurora-A kinase in rat1 and mouse NIH-3T3 fibroblasts led to *in vitro* transformation and tumour formation in nude mice [17-18], indicating the potential of Aurora-A kinase as an oncogene. Also, its ectopic expression in near-diploid human breast epithelial cells caused centrosome amplification [18] with induction of aneuploidy. It had been shown that overexpression of Aurora-A and centrosome amplification were the early events in tumorigenesis in a rat mammary carcinogenesis [34]. A clinical study also showed that Aurora-A overexpression and activation was an early pathology event in human ovarian tumorigenesis [35]. Thus, Aurora-A overexpression might accelerate or potentiate the multistep tumorigenesis.

Interestingly, kinase activity of Aurora-A is not essential for the induction of aneuploidy and centrosome amplification associated with Aurora-A overexpression, however the oncogenic transformation requires the active kinase [36].

1.3.3. Mechanisms of Aurora-A-induced Tumorigenesis

1.3.3.1 Abrogation of post-mitotic G1 checkpoint

Recent studies have shown that Aurora-A overexpression does not directly trigger centrosome amplification, rather it causes abnormal mitotic spindle formation and cytokinesis failure, leading to tetraplodization [37]. Normal non-transformed cells have the functional p53-RB-dependent checkpoint, known as “post-mitotic G1 checkpoint”, which detects tetraploidy and induces G1 arrest. When Aurora-A kinase is overexpressed in cells that lack p53 and therefore is defective in G1 checkpoint, the newly generated tetraploid cells still progress through the mitosis and thus acquire multiple centrosomes and genomic instability. This is summarized in Figure 1-5.

Interestingly, the Aurora-A overexpression-induced tetraploidization or centrosome amplification does not require its kinase activity but the cellular transformation is still dependent on the kinase activity [17-18, 37].

Figure 1-5: (Figure adapted from ref [14])

Diagram Depicting the Predicted Tumorigenesis by Aurora-A Overexpression

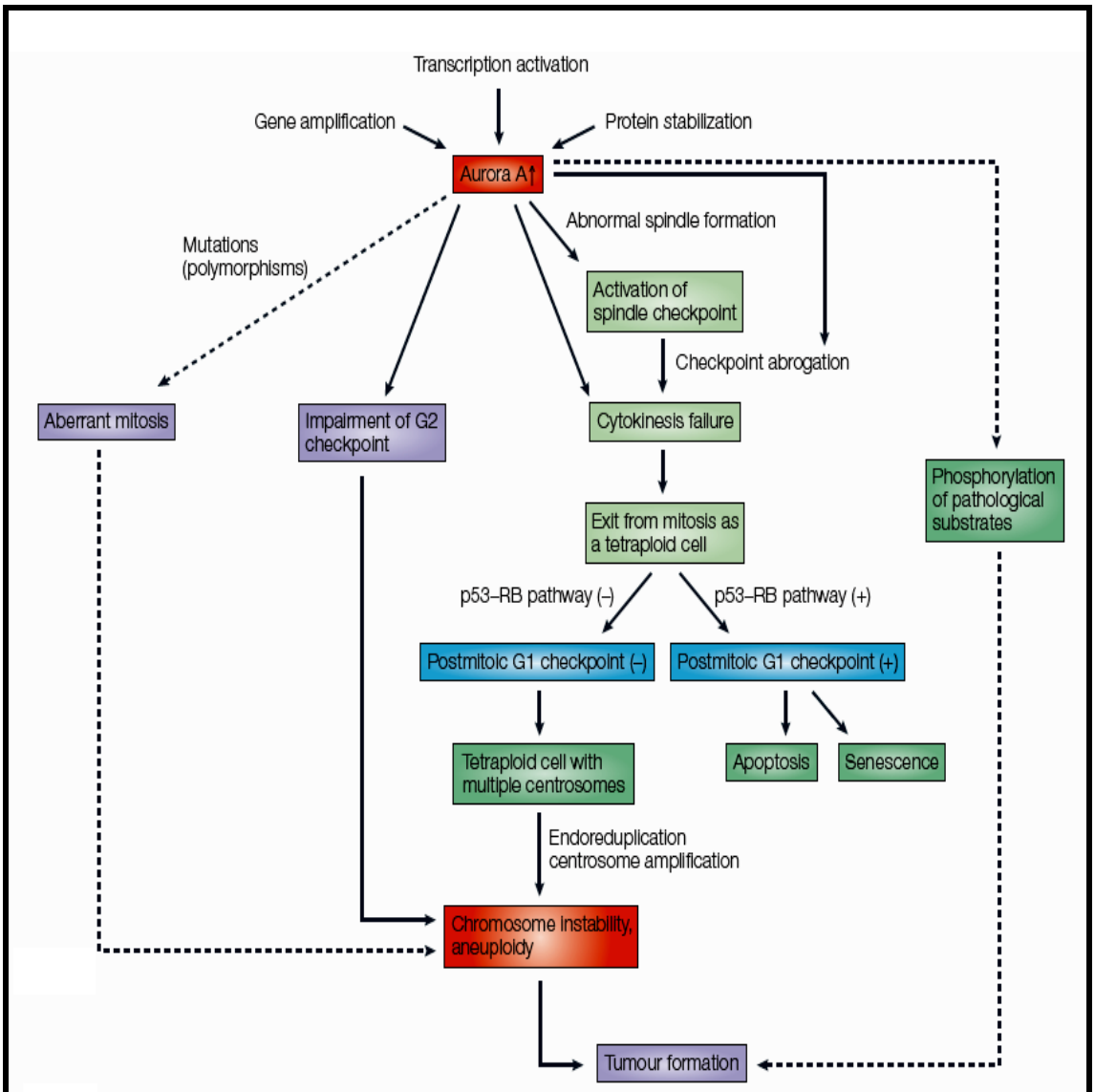


Diagram depicting the predicted tumorigenesis by Aurora-A overexpression. Increases in Aurora-A levels, through gene amplification, transcriptional upregulation or protein stabilization, induces abnormal spindle formation and cytokinesis failure. In normal cells, spindle defects would activate the spindle checkpoint and the cells would undergo arrest at metaphase, but Aurora-A overexpressing cells bypass this checkpoint and instead undergo cytokinesis failure, resulting in formation of tetraploid cells. In cells with intact p53–RB signalling, tetraploidy activates the postmitotic G1 checkpoint, leading to apoptosis or senescence. In cells with impairments in the p53–RB pathway, tetraploid cells tend to have centrosome amplification, which induces chromosome instability. Furthermore, the DNA-damage-induced G2 checkpoint is impaired in Aurora-A overexpressing cells, which also contributes to genomic instability. Overexpression of mutated forms of Aurora-A and the phosphorylation of inappropriate substrates are speculative pathways (dashed lines) by which Aurora-A might also induce tumorigenesis.

1.3.3.2. p53 Inactivation

Few studies had clearly demonstrated the functional link between the Aurora-A and p53. p53 can interact with Aurora-A and inhibit its kinase/oncogenic function in a transactivation-independent manner [38]. On the other hand, Aurora-A kinase directly phosphorylates p53 at Ser315, facilitating the MDM2-degradation of p53 [39]. Moreover, phosphorylation of p53 at the alternative site, Ser215 by Aurora-A, leads to the inhibition of its transcriptional activity [40]. Therefore, deregulation of this mutual suppression mechanism between Aurora-A and p53 can thus induce checkpoint disruption and chromosome instability.

An Aurora-A transgenic mouse model, in which Aurora-A was conditionally overexpressed in mammary epithelial cells, was generated [41] and had provided further insight into the relationship between Aurora-A and p53. In these cells, cytokinesis failed, leading to significant increase of binucleated cells, which the activated the post-mitotic G1 checkpoint and were arrested in G1 and subsequently underwent apoptosis. Interestingly, the level of the p53 protein, a regulator of this post-mitotic G1 checkpoint, was also increased and malignant tumour formation was not observed after long latency. However, apoptosis was inhibited by deletion of p53, suggesting that tumorigenesis might require additional factors, like p53 inactivation and expression of anti-apoptotic proteins.

In vivo evidence from a recent clinical observation [31] demonstrated that Aurora-A

overexpression and p53 inactivation played a cooperative role in tumorigenesis. Not only Aurora-A overexpression correlates with p53 mutation in hepatocellular carcinoma, tumours which harbour both Aurora-A overexpression and p53 mutation, also have worse prognosis than those with p53 mutation alone.

1.3.3.2 Overriding Spindle Assembly Checkpoint

Besides the functional link between Aurora-A and p53-dependent G1 checkpoint, Aurora-A overexpression had been found to override the BUB1-dependent spindle assembly checkpoint, activated either by taxol (Paclitaxel) [36, 42] or nocodazole (microtubule depolymerizing drug) [43]. Eventually, cells inappropriately entered into anaphase in the presence of defective spindle formation, leading to polyploidization.

Normally upon taxol treatment, cells arrest at metaphase and eventually undergo apoptosis, however, cells that overexpress Aurora-A, have acquired increased resistance to taxol-induced apoptosis [36]. Study with nocodazole had demonstrated that Aurora-A overexpression caused checkpoint override in the presence of nocodazole by disrupting the binding of BubR1 to Cdc20, leading to chromosomal instability (CIN) phenotype [43].

On the other hand, signals generated from the DNA damage had been found to inhibit Aurora-A kinase activity and induce G2 arrest. Aurora-A overexpression disrupted the DNA damage induced G2 checkpoint, leading to premature mitotic entry in the presence of DNA damage. Therefore, checkpoint disruption through Aurora-A overexpression probably leads to

cell transformation [44-46].

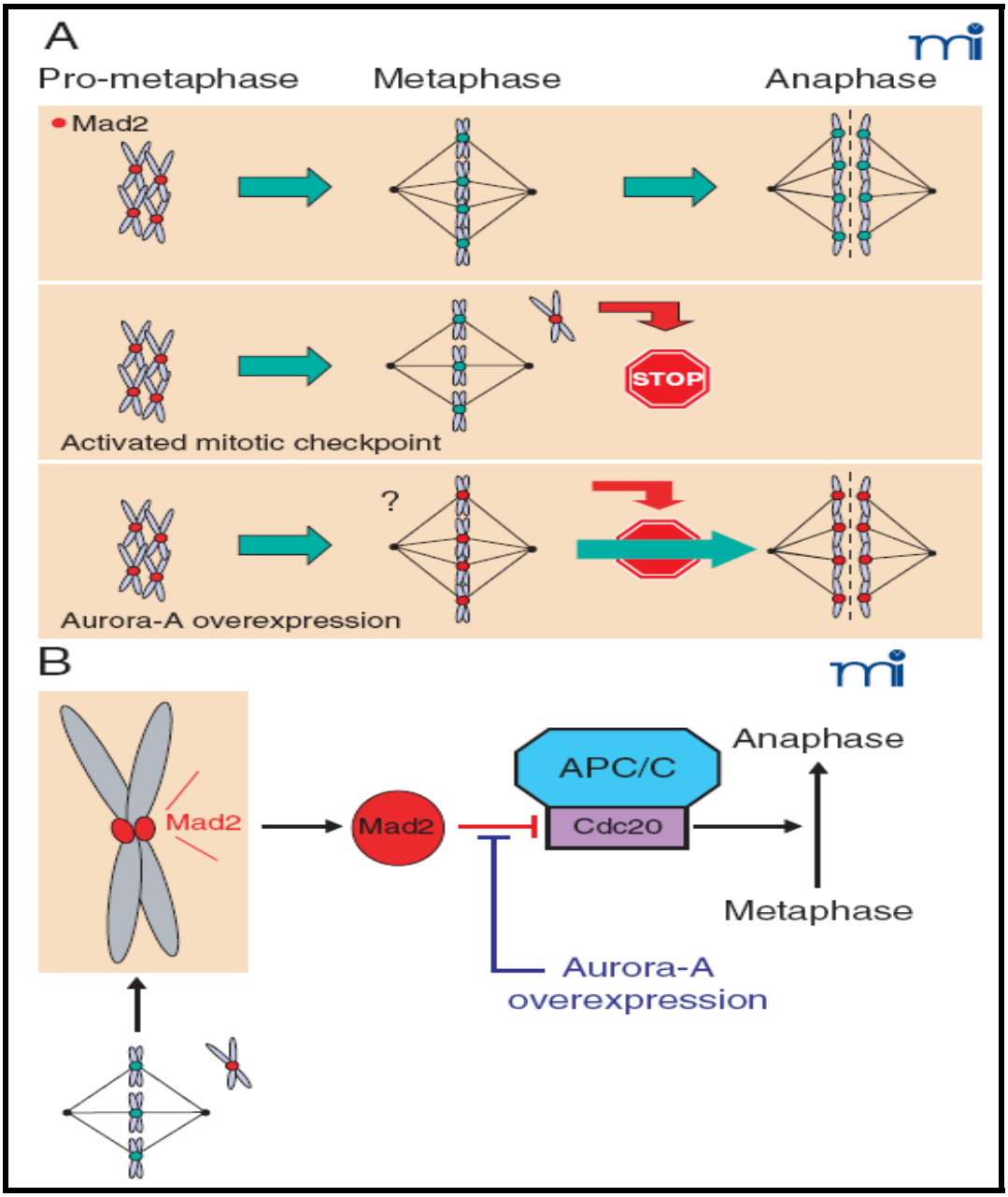
Aurora-A overexpression abrogates the activated mitotic checkpoint signaled by Mad2 [42], as shown in Figure 1-6. Mad2 inhibits Cdc20, an activator of Anaphase Promoting Complex/Cyclosome (APC/C) complex, which targets protein for degradation and thus triggers the metaphase-anaphase transition. Retainment of Mad2 on the kinetochore signals to the cells the presence of unattached chromosome and blocks the metaphase-anaphase transition. Only when the kinetochore captures the microtubule, the Mad2 will dissociate from the kinetochore, thus relieving its inhibition on Cdc20, which can then activate the APC/C. However, when Aurora-A is overexpressed, it acts downstream of Mad2 and upstream of Cdc20, thereby interfering the Mad2-Cdc20 interaction and overriding the checkpoint. The Mad2-Cdc20 interaction may be interfered by binding of Aurora-A kinase to Cdc20, as their *in vivo* interaction had been previously shown [47].

Aurora-B kinase, however, is also implicated in the spindle assembly checkpoint [48]. Inhibition of Aurora-B function impairs the retainment of the checkpoint proteins at the kinetochore and thus overriding of the taxol-sensitive spindle checkpoint [49-51]. One study had shown that phosphorylation of CENP-A by Aurora-A was necessary for the recruitment of Aurora-B to the inner centromere in prometaphase [52]. Since Aurora-A plays a role in recruitment of Aurora-B, the effect of Aurora-A on spindle assembly checkpoint may be indirect.

Figure 1-6: (Figure adapted from ref [36])

Figure 1-6

Mad2 binding to kinetochores during metaphase-anaphase transition in HeLa cells. A. (Top) In prophase every kinetochore is decorated with Mad2. Once a kinetochore is captured by microtubules, Mad2 labelling disappears. Metaphase-to-anaphase transition will be triggered only when all kinetochores are captured. However, any kinetochore remaining decorated with Mad2 (i.e., unattached chromosome) will activate the mitotic checkpoint leading to cell cycle arrest (Middle). In the presence of overexpressed aurora-A, the cell ignores the Mad2 signal and triggers metaphase exit (Bottom). **B.** Mad2 is an inhibitor of Cdc20, a known APC/C activator. Overexpression of aurora-A interferes with the Mad2–Cdc20 signal that promotes progression to anaphase *(14)*.



1.3.3.3 Aurora-A Kinase as Tumour Susceptibility Gene

Moreover, Aurora-A was discovered to be a low-penetrance tumour susceptibility gene or tumour modifier gene for multiple cancer cell types [53-61]. Two polymorphisms of Aurora-A (Phe-31-IIe and Val-57-IIe) are involved in the human tumour susceptibility. The allelic variant, IIe31, is frequently amplified in human colon [53], breast [58], esophageal [57] and ovarian tumours [59]. The co-existence of two polymorphisms (IIe31 and Val57) correlates well with the increased risk of breast cancer [55-56].

IIe31 allele was shown to transform more potently than the common Phe31 allele. Study had identified Aurora-A kinase as the direct substrate for ubiquitination by UBE2N, an E2 ubiquitin-conjugating enzyme, and interestingly, the “strong” IIe31 variant bound the UBE2N far less efficiently than the “weak” Phe31 variant and thus had compromised ubiquitination, which may probably lead to subsequent impaired degradation and inactivation, with induction of cellular transformation [53].

1.3.3.4 Enhanced Cell Migration

Another significant study had provided mechanistic insights into the distinct role of Aurora-A kinase in cellular transformation by promoting the cell migration. Aurora-A phosphorylates one of its downstream substrates, RalA on Ser194, and the Ser194 phosphorylation leads to RalA activation, therefore enhancing the transforming activity of Ras and Raf by promoting the cell motility and anchorage-independent growth [62].

1.3.3.5 Transforming target-HURP

Other transforming target of Aurora-A kinase, such as HURP (Hepatoma Upregulated Protein), were also isolated. HURP is a mitotic protein [63]. Phosphorylation of HURP by Aurora-A leads to its protein stabilization. Eventually, the accumulated intracellular HURP promotes the serum- and anchorage-independent growth.

1.4. References

1. Nigg E.A. **Mitotic kinases as regulators of cell division and its checkpoints.** Nat. Rev. Mo.l Cell. Biol. (2001) 2(1): 21-32.

2. Vernos I., Karsenti E. **Chromosomes take the lead in spindle assembly.** Trends Cell Biol. (1995) 5(8): 297-301.

3. Giet R., Petretti C., Prigent C. **Aurora kinases, aneuploidy and cancer, a coincidence or a real link?** Trends Cell Biol. (2005) 15(5): 241-50.

4. Morgan D.O. **Principles of CDK regulation.** Nature. (1995) 374(6518): 131-4.

5. Nigg E.A. **Polo-like kinases: positive regulators of cell division from start to finish.** Curr Opin Cell Biol. (1998) 10(6): 776-83.

6. Mayor T, Meraldi P, Stierhof YD, Nigg EA, Fry AM. **Protein kinases in control of the centrosome cycle.** FEBS Lett. (1999) 452(1-2): 92-5.

7. Straight A.F. **Cell cycle: checkpoint proteins and kinetochores.** Curr Biol. (1997) 7(10): R613-6.

8. Giet R, Prigent C. **Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases.** J Cell Sci. (1999) 112 (Pt 21): 3591-601.

9. Bischoff JR, Plowman GD. **The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis.** Trends Cell Biol. (1999) 9(11): 454-9.

10. Chan CS, Botstein D. **Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast.** Genetics. (1993) 135(3): 677-91.

11. Carmena M., Earnshaw W.C. **The cellular geography of aurora kinases.** Nat Rev Mol Cell Biol. (2003) 4(11): 842-54.

12. Katayama H., Brinkley WR, Sen S**. The Aurora kinases: role in cell transformation and tumorigenesis.** Cancer Metastasis Rev. (2003) 22(4): 451-64.

Ke YW, Dou Z, Zhang J, Yao XB**. Function and regulation of Aurora/Ipl1p kinase family in cell division.** Cell Res. (2003) 13(2): 69-81.

13. Marumoto T, Zhang D, Saya H. **Aurora-A - a guardian of poles**. Nat Rev Cancer. (2005) 5(1): 42-50.

14. Andrews PD, Knatko E, Moore WJ, Swedlow JR. **Mitotic mechanics: the auroras come into view.** Curr Opin Cell Biol. (2003) 15(6): 672-83.

15. Ducat D, Zheng Y. **Aurora kinases in spindle assembly and chromosome segregation.** Exp Cell Res. (2004) 301(1): 60-7.

16. Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B, Schryver B, Flanagan P, Clairvoyant F, Ginther C, Chan CS, Novotny M, Slamon DJ, Plowman GD. **A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers.** EMBO J. (1998) 17(11):3052-65.

17. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR, Sen S. **Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation.** Nat Genet. (1998) 20(2):189-93.

18. Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, Borg A, Isola JJ. **Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer**. Clin Cancer Res. (2000) 6(5):1833-9.

19. Watanabe T, Imoto I, Katahira T, Hirasawa A, Ishiwata I, Emi M, Takayama M, Sato A, Inazawa J. **Differentially regulated genes as putative targets of amplifications at 20q in ovarian cancers.** Jpn J Cancer Res. (2002) 93(10):1114-22.

20. Rojanala S, Han H, Munoz RM, Browne W, Nagle R, Von Hoff DD, Bearss DJ. **The mitotic serine threonine kinase, Aurora-2, is a potential target for drug development in human pancreatic cancer.** Mol Cancer Ther. (2004) 3(4):451-7.

21. Tanaka T, Kimura M, Matsunaga K, Fukada D, Mori H, Okano Y. **Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast.** Cancer Res. (1999) 59(9):2041-4.

22. Takahashi T, Futamura M, Yoshimi N, Sano J, Katada M, Takagi Y, Kimura M, Yoshioka T, Okano Y, Saji S. **Centrosomal kinases, HsAIRK1 and HsAIRK3, are overexpressed in primary colorectal cancers.** Jpn J Cancer Res. (2000) 91(10):1007-14.

23. Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, Fiorica JV, Nicosia SV, Cheng JQ. **Activation and overexpression of centrosome kinase BTAK/Aurora-A in human ovarian cancer.** Clin Cancer Res. (2003) 9(4):1420-6.

24. Li D, Zhu J, Firozi PF, Abbruzzese JL, Evans DB, Cleary K, Friess H, Sen S. **Overexpression of oncogenic STK15/BTAK/Aurora A kinase in human pancreatic cancer.** Clin Cancer Res. (2003) 9(3):991-7.

25. Katayama H, Ota T, Jisaki F, Ueda Y, Tanaka T, Odashima S, Suzuki F, Terada Y, Tatsuka M. **Mitotic kinase expression and colorectal cancer progression.** J Natl Cancer Inst. (1999) 91(13):1160-2.

26. Tatsuka M, Katayama H, Ota T, Tanaka T, Odashima S, Suzuki F, Terada Y. **Multinuclearity and increased ploidy caused by overexpression of the aurora- and Ipl1-like midbody-associated protein mitotic kinase in human cancer cells.** Cancer Res. (1998) 58(21):4811-6.

27. Kamada K, Yamada Y, Hirao T, Fujimoto H, Takahama Y, Ueno M, Takayama T, Naito A, Hirao S, Nakajima Y. **Amplification/overexpression of Aurora-A in human gastric carcinoma: potential role in differentiated type gastric carcinogenesis.** Oncol Rep. (2004) 12(3):593-9.

28. Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, Masuda K, Shimomura K, Nakamura Y, Inazawa J, Abe T, Yamagishi H. **Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation.** Br J Cancer. (2001) 84(6):824-31.

29. Buschhorn HM, Klein RR, Chambers SM, Hardy MC, Green S, Bearss D, Nagle RB. **Aurora-A over-expression in high-grade PIN lesions and prostate cancer.** Prostate. (2005) 64(4):341-6.

30. **Overexpression and amplification of Aurora-A in hepatocellular carcinoma**. Clin Cancer Res. (2004) 10(6):2065-71.

31. Fraizer GC, Diaz MF, Lee IL, Grossman HB, Sen S. **Aurora-A/STK15/BTAK enhances chromosomal instability in bladder cancer cells.** Int J Oncol. (2004) 25(6):1631-9.

32. Warner SL, Bearss DJ, Han H, Von Hoff DD. **Targeting Aurora-2 kinase in cancer.** Mol Cancer Ther. (2003) 2(6):589-95.

33. Goepfert TM, Adigun YE, Zhong L, Gay J, Medina D, Brinkley WR. **Centrosome amplification and overexpression of aurora A are early events in rat mammary carcinogenesis.** Cancer Res. (2002) 62(14):4115-22.

34. Hu W, Kavanagh JJ, Deaver M, Johnston DA, Freedman RS, Verschraegen CF, Sen S. **Frequent overexpression of STK15/Aurora-A/BTAK and chromosomal instability in tumorigenic cell cultures derived from human ovarian cancer.** Oncol Res. (2005) 15(1):49-57.

35. Dutertre S, Prigent C. **Aurora-A overexpression leads to override of the microtubule-kinetochore attachment checkpoint.** Mol Interv. (2003) 3(3):127-30.

36. Meraldi P, Honda R, Nigg EA. **Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells.** EMBO J. (2002) 21(4):483-92.

37. Chen SS, Chang PC, Cheng YW, Tang FM, Lin YS**. Suppression of the STK15 oncogenic activity requires a transactivation-independent p53 function.** EMBO J. (2002) 21(17):4491-9.

38. Katayama H, Sasai K, Kawai H, Yuan ZM, Bondaruk J, Suzuki F, Fujii S, Arlinghaus RB, Czerniak BA, Sen S. **Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53.** Nat Genet. (2004) 36(1):55-62.

39. Liu Q, Kaneko S, Yang L, Feldman RI, Nicosia SV, Chen J, Cheng JQ. **Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215.** J Biol Chem. (2004) 279(50):52175-82.

40. Zhang D, Hirota T, Marumoto T, Shimizu M, Kunitoku N, Sasayama T, Arima Y, Feng L, Suzuki M, Takeya M, Saya H. **Cre-loxP-controlled periodic Aurora-A**

overexpression induces mitotic abnormalities and hyperplasia in mammary glands of mouse models. Oncogene. (2004) 23(54):8720-30.

41. Anand S, Penrhyn-Lowe S, Venkitaraman AR. **AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol.** Cancer Cell. (2003) 3(1):51-62.

42. Jiang Y, Zhang Y, Lees E, Seghezzi W. **Aurora-A overexpression overrides the mitotic spindle checkpoint triggered by nocodazole, a microtubule destabilizer.** Oncogene. (2003) 22(51):8293-301.

43. Marumoto T, Hirota T, Morisaki T, Kunitoku N, Zhang D, Ichikawa Y, Sasayama T, Kuninaka S, Mimori T, Tamaki N, Kimura M, Okano Y, Saya H. **Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells.** Genes Cells. (2002) 7(11):1173-82.

44. Krystyniak A, Garcia-Echeverria C, Prigent C, Ferrari S. **Inhibition of Aurora-A in response to DNA damage.** Oncogene. (2006) 25(3):338-48.

45. Cazales M, Schmitt E, Montembault E, Dozier C, Prigent C, Ducommun B. **CDC25B phosphorylation by Aurora-A occurs at the G2/M transition and is inhibited by DNA damage.** Cell Cycle. (2005) 4(9):1233-8.

46. Farruggio DC, Townsley FM, Ruderman JV. **Cdc20 associates with the kinase aurora2/Aik.** Proc Natl Acad Sci U S A. (1999) 96(13):7306-11

47. Morrow CJ, Tighe A, Johnson VL, Scott MI, Ditchfield C, Taylor SS. **Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20.** J Cell Sci. (2005) 118(Pt 16):3639-52.

48. Kallio MJ, McCleland ML, Stukenberg PT, Gorbsky GJ. **Inhibition of aurora B kinase blocks chromosome segregation, overrides the spindle checkpoint, and perturbs microtubule dynamics in mitosis**. Curr Biol. (2002) 12(11):900-5.

49. Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T, Mortlock A, Keen N, Taylor SS. **Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores.** J Cell Biol. (2003) 161(2):267-80.

50. Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, Heckel A, van Meel J, Rieder CL, Peters JM. **The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint.** J Cell Biol. (2003) 161(2):281-94.

51. Kunitoku N, Sasayama T, Marumoto T, Zhang D, Honda S, Kobayashi O, Hatakeyama K, Ushio Y, Saya H, Hirota T. **CENP-A phosphorylation by Aurora-A in prophase is required for enrichment of Aurora-B at inner centromeres and for kinetochore function.** Dev Cell. (2003) 5(6):853-64.

52. Ewart-Toland A, Briassouli P, de Koning JP, Mao JH, Yuan J, Chan F, MacCarthy-Morrogh L, Ponder BA, Nagase H, Burn J, Ball S, Almeida M, Linardopoulos S, Balmain A. **Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human.** Nat Genet. (2003) 34(4):403-12.

53. Kimura MT, Mori T, Conroy J, Nowak NJ, Satomi S, Tamai K, Nagase H. **Two functional coding single nucleotide polymorphisms in STK15 (Aurora-A) coordinately increase esophageal cancer risk.** Cancer Res. (2005) 65(9):3548-54.

54. Ewart-Toland A, Dai Q, Gao YT, Nagase H, Dunlop MG, Farrington SM, Barnetson RA, Anton-Culver H, Peel D, Ziogas A, Lin D, Miao X, Sun T, Ostrander EA, Stanford JL, Langlois M, Chan JM, Yuan J, Harris CC, Bowman ED, Clayman GL, Lippman SM, Lee JJ, Zheng W, Balmain A. **Aurora-A/STK15 T+91A is a general low penetrance cancer susceptibility gene: a meta-analysis of multiple cancer types.** Carcinogenesis. (2005) 26(8):1368-73.

55. Dai Q, Cai QY, Shu XO, Ewart-Toland A, Wen WQ, Balmain A, Gao YT, Zheng W. **Synergistic effects of STK15 gene polymorphisms and endogenous estrogen exposure in the risk of breast cancer.** Cancer Epidemiol Biomarkers Prev. (2004) 13(12):2065-70.

56. Miao X, Sun T, Wang Y, Zhang X, Tan W, Lin D. **Functional STK15 Phe31Ile polymorphism is associated with the occurrence and advanced disease status of esophageal squamous cell carcinoma.** Cancer Res. (2004) 64(8):2680-3.

57. Sun T, Miao X, Wang J, Tan W, Zhou Y, Yu C, Lin D. **Functional Phe31Ile polymorphism in Aurora A and risk of breast carcinoma.** Carcinogenesis. (2004) 25(11):2225-30.

58. Dicioccio RA, Song H, Waterfall C, Kimura MT, Nagase H, McGuire V, Hogdall E, Shah MN, Luben RN, Easton DF, Jacobs IJ, Ponder BA, Whittemore AS, Gayther SA, Pharoah PD, Kruger-Kjaer S. **STK15 polymorphisms and association with risk of invasive ovarian cancer.** Cancer Epidemiol Biomarkers Prev. (2004) 13(10):1589-94.

59. Egan KM, Newcomb PA, Ambrosone CB, Trentham-Dietz A, Titus-Ernstoff L, Hampton JM, Kimura MT, Nagase H. **STK15 polymorphism and breast cancer risk in a population-based study.** Carcinogenesis. (2004) 25(11):2149-53.

60. Cox DG, Hankinson SE, Hunter DJ. **Polymorphisms of the AURKA (STK15/Aurora Kinase) Gene and Breast Cancer Risk (United States).** Cancer Causes Control. (2006) 17(1):81-3.

61. Wu JC, Chen TY, Yu CT, Tsai SJ, Hsu JM, Tang MJ, Chou CK, Lin WJ, Yuan CJ, Huang CY. **Identification of V23RalA-Ser194 as a critical mediator for Aurora-A-induced cellular motility and transformation by small pool expression screening.** J Biol Chem. (2005) 280(10):9013-22

62. Yu CT, Hsu JM, Lee YC, Tsou AP, Chou CK, Huang CY. **Phosphorylation and stabilization of HURP by Aurora-A: implication of HURP as a transforming target of Aurora-A.** Mol Cell Biol. (2005) 25(14):5789-800.

63. Keen N, Taylor S. **Aurora-kinase inhibitors as anticancer agents.** Nat Rev Cancer. (2004) 4(12):927-36.

64. Meraldi P, Honda R, Nigg EA. **Aurora kinases link chromosome segregation and cell division to cancer susceptibility.** Curr Opin Genet Dev. (2004) 14(1):29-36.

(Introduction and Literature Review)

Chapter 2

Negative Regulation of Aurora-A Kinase

2.1. Negative Regulation of Aurora-A Kinase

Given the strong implication of Aurora-A in tumorigenesis, Aurora-A kinase serves as a potential therapeutic target of great interest. Understanding its normal regulation would be very essential to identify and develop inhibitors for Aurora-A kinase. Aurora-A kinase can be regulated at two different levels: its kinase activity and protein stability [1].

2.1.1. Regulation at the Kinase Activity Level

2.1.1.1. PP1 Inhibition of TPX-2-induced Aurora-A Kinase Activation

As we know, Aurora-A kinase activity is cell cycle-regulated and peaks in mitosis. Regulation of human Aurora-A kinase activity involves phosphorylation and dephosphorylation at three major potential phosphorylation sites, Thr288, Ser51 and Ser342 [2]. Phosphorylation of Thr288 in the activation loop of human Aurora-A activates the kinase activity. Thr288 is in a protein kinase A (PKA) consensus motif and also an autophosphorylation site [3]. PKA can phosphorylate and activate Aurora-A *in vitro* [1].

On the other hand, Ser51 in the N-terminal A box of human Aurora-A is also phosphorylated during M phase and this negatively regulates Aurora-A degradation till the end of mitosis [4]. However, there is no effect of Ser51 phosphorylation on Aurora-A kinase activity. Finally, the role of Ser342 phosphorylation on human Aurora-A kinase activity is still unclear although crystal structure of Aurora-A kinase [14] has suggested that phosphorylation at this site could affect the conformation of Aurora-A, therefore playing a regulatory or

structural role in Aurora-A activation. It is also found that Ser342 is not essential for Aurora-A kinase activity but Ser342 phosphorylation (*Xenopus* S349D mutant) blocks kinase activation [2]. Interestingly, Ser342 is located immediately adjacent to Aurora-A binding motif of type I phosphatase PP1, a negative regulator of Aurora-A activity [5-7]. Probably, *in vivo* phosphorylation of Ser342 enhances PP1 binding to Aurora-A, preventing phosphorylation of Thr288.

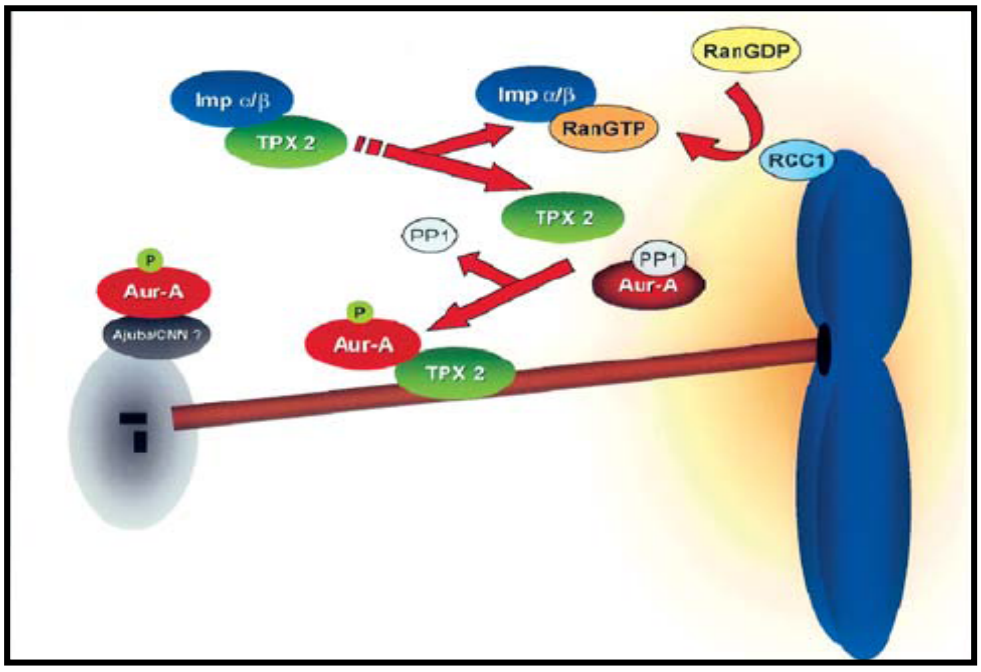
Aurora-A and PP1 are both enriched on centrosomes. They can interact directly *in vivo* and each can negatively regulate the other in a regulatory feedback loop [7]. A thorough structural study had shown that prior to the Aurora-A activation, the T loop of Aurora-A is in a flexible conformation, exposing the Thr288 for continuous interaction with PP1, therefore maintaining the Thr288 in the dephosphorylated form.

TPX2 (Target Protein for Xenopus kinesin-like protein 2) is currently the best-understood substrate that activates Aurora-A [8-13]. Once Aurora-A associates with the activator substrate TPX2, the activation loop changes to a more compact conformation so that the Thr288 is now hidden and protected from PP1-catalyzed dephosphorylation. This triggers Aurora-A autophosphorylation, leading to its activation. *In vitro* study had shown that the activated Aurora-A could also phosphorylate and inhibit PP1. Aurora-A has two PP1-binding motifs (KVxF) in the catalytic kinase domain, one includes the catalytic lysine residues (KVLF), the other is immediately adjacent to Ser342 (KVEF).

TPX2 is a microtubule-associated protein and plays an important role in small GTPase Ran-mediated mitotic spindle assembly process. Intriguingly, TPX2 is required for binding and localizing Aurora-A to the spindle poles, thereby stimulating the autophosphorylation and subsequent activation of Aurora-A, as shown in Figure 2-1.

Figure 2-1: A model linking Ran-GTP to Aurora-A Activation on Spindle Apparatus (Figure adapted from ref [15])

TPX2 establishes a link between Ran-GTP and the activation of Aurora-A on mitotic spindle. TPX2 is inhibited in the interphase by binding of importin-α/β. The active GTP-bound Ran stimulates Aurora-A kinase activity by displacing TPX2 from importin α/β. The free TPX2 in turn binds efficiently to Aurora-A, resulting in autophosphorylation and activation of Aurora-A. The kinase is now in its active conformation for substrate binding and catalysis as well as protection from PPI inactivation. The local concentration of Ran-GTP is controlled by chromatin-associated exchange factor, RCC1 (RanGEF). While TPX2 acts primarily on Aurora-A that is associated with spindle microtubules, CNN (centrosome component centrosomin) and Ajuba regulate the centrosome-associated pool of Aurora-A.



2.1.1.2. Inhibition by p53

Another interesting negative regulator of Aurora-A kinase is the well-known tumour suppressor, p53. In response to DNA or spindle damage or inappropriate activation of

oncogenes, p53 plays an important role in maintaining the genomic stability through transcriptional activation of target genes, which can induce G1 and/or G2 checkpoint arrests. p53 is localized to the centrosomes during mitosis, downregulation or loss of p53 leads to centrosome amplification and defective spindle formation. Significantly, overexpression of Aurora-A also induces the similar defects, which are further exacerbated in the p53 mutant cells [16].

Aurora-A was isolated as a p53-interacting partner in a two-hybrid screen using a transactivation-defective p53 as the bait. An *in vitro* study had shown that p53 interacted with the N-terminal non-catalytic domain of Aurora-A and inhibited its kinase activity. This negative regulation of Aurora-A by p53 could suppress the Aurora-A-induced centrosome amplification and cellular transformation in a transactivation-independent manner [17].

As described previously, Aurora-A phosphorylates p53, leading to the inactivation of p53 transcriptional activity and enhanced MDM2-mediated p53 degradation [18]. A fine-tuned balance of this mutual suppression mechanism between Aurora-A and p53 is therefore essential. Overexpression or hyperactivation of Aurora-A may override the p53-mediated negative regulation, leading to checkpoint defects, chromosome instability and subsequent cell transformation.

2.1.1.3 Inhibition by RasGAP

In another study, Aurora-A was identified as the SH3 (Src Homology 3) binding protein for

the RasGAP (GTPase-Activating Protein) [19]. Interestingly, RasGAP functions normally as a negative regulator of Ras by stimulating the GTP hydrolysis and Ras deactivation. RasGAP interacts with the kinase domain of Aurora-A and inhibits its kinase activity. Instead, in tumour cells where oncogenic Ras was expressed, RasGAP acted as a Ras effector and no longer negatively regulated its downstream effector, Aurora-A, leading to hyperactivation of Aurora-A. Aurora-A, RasGAP and survivin existed as a ternary complex, regulating the balance between cell division and apoptosis. A recent study had demonstrated that overexpression of Aurora-A augmented the G12V-mutated HRAS-induced oncogenic transformation and interestingly, expression level of Aurora-A determined the susceptibility to in vitro oncogenic transformation [46]. In addition, Ajuba, an Aurora-A activator [47], interacts with Grb2 and affects Ras signaling [48], and presumably, the Aurora-A/Ajuba complex may also modulate Ras signaling.

2.1.2. Regulation at the Protein Stability through Ubiquitination

Protein degradation plays an essential role in the regulation of many fundamental cellular physiological processes, such as cell cycle, immune and inflammation response, development, differentiation and transformation [20]. In particular, the role of protein degradation in cell cycle control is to ensure the periodic expression of various cell cycle proteins as aberrant protein degradation could lead to uncontrolled cell cycle and cancers.

2.1.2.1. Ubiquitin-Proteasome System

The ubiquitin-proteasome system has evolved as the key machinery in the selective degradation of most intracellular short-lived regulatory or abnormal proteins [21]. Target proteins are covalently tagged with multiple ubiquitins, forming the polyubiquitin chain, which not only serves as the recognition signal for 26S proteasome, but also assists in the unfolding of target proteins. Ubiquitination is a multistep process where the ubiquitin is covalently attached to the acceptor lysine residues and this requires the ubiquitin-activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3), where E3 confers the substrate specificity. E1 activates the ubiquitin and the activated ubiqutin is transferred from E1 to ubiquitin-conjugating E2, which can then transferred the ubiquitin to substrates by itself, or in cooperation with an ubiquitin ligase. Normally there are only one isoform of E1 and multiple isoforms of E2s and E3s. The multitude of E2 and E3 enzymes allows elaboration of ubiquitination pathway *in vivo* and therefore ensures the specific and regulated turnover of a wide array of substrates. Once after the K48 (lysine) of first ubiquitin is covalently attached to the protein, additional ubiquitins can be linked to form a substrate-tethered polyubiquitin chain. A tetra-ubiquitin chain serves as the minimal targeting signal for degradation.

2.1.2.2. Cdh1-mediated Cell Cycle-dependent Degradation

Aurora-A kinase represents one of the many mitotic proteins, where its protein level is

temporally regulated by the ubiquitin-dependent proteolysis at the end of mitosis before the cells progress into the G1 phase of next cell cycle [1, 22]. Aurora-A kinase is ubiquitinated by Cdh1-activated (APC/C), an E3 ubiquitin ligase through the recognition of C-terminal destruction box (D3 box) and non-catalytic N-terminal A box [23-26]. The Cdh1-dependent degradation of Aurora-A does not require the KEN sequence, an APC/C recognition signal, which is located at the N-terminal of Aurora-A. The A box contains the highly conserved Ser51 among the Aurora-A orthologs. Ser51 is normally phosphorylated in mitosis and dephosphorylated upon mitotic exit [4]. The A box is initially thought to be specific for Aurora-A orthologs, but a recent study had also identified the A box in Aurora-B and degradation of Aurora-B requires the intact KEN and A boxes [45].

2.1.2.3. Chfr-mediated Degradation

Besides the cell cycle-dependent degradation of Aurora-A mediated by Cdh1, Aurora-A kinase is also targeted for ubiquitin-dependent degradation by Chfr (Checkpoint protein with FHA and Ring domain), an E3 ubiquitin ligase [27]. Chfr is a newly identified early mitotic checkpoint protein, which responds to mitotic stress by delaying the progression to metaphase [28]. Its N-terminal FHA domain plays a role in phosphoprotein interaction [29], whereas its Ring domain is involved in protein ubiquitination [30]. As an E3 ubiquitin ligase, Chfr directly interacts with Aurora-A and ubiquitinates Aurora-A *in vivo*. Therefore, Chfr is a tumour suppressor and Chfr-mediated degradation of Aurora-A controls the expression level

of Aurora-A and maintains the chromosomal stability.

2.1.2.4. hDCD4-mediated Degradation

Another ubiquitin ligase, FBXW7/hCDC4 is also implicated in the regulation of Aurora-A stability. The study had shown that mouse embryo fibroblast from Fbxw7-deficient mice or wild-type mouse expressing Fbxw7 siRNA, had significantly higher level of Aurora-A kinase [31]. However, the mechanism of which is still unclear.

2.2. Aurora Kinase Inhibition by Small Molecule Inhibitors

2.2.1. Aurora-A Kinase as Anti-Cancer Target

Ideally, a good target for cancer therapy should be a specific gene, or protein or process that is different between the normal and cancer cells [32]. So, are the Aurora kinases good targets for anticancer drug development?

Aurora-A is a potential oncogene with various implicated roles in tumorigenesis. As mentioned in the earlier chapter, Aurora-A overexpression causes centrosome amplification, multipolar spindle, aneuploidy and oncogenic transformation. Amplification of AURKA locus and Aurora-A overexpression correlates with the chromosome instability in a wide range to human tumours. Amplification of AURKA locus also correlates with poor prognosis [33].

On the other hand, Aurora-B is also overexpressed in multiple human tumour cell lines and primary colorectal cancers [34]. Impaired Aurora-B function could lead to CIN (gain or loss

of chromosomes) phenotype, generating the genetic heterogeneity of cancers and also promoting tumorigenesis. Survivin, part of the Aurora-B complex (chromosome passenger protein), plays the protective role against apoptosis and/or mitotic catastrophe [35]. Similarly, Aurora-C kinase is often overexpressed in primary colorectal cancer and various tumour cell lines [36]. Given these facts, Aurora kinases undeniably become popular targets for cancer drugs design by many pharmaceutical companies.

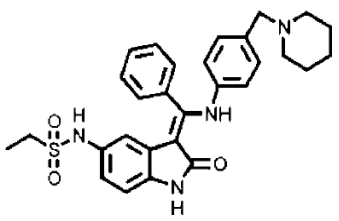
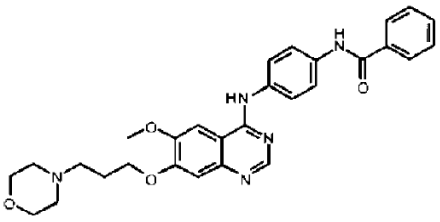
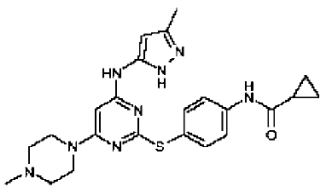
The previous clinical success of targeted therapy for chronic myelogenous leukemia (CML) using Gleevec [37], a small molecule kinase inhibitor that targets BCR-ABL, x-Kit and PDGF receptor kinase, has boosted confidence that small molecule inhibitors of specific kinase have the potential to be the highly effective anticancer drugs.

2.2.2. Development of Small Molecule Aurora Kinase Inhibitor

To date, three Aurora kinase small-molecule inhibitors have been developed and characterized to quite a good extent—ZM447439 [38], Hesparadin [39] and VX-680 [40]. Table 2-1 shows their comparisons in term of structure, specificity and phenotype in treated cells [32, 41]. However, none of these inhibitors selectively inhibits a single kinase. ZM447439 was founded by AstraZeneca through the screening of a 250000 compound library using Aurora-A kinase and a model peptide substrate. ZM447439 inhibited Aurora-A and –B kinases at least 10-fold more potently than other 13 kinases tested. Its inhibition of Aurora-C had not yet been tested. On the other hand, Hesparadin developed by Boehringer Ingelheim,

specifically inhibited Aurora-B of the family. It was still unclear whether hesparadin can inhibit Aurora-A and –C. VX-680 was the most recently discovered drug by Vertex Pharmaceuticals, designed from a knowledge-based chemical synthesis programme according to the crystal structure of Aurora kinase, which consists of several unique structural features. VX-680 potently inhibited all three Aurora kinases, with highest selectivity for Aurora-A kinase. On the other hand, VX-680 displayed more than 100-fold selectivity for Aurora-A kinase over a diverse panel of 55 other kinases tested.

Table 2-1: Aurora-Kinase Inhibitors (Table adapted from ref [32])

Company Boehringer Ingelheim	*Generic name* Hesperadin	*Compound name* Sulphonylamino substituted 3-(aminomethylide)-2-indolinone
Specificity	*Aurora kinases inhibited (IC_{50})* Aurora B (50 nM in cells; > 200 μM *in vitro*) *Off target kinases inhibited (IC_{50})* AMPK, LCK, MKK1, MAPKAP-K1, CHK1 and PHK (1 μM) CycB/CDK1 (2.8 μM) (25 tested)	*Structure* 
Phenotype	Decreased H3 phosphorylation Chromosome segregation and cytokinesis defects leading to polyploidization to > 32*N* content Increased syntelic Kt–MT attachments Overcame spindle checkpoint arrest triggered by taxol but not nocodazole	
Company Astra Zeneca	*Generic name* ZM447439	*Compound name* (4-(4-(*N*-benzoylamino)anilino)-6-methoxy-7-(3-(1-morpholino) propoxy)quinazoline
Specificity	*Aurora kinases inhibited (IC_{50})* Aurora A (0.11 μM) Aurora B (0.13 μM) *Off-target kinases inhibited (IC_{50})* MEK (1.79 μM), SRC (1.03 μM) and LCK (0.88 μM) (13 tested)	*Structure* 
Phenotype	Decreased H3 phosphorylation Endoreduplication then arrested with 4*N*/8*N* DNA content Arrest was p53-dependent. Inhibited colony formation in proliferation assay Compromises spindle checkpoint, causing failure to recruit BubRI, Mad2 and CENP-E	
Company Vertex	*Generic name* VX-680	*Compound name* Cyclopropane carboxylic acid {4-[4-(4-methyl-piperazin-1-yl)-pyrimidin-2ylsulphanyl]-phenyl}-amide
Specificity	Aurora kinases inhibited (IC_{50}) Aurora A (0.6 nM) Aurora B (18 nM) Aurora C (4.6 nM) *Off-target kinases inhibited (IC_{50})* FLT-3; 30 nM (55 tested)	*Structure* 
Phenotype	Accumulation of cells with > 4*N* content Inhibited the proliferation of a panel of cancer cell lines Loss of H3 phosphorylation *in vivo* Fourfold increase in apoptosis *in vivo* Effective killing of AML tumour cells from patients Tumour killing in xenograft models	

2.2.3. Action of Aurora Kinase Inhibitors

All these three reversible inhibitors selectively target the enzymatic activity of Aurora kinases by occupying the catalytic ATP-binding site. All three inhibit phosphorylation of histone H3 on serine 10 and cause cytokinesis defect without inducing any mitotic arrest, which is clearly different from the classic “anti-mitotic” drug. The drug-treated tumour cells enter and exit from mitosis normally and endo-reduplicate their genomes, leading to tetraploidy. Depending on the p53 status of the cells, cells treated with Aurora inhibitors can have different fates upon longer exposure. In the event of DNA damage or abnormal mitosis, the p53 can induce the post-mitotic G1 checkpoint and arrest the cells in G1 or S/G2 phase to allow repair or alternatively induce apoptosis. This serves as a secondary defense to the spindle checkpoint to prevent further genome instability. Therefore in most tumour cells that usually lack the functional p53, they will not undergo cell cycle arrest upon drug treatment. Instead, they continue to divide in more additional cycles and become massively polyploidy, leading to mitotic catastrophe and eventual apoptosis [32, 41-42].

These Aurora inhibitors are only selective against tumour cells, not on the viability of the non-cycling primary human cells, which usually have low or undetectable level of Aurora expression and kinase activity [32,41-42]. Thus, Aurora inhibition is a good target for cancer intervention, where the dividing and non-dividing cells can be discriminated by the Aurora inhibitors, unlike some of the conventional chemotherapeutic drugs that target microtubules

[43], such as taxanes, epothilones, discodermolides, Vinca alkaloids, nocodazole, colchicines, etc. With such discrimination, toxic side effect can be further minimized or avoided. For instance, treatment with taxanes (e.g. taxol) causes peripheral neuropathy due to its effect on the neuronal microtubule network [42].

2.2.4. VX-680-The Most P otent Among All

With respect to their tumor killing potential, VX-680 seems to be the most promising one to further progress into clinical development. VX-680 blocked the cell cycle progression and induces apoptosis in diverse range of tumour cell types. Profoundly, VX-680 effectively killed three different tumour cell types (leukemia, colon and pancreas) in the *in vivo* xenograft models. These three tumour cell types were usually refractory to conventional treatment in human cancer patients, however, their significant inhibition could be achieved with VX-680 at well-tolerated doses without any signs of mechanism-independent toxicity. *In vitro* studies had shown that VX-680 could completely abolish the colony formation of primary AML cells isolated from patients who are refractory to the standard therapies. VX-680 also potently inhibited the tumour growth *in vivo* in the human AML xenograft model, though the efficacy was accompanied with some level of toxicity to the bone-marrow cells, neutropenia and some body weight loss, but they recovered after drug withdrawal. The existence of other toxic side effects and/or long-term survival rates in these treated mice was not addressed yet and this should be the essential next step towards clinical trials. Therefore, targeted therapy using this

Aurora-A inhibitor, VX-680 could be a new potential approach to kill tumours that are refractory to the conventional therapies [40].

Interestingly, although VX-680 can inhibit Aurora-A kinase more potently than other members of the family, the phenotypes or responses observed in VX-680-treated cells most closely resemble those described for Aurora-B inhibition, rather than those observed after genetic disruption of Aurora-A. Recently, a study had addressed this question on why treatment of cells with dual-specific Aurora A and B kinase inhibitors produced phenotypes identical to Aurora-B inactivation. It was found that Aurora-B inhibition bypassed the mitotic requirement for Aurora-A and leads to polyploidy. Inactivation of Aurora-A activates spindle checkpoint kinase BubR1 and mitotic arrest in the Aurora B-dependent manner [44].

2.2.5. Future Outlook

Despite the encouraging demonstrations that these small molecule Aurora kinase inhibitors are effective in cancer killing, we should be very careful about their overall efficacy when applying clinically in human cancer treatment. Interestingly to note that these inhibitors treatment only slightly increased the proportion of apoptotic population, and moreover, the mechanism of how these inhibitors cause cell death via polyploidization was still an unanswered doubt. Does this mode of polyploidization-induced apoptosis also happen *in vivo*? What is the long-term effect of their inhibition? Will this induced polyploidization instead cause greater probability of genetic heterogeneity and hence potential risk for tumour

evolution? Again, nothing is understood as to what extent of massive chromosome loss would be sensed in the cancer cell to induce the apoptosis, not survival. Hence, the pharmacological properties of these inhibitors in the clinical setting are still unclear yet. Most probably, second or third generation inhibitors with even less toxicity or optimized biological properties will be developed. One important future challenge before the entrance of these inhibitors into clinical trials would be the ability to monitor the kinase suppression in treated patients by biomarkers. Phosphorylation status of histone H3 at Ser10 would be a useful biomarker for Aurora-B inhibition, whereas specific biomarkers for Aurora-A inhibition are yet to be identified. Finally, another aspect of future study would be the understanding of the tumour types that are responsive to these inhibitors. Various factors can determine and modify the sensitivity, such as p53 status of the tumour, kinase selectivity profile of the drug, proliferation rate of tumour and the complexicity of the tumour genotype [32, 41].

2.3. References

1. Walter AO, Seghezzi W, Korver W, Sheung J, Lees E. **The mitotic serine/threonine kinase Aurora2/AIK is regulated by phosphorylation and degradation**. Oncogene. (2000) 19(42):4906-16.

2. Littlepage LE, Wu H, Andresson T, Deanehan JK, Amundadottir LT, Ruderman JV. **Identification of phosphorylated residues that affect the activity of the mitotic kinase Aurora-A.** Proc Natl Acad Sci U S A. (2002) 99(24):15440-5.

3. Ferrari S, Marin O, Pagano MA, Meggio F, Hess D, El-Shemerly M, Krystyniak A, Pinna LA**. Aurora-A site specificity: a study with synthetic peptide substrates.** Biochem J. (2005) 390(Pt 1):293-302.

4. Littlepage LE, Ruderman JV. **Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit.** Genes Dev. (2002) 16(17):2274-85.

5. Francisco L, Wang W, Chan CS**. Type 1 protein phosphatase acts in opposition to IpL1 protein kinase in regulating yeast chromosome segregation.** Mol Cell Biol. (1994) 14(7):4731-40.

6. Francisco L, Chan CS. **Regulation of yeast chromosome segregation by Ipl1 protein kinase and type 1 protein phosphatase**. Cell Mol Biol Res. (1994) 40(3):207-13.

7. Katayama H, Zhou H, Li Q, Tatsuka M, Sen S. **Interaction and feedback regulation between STK15/BTAK/Aurora-A kinase and protein phosphatase 1 through mitotic cell division cycle.** J Biol Chem. (2001) 276(49):46219-24.

8. Kufer TA, Sillje HH, Korner R, Gruss OJ, Meraldi P, Nigg EA. **Human TPX2 is required for targeting Aurora-A kinase to the spindle.** J Cell Biol. (2002) 158(4):617-23.

9. Tsai MY, Wiese C, Cao K, Martin O, Donovan P, Ruderman J, Prigent C, Zheng Y. **A Ran signalling pathway mediated by the mitotic kinase Aurora A in spindle assembly.** Nat Cell Biol. (2003) 5(3):242-8.

10. Eyers PA, Erikson E, Chen LG, Maller JL. **A novel mechanism for activation of the protein kinase Aurora A.** Curr Biol. (2003) 13(8):691-7.

11. Bayliss R, Sardon T, Vernos I, Conti E. **Structural basis of Aurora-A activation by TPX2 at the mitotic spindle.** Mol Cell. (2003) 12(4):851-62.

12. Trieselmann N, Armstrong S, Rauw J, Wilde A. **Ran modulates spindle assembly by regulating a subset of TPX2 and Kid activities including Aurora A activation**. J Cell Sci. (2003) 116(Pt 23):4791-8.

13. Eyers PA, Maller JL. **Regulation of Xenopus Aurora A activation by TPX2**. J Biol Chem. (2004) 279(10):9008-15.

14. Cheetham GM, Knegtel RM, Coll JT, Renwick SB, Swenson L, Weber P, Lippke JA, Austen DA. **Crystal structure of aurora-2, an oncogenic serine/threonine kinase.** J Biol Chem. (2002) 277(45):42419-22.

15. Kufer TA, Nigg EA, Sillje HH. **Regulation of Aurora-A kinase on the mitotic spindle.** Chromosoma. (2003) 112(4):159-63.

16. Meraldi P, Honda R, Nigg EA. **Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells.** EMBO J. (2002) 21(4):483-92.

17. Chen SS, Chang PC, Cheng YW, Tang FM, Lin YS. **Suppression of the STK15 oncogenic activity requires a transactivation-independent p53 function.** EMBO J. (2002) 21(17):4491-9.

18. Katayama H, Sasai K, Kawai H, Yuan ZM, Bondaruk J, Suzuki F, Fujii S, Arlinghaus RB, Czerniak BA, Sen S. **Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53.** Nat Genet. (2004) 36(1):55-62.

19. Gigoux V, L'Hoste S, Raynaud F, Camonis J, Garbay C. **Identification of Aurora kinases as RasGAP Src homology 3 domain-binding proteins.** J Biol Chem. (2002) 277(26):23742-6.

20. Ciechanover A. **The ubiquitin-proteasome pathway: on protein death and cell life.** EMBO J. (1998) 17(24):7151-60.

21. Glickman MH, Ciechanover A. **The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction.** Physiol Rev. (2002) 82(2):373-428.

22. Honda K, Mihara H, Kato Y, Yamaguchi A, Tanaka H, Yasuda H, Furukawa K, Urano T. **Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway.** Oncogene. (2000) 19(24):2812-9.

23. Arlot-Bonnemains Y, Klotzbucher A, Giet R, Uzbekov R, Bihan R, Prigent C. **Identification of a functional destruction box in the Xenopus laevis aurora-A kinase pEg2.** FEBS Lett. (2001) 508(1):149-52.

24. Castro A, Arlot-Bonnemains Y, Vigneron S, Labbe JC, Prigent C, Lorca T. **APC/Fizzy-Related targets Aurora-A kinase for proteolysis.** EMBO Rep. (2002) 3(5):457-62.

25. Castro A, Vigneron S, Bernis C, Labbe JC, Prigent C, Lorca T. **The D-Box-activating domain (DAD) is a new proteolysis signal that stimulates the silent D-Box sequence of Aurora-A.** EMBO Rep. (2002) 3(12):1209-14.

26. Taguchi S, Honda K, Sugiura K, Yamaguchi A, Furukawa K, Urano T. **Degradation of human Aurora-A protein kinase is mediated by hCdh1.** FEBS Lett. (2002) 519(1-3):59-65.

27. Yu X, Minter-Dykhouse K, Malureanu L, Zhao WM, Zhang D, Merkle CJ, Ward IM, Saya H, Fang G, van Deursen J, Chen J. **Chfr is required for tumor suppression and Aurora A regulation.** Nat Genet. (2005) 37(4):401-6.

28. Scolnick DM, Halazonetis TD. **Chfr defines a mitotic stress checkpoint that delays entry into metaphase.** Nature. (2000) 406(6794):430-5.

29. Stavridi ES, Huyen Y, Loreto IR, Scolnick DM, Halazonetis TD, Pavletich NP, Jeffrey PD. **Crystal structure of the FHA domain of the Chfr mitotic checkpoint protein and its complex with tungstate.** Structure. (2002) 10(7):891-9.

30. Chaturvedi P, Sudakin V, Bobiak ML, Fisher PW, Mattern MR, Jablonski SA, Hurle MR, Zhu Y, Yen TJ, Zhou BB. **Chfr regulates a mitotic stress pathway through its RING-finger domain with ubiquitin ligase activity.** Cancer Res. (2002) 62(6):1797-801.

31. Mao JH, Perez-Losada J, Wu D, Delrosario R, Tsunematsu R, Nakayama KI, Brown K, Bryson S, Balmain A. **Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene.** Nature. (2004) 432(7018):775-9.

32. Andrews PD. **Aurora kinases: shining lights on the therapeutic horizon?** Oncogene. (2005) 24(32):5005-15.

33. Isola JJ, Kallioniemi OP, Chu LW, Fuqua SA, Hilsenbeck SG, Osborne CK, Waldman FM. **Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer.** Am J Pathol. (1995) 147(4):905-11.

34. Katayama H, Ota T, Jisaki F, Ueda Y, Tanaka T, Odashima S, Suzuki F, Terada Y, Tatsuka M. **Mitotic kinase expression and colorectal cancer progression.** J Natl Cancer Inst. 1999 Jul 7;91(13):1160-2.

35. Wheatley SP, McNeish IA. **Survivin: a protein with dual roles in mitosis and apoptosis.** Int Rev Cytol. (2005) 247:35-88.

36. Takahashi T, Futamura M, Yoshimi N, Sano J, Katada M, Takagi Y, Kimura M, Yoshioka T, Okano Y, Saji S. **Centrosomal kinases, HsAIRK1 and HsAIRK3, are overexpressed in primary colorectal cancers**. Jpn J Cancer Res. (2000) 91(10):1007-14.

37. Sawyers CL. **Finding the next Gleevec: FLT3 targeted kinase inhibitor therapy for acute myeloid leukemia.** Cancer Cell. (2002) 1(5):413-5.

38. Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T, Mortlock A, Keen N, Taylor SS. **Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores**. J Cell Biol. (2003) 161(2):267-80.

39. Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, Heckel A, van Meel J, Rieder CL, Peters JM. **The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint.** J Cell Biol. (2003) 161(2):281-94.

40. Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama

T, Graham JA, Demur C, Hercend T, Diu-Hercend A, Su M, Golec JM, Miller KM. **VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo.** Nat Med. (2004) 10(3):262-7.

41. Keen N, Taylor S. **Aurora-kinase inhibitors as anticancer agents.** Nat Rev Cancer. (2004) 4(12):927-36.

42. Warner SL, Bearss DJ, Han H, Von Hoff DD. **Targeting Aurora-2 kinase in cancer.** Mol Cancer Ther. (2003) 2(6):589-95.

43. Mollinedo F, Gajate C. **Microtubules, microtubule-interfering agents and apoptosis.** Apoptosis. (2003) 8(5):413-50.

44. Yang H, Burke T, Dempsey J, Diaz B, Collins E, Toth J, Beckmann R, Ye X. **Mitotic requirement for aurora A kinase is bypassed in the absence of aurora B kinase.** FEBS Lett. (2005) 579(16):3385-91.

45. Nguyen HG, Chinnappan D, Urano T, Ravid K. **Mechanism of Aurora-B degradation and its dependency on intact KEN and A-boxes: identification of an aneuploidy-promoting property.** Mol Cell Biol. (2005) 25(12):4977-92.

46. Tatsuka M, Sato S, Kitajima S, Suto S, Kawai H, Miyauchi M, Ogawa I, Maeda M, Ota T, Takata T. **Overexpression of Aurora-A potentiates HRAS-mediated oncogenic transformation and is implicated in oral carcinogenesis.** Oncogene. (2005) 24(6):1122-7.

47. Hirota T, Kunitoku N, Sasayama T, Marumoto T, Zhang D, Nitta M, Hatakeyama K, Saya H. **Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells.** Cell. (2003) 114(5):585-98.

48. Goyal RK, Lin P, Kanungo J, Payne AS, Muslin AJ, Longmore GD. **Ajuba, a novel LIM protein, interacts with Grb2, augments mitogen-activated protein kinase activity in fibroblasts, and promotes meiotic maturation of Xenopus oocytes in a Grb2- and Ras-dependent manner.** Mol Cell Biol. (1999) 19(6):4379-89.

(Introduction and Literature Review)

Chapter 3

Ubiquitin-Independent Protein Degradation Pathway

3.1. Architecture of Proteasome Degradation System: 20S and 26S

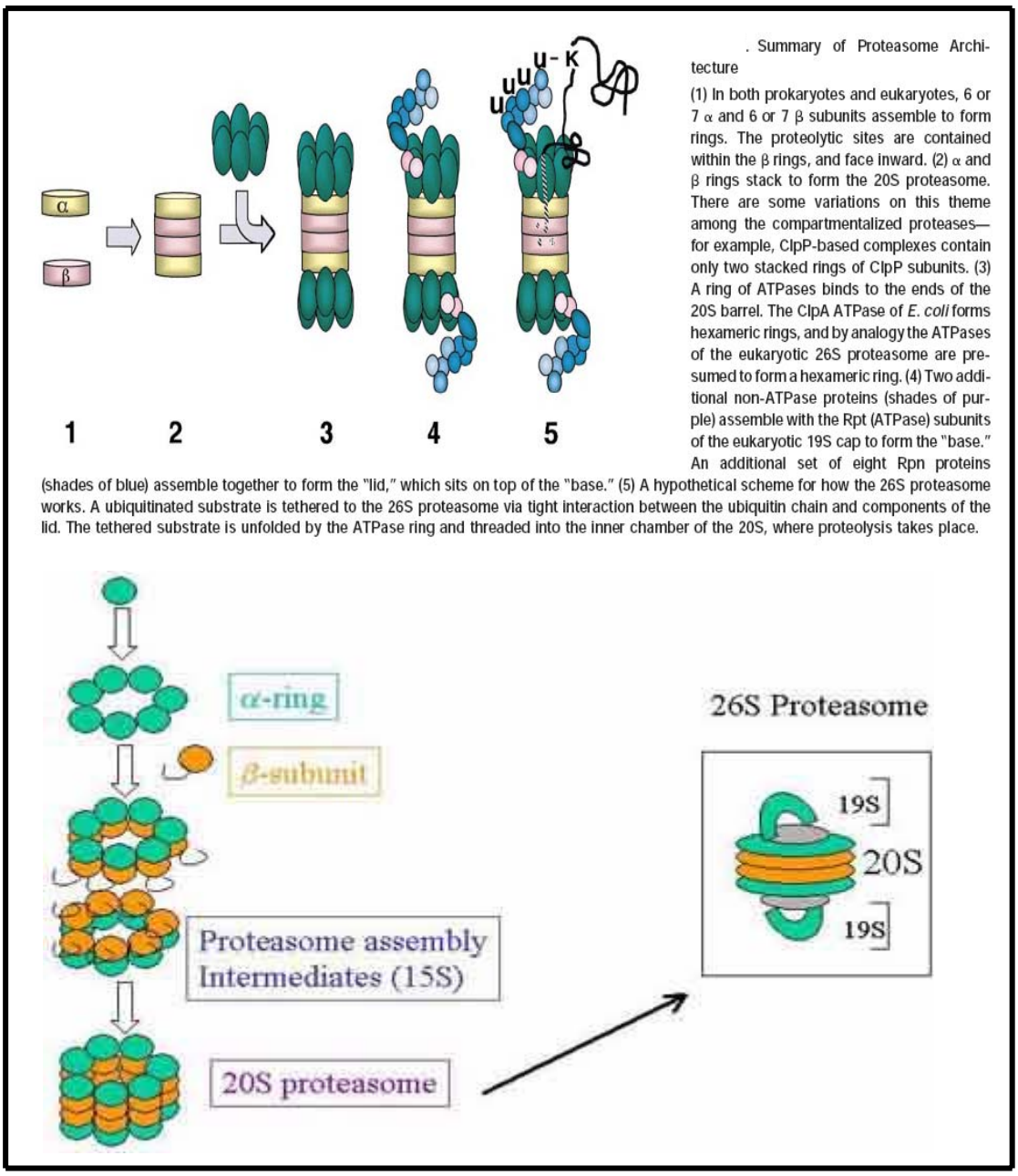
In eukaryotic cells, the multicatalytic proteinase complex, proteasome, is the main proteolytic weapon for the extralysosomal proteolysis. The 20S proteasome is 700 KDa barrel-like structure formed by four stacked 7-subunits rings, 2 α-rings and 2 β-rings, as shown Figure 3-1. Only the inner β-rings contain the active catalytic site for proteolysis, directed toward the inner chamber. The outer α-rings control the opening of the chamber and play the role in the conditional degradation of cellular proteins [1]. The 20S proteasome is present up to 1% of the total cellular protein. Capping of 19S regulatory complexes at both ends of the 20S core forms the 26S proteasome (Figure 3-1) [2-3]. The 19S assembly consists of a “base” and a “lid”. The base contains ATPases and binds to the end of 20S cylinder to catalyze the substrate unfolding. The lid binds the base and functions in the substrate recognition and insertion [4].

Generally, substrates destined for degradation must first be recognized and associated with the proteasome [5], followed by ATP-dependent unfolding and denaturation of the substrates if necessary [6]. In the meantime, the pore of proteasomal core is opened to allow substrate entry [7-8]. Before the substrate entry, the degradation tag must be cleared and recycled. Ubiquitins are recovered from the Ub-protein conjugates through the catalytic activity of the Ub-isopeptidase [9-10]. Only then, the unfolded substrate can be inserted into the pore. Further processive translocation leads to complete transfer of the polypeptide chains to the

inner catalytic chamber and subsequent proteolysis to small peptides [11-14].

Figure 3-1: Architecture of 20S and 26S Proteasome

(Figure adapted from ref [15-16])



3.2. Alternative Ubiquitin-Independent Degradation Pathway

In the past 20 years, most of the researches on extralysosomal proteolysis have focused on the role of 26S proteasome in the ubiquitin-dependent degradation pathway. Therefore, it has been long thought that all the substrates of 26S proteasome are to be marked with

polyubiquitin tag before being targeted for degradation. However, this has led us to underestimate a globally existed class of proteasomal substrates that can be degraded without the prior ubiquitin tagging, either by the 26S proteasome in the presence of ATP or by the 20S proteasome directly [8]. In the recent few years, a lot of evidences had shown that the 20S proteasome and 26S proteasome could degrade some proteins in an ubiquitin-independent manner [18]. It will be interesting to know what substrates have been identified that are not dependent on ubiquitin for degradation and how are they processed without the ubiquitin tag? What are the differences and/or similarities in the characteristics of proteasomal degradation for the ubiquitin-dependent and independent substrates?

For some proteins, the polyubiquitination and degradation processes can be uncoupled and thus no prior modification is required for degradation. The number of proteins recognized as targets of this ubiquitin-independent proteasomal degradation will continue to increase in the future.

On the other hand, some proteins can be modified by SUMO (Small Ubiquitin-like Modifier), a protein of 97 amino acids, which is structurally similar to ubiquitin [58]. Similar to ubiquitin, SUMO is also covalently attached to certain lysine residues of specific target proteins [59]. However, in contrast to ubiquitination, sumoylation does not always promote protein degradation, instead plays important roles in regulating the subcellular localization, protein-protein interaction, DNA binding or transactivation functions of the protein substrates

[59-61]. With respect to the role in regulation of protein degradation, sumoylation has been found to regulate the ubiquitination of proteins, such as NFκB inhibitor IκBα, PCNA, Smad4, and Mdm2, either directly by competition for the modified lysine or indirectly, the effects of sumoylation could be mediated via control of protein level in general or turnover of specific subpopulations of a protein in a cell [59, 62-63].

3.3. Evidences for the Existence of Ubiquitin-Independent Degradation

3.3.1. Lysine-less Substrate

There are a few approaches that can be used to assess if the ubiquitination is essential for a protein's degradation, one of which is the mutation of the acceptor lysine residues in the target substrate, thereby inhibiting the formation of polyubiquitin chain in cis [15]. However, lysine residues are commonly found in most proteins and thus replacement of many (if not all) of the lysine residues in target substrates is required for elimination of all the possible ubiquitination target sites. Sheaff and coworkers had created the lysine-free p21 mutant, which was still fully functional in its activity, however, could still be degraded efficiently by the proteasome [18].

3.3.2. Lysine-less or Mutant Ubiquitin

On the other hand, inhibition of ubiquitination in trans via overexpression of a mutant lysine-free ubiquitin leads to the chain-terminating effect on the ubiquitin chain extension [15]. Again, overexpression of this lysine-free ubiquitin mutant stabilizes the cyclin E, a positive control protein that depends on ubiquitin conjugation for its degradation, but has no

effect on the turnover of wild-type p21 [15, 18]. Though lysine K11, K29, K48 and K63 of the ubiquitin could be used for formation of polyubiquitin chain, only the K48-linked multiubiquitin chains had been shown to be functional in targeting substrates for degradation [19]. Thus, overexpression K48R ubiquitin mutant is also used as an alternative approach for inhibition of polyubiquitination. Incorporation of K48R mutant blocks further ubiquitin chain extension.

3.3.3. N-terminal Fusion of Epitope

Interestingly, some lysine-free proteins can still be targeted for ubiquitin-dependent degradation via ubiquitination at the amino-terminal free amine group [20-21], such as MyoD [20]. Fusion of epitope to the N-terminus stabilizes MyoD but fails to stabilize both the wild type and lysine-free p21 [18].

3.3.4. Inactivation of E1 Ubiquitin-Activating Enzyme

Another strategy of polyubiquitination inhibition in trans is thermal inactivation of the temperature-sensitive mutant of E1 ubiquitin-activating enzyme at the non-permissive temperature [15, 22]. Again, the p21 degradation is not inhibited in these mutant cells harboring the temperature-sensitive mutation in E1 [18], implying the *in vivo* existence of ubiquitin-independent proteasomal degradation for p21.

3.4. Proteasome-Dependence of Ubiquitin-Independent Degradation Pathway

3.4.1 26S Proteasome in Ub-independent Protein Degradation

How does the 26S proteasome recognize its substrates if they are not tagged with ubiquitin? It is still possible for the 26S proteasome to catalyze the Ub-independent degradation provided another protein can take over the targeting role of ubiquitin or protein substrates themselves harbour the degradation signal sequence [8]. Ornithine decarboxylase (ODC) was the first example of protein shown to be degraded by the 26S proteasome in the Ub-independent manner [23-25]. Its ATP-dependent degradation requires the antizyme (AZ), which promotes the targeting of ODC to proteasome. Furthermore, the presence of a degradation signal sequence in the C-terminal region of ODC enhances its recognition by the 26S proteasome. The detailed mechanism of how the antizyme mediates the ODC degradation will be discussed in the following chapter.

Other proteins, which are degraded through the Ub-independent, 26S proteasome-dependent pathway, include c-Jun [26], calmodulin [27], troponin C [28], cyclin-dependent kinase inhibitor p21 [18] and tumor suppressor protein p53 [29]. Some proteins, such as p21 and p53 can be targeted for degradation by 26S proteasome in both the Ub-dependent and Ub-independent manner. Recently, p21 was also shown to be susceptible to degradation directly by the 20S proteasome [30]. In the case of p53, its Ub-independent degradation is negatively regulated by the NADPH quinone oxidoreductase I (NQO1).

Inhibition of the NQO1 activates the Ub-independent degradation of p53 [29].

3.4.2. 20S Proteasome in Ub-Independent Protein Degradation

The free 20S proteasome forms the major portion of the total proteasomes (20S, 26S and 20S-11S-REG) present in the cells [31]. Native unfolded proteins, short or long-lived proteins, oxidized, misfolded, mutated or damaged proteins are all susceptible to the 20S proteasome-mediated Ub-independent degradation [8].

3.4.2.1. Preferred Substrates

Multiple native and oxidized proteins, such as oxidized glutamine synthetase, calmodulin, casein, superoxide dismutase, hemoglobin, myoglobin, serum albumin and oxidized histones, serve as good substrates for the 20S proteasomal degradation [32-35]. Protein oxidation increases the surface hydrophobicity [33], and therefore the oxidized substrates are targeted better by the 20S proteasome for degradation, as compared to the 26S proteasome even in the presence of ATP and ubiquitin. Interestingly, studies on intact cells have shown that mild oxidation impairs the ubiquitin-dependent system and the activity of the 26S proteasome without affecting that of the 20S proteasome [36].

The role of the 20S proteasome in the degradation of the oxidized protein is strengthened by two different studies. Firstly, genetically modified yeast, lacking the regulatory complex and thus was defective in 26S proteasome assembly, could degrade the oxidized protein more

efficiently as compared to the wild type yeast [37]. On the other hand, mutant cells harboring the thermal inactivation of E1 Ub-activating enzyme and displaying markedly compromised ubiquitination at the restrictive temperature, however, degraded oxidized proteins in the proteasome-dependent manner [38].

3.4.2.2. Mechanisms

Since the 19S or 11S regulatory complexes does not participate in the 20S proteasome-mediated degradation process, alternative mechanisms that lead to protein targeting, protein unfolding and catalytic pore opening must have evolved [8]. These mechanisms are discussed below and summarized in Table 3-1

Table 3-1:
Proposed Mechanisms for Ubiquitin-Independent Proteolysis by 20S Proteasome
(Table adapted from ref [8])

Mechanism	Examples
Targeting	
By an accessory factor	Tax pp71
By the substrate	$p21^{Cip1}$ Oxidized proteins
Unfolding	
By disruption of disulfide bonds	Lysozyme
Gating (opening access to the catalytic chamber)	
By the substrate	$p21^{Cip1}$
By an accessory factor	Histones H3 and H4 Hydrophobic peptides
Activation	
By an allosteric change in the active site	Histones H3 and H4

3.4.2.2.1. Protein Targeting

Like the 26S proteasome-mediated Ub-independent degradation, targeting of a substrate to the 20S proteasome can be facilitated by an accessory protein or by a sequence within the substrate itself. An example of accessory factor that promotes substrate targeting is Tax [39], a protein encoded by human T cell leukemia virus. Tax enhances the binding of IκB to the 20S proteasome subunit HsN3, leading to the constitutive Ub- and phosphorylation-independent degradation of IκB. The human cytomegalovirus protein, pp71, represents another example of accessory factor that targets the hypophosphorylated member of Rb (retinoblastoma) tumor suppressor for the Ub-independent degradation [40].

On the other hand, example of substrate that targets itself to the 20S proteasome is p21. C-terminus of p21 binds to the C8 α-subunit of the 20S proteasome, promoting its own degradation. This mechanism is novel as the α-subunit of the proteasome is first documented to play a role in protein degradation and the degradation is direct without the help from 19S regulatory complex and ubiquitin [41]. Another example comes from the studies on the bacterial proteasome, such as the CIp complexes from *E.coli* [42]. CIpY, which is distantly related to the ATPases of the eukaryotic 19S cap, sandwiches the CIpQ, which is related to the proteolytically active eukaryotic 20S core. There is a conserved SSD (sensor and substrate discrimination) domain in CIpY [43], which can directly interact with degradation-targeting signal sequence embedded within the substrates, such as the unfolded citrate synthase [44].

3.4.2.2.2. Protein Unfolding

Protein unfolding is essential to initiate the transfer of protein substrate to the catalytic core of the proteasome [44]. In the case of 26S proteasome, substrate unfolding is mediated by the ATPases in the 19S regulatory cap, which is now absent in the 20S proteasome.

Since the conformation of many proteins is formed and maintained by the disulfide bonds, therefore disruption of these disulfide linkages may promote protein unfolding [8]. Lysozyme, a model substrate for Ub-dependent degradation pathway is resistant to 20S proteasome-mediated degradation *in vitro*, however, it becomes susceptible to degradation once its disulfide linkages are reduced [45]. The reduction of disulfide linkages is also observed for Ig-μ heavy chain before it is transferred from endoplasmic reticulum (ER) to cytoplasm for degradation, implying the existence of this mechanism [46].

TCRα chains that are unprocessed and fail to assemble in the ER, are transported back to cytoplasm and degraded by the 20S proteasome in the Ub-independent manner [47].

3.4.2.2.3. Opening of Gated Catalytic Chamber

The N-terminal tails of the outer α-subunit ring occlude the opening of the proteolytically active chamber of the 20S proteasome, preventing uncontrolled protein degradation [48]. This opening of access to the catalytic chamber could be effected either by an accessory factor or the substrate itself [8]. Recent studies had shown that histone H3 and H4 accelerated the 20S proteasomal degradation of casein and lysozyme [45]. Other accessory factors identified

include the cardiolipin and hydrophobic peptides [49-50]. On the other hand, p21 and α-synuclein were recently found to promote the gating of the proteasome themselves [30]. Their unfolded forms were degraded efficiently by the latent closed channel form of the 20S proteasome.

3.4.2.2.4. Allosteric Activation At the Active Site

The proteolytic activity of proteasome depends on the structural integrity of the proteasome, which relies much on the interaction between subunits [8]. Different proteasome activators (REGγ, PA200 [51-52] and histone H3 [45]) or inhibitors (e.g. Hsp90 [53] and Ritonavir [54]) can differentially regulate the allosteric interaction between the proteasomal subunits, leading to changes in the active site geometry and selective activation of distinct catalytic center.

3.5. Evolution of Ubiquitination to Complement Ub-Independent Degradation

Lower organisms (prokaryotes and archeae) employ only the Ub-independent mechanism to regulate their selective proteasomal degradation. However, the evolution has adopted the ubiquitination as an additional mechanism in the eukaryotic proteolytic pathways [15]. What is the central role for the evolution of ubiquitination system? Ubiquitin-mediated targeting (1) expands the range of substrates that can be selectively degraded, (2) allows greater flexibility in regulating proteolysis, (3) increases the specificity of substrate targeting, and (4) enhances the biochemical repertoire of the proteasome [15].

3.5.1. Expanded Substrate Repertoire

This acquired ubiquitin-mediated targeting tool in turn widens the range of substrates that can be selectively degraded through the evolvement of multiple E2 and E3 enzymes [11]. Different combinations of E2s and E3s, each with different substrate specificity, definitely diversify the substrate repertoire as they can expand the range of substrate targeting motifs and pathways [15].

3.5.2. Flexibility in Proteolysis Regulation

With the expanded substrate range, the ubiquitination allows greater flexibility in regulation of proteolysis [15]. The turnover of proteins can be differentially regulated in response to a wide variety of signals, such as phosphorylation or mitosis-triggered ubiquitination and degradation [55]. Moreover, the requirement of a multiubiquitin chain enables additional fine-tuning of substrate selection where a protein's stability can be controlled by changes in the rate of either ubiquitination or deubiquitination [19].

3.5.3. Enhanced Functional Repertoire of Proteasome

On the other hand, ubiquitination enhances the functional repertoire of the proteasome [15]. Most of the 26S proteasome substrates, like cyclins, Cdk inhibitors, securin and IκB, exist as heteromeric assemblies and must be efficiently degraded for proper functioning of the regulatory switch. However, the prokaryotic proteasomal system may be too rapid and thus

does not allow efficient unfolding of multidomain polypeptide chains and disassembly of multisubunit complexes. Substrate ubiquitination, however, allows enough time for the 19S ATPases to unfold and translocate the substrate before it enters into the proteolytic core [15].

3.6. Conclusion

In conclusion, for both Ub-dependent and Ub-independent degradation, conformational flexibility of the protein substrates is important for their proteasomal recognition and processing [17]. As we know, the Ub-dependent degradation pathway requires the prior unfolding of the substrates before being delivered into the proteasomal core for downstream processing [17]. Similarly, those proteins that are targeted for Ub-independent degradation are usually globally loosely folded [56] or consist of a disordered domain [57], which is necessary for interaction with proteasome.

At present, our understanding for the Ub-independent mode of degradation is still very limited. This is partly hindered by the difficulty in demonstrating the *in vivo* physiological function of this degradation system. Hopefully, future identification of more substrates and/or players in this Ub-independent pathway will help us to appreciate the physiologic and metabolic significance of this pathway.

3.7. References

1. Groll M., Ditzel L., Lowe J., Stock D., Bochtler M. Bartunik H.D. et al **Structure of 20S proteasome from yeast at 2.4A resolution.** Nature (1997) 386: 463-471

2. Hershko A., Ciechanover A. **The Ubiquitin System.** Annu. Rev. Biochem. (1998) 67: 425-479

3. Voges D., Zwickl P., Baumeister W. **The 26S proteasome: a molecular machine designed for controlled proteolysis.** Annu. Rev. Biochem. (1999) 68: 1015-1068

4. Glickman M., Rubin D., Coux O., Wefes I., Pfeifer G., Cjeka Z., Baumeister W., Fried V., Finley D. **A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and elF3**. Cell (1998) 94: 615-623

5. Lam Y.A., Lawson T.G., Velayutham M. Zweier J.L., Pickart C.M. **A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal.** Nature (2002) 416: 763-767

6. Liu C.W., Millen L., Roman T.B., Xiong H., Gilbert H.F., Noiva R. **Conformational remodeling of proteasomal substrates by PA700, the 19S regulatory complex of the 26S proteasome.** J. Biol. Chem. (2002) 277: 26815-26820

7. Kohler A., Cascio P., Leggett D.S., Woo K.M., Goldberg A.L., Finley D. **The axial channel of the proteasome is gated by the Rpt2 ATPase and controls both substrate entry and product release.** Mol. Cell. (2001) 7: 1143-1152

8. Orlowski M., Wilk S**. Ubiquitin-independent proteolytic function of the proteasome**. Arch. Biochem. Biophys. (2003) 415: 1-5

9. Verma R., Aravind L., Oania R., McDonald W.H., Yates J.R., Koonin E.V., et al. **Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome.** Science (2002) 298: 611-615

10. Yao T., Cohen R.E. **A cryptic protease couples deubiquitination and degradation by the proteasome.** Nature (2002) 419: 403-407

11. Glickman M.H., Ciechanover A**. The ubiqutin-proteasome proteolytic pathway: Destruction for the sake of construction.** Physiol. Rev. (2001) 82: 373-428

12. Lee C., Prakash S., matouschek A. **Concurrent translocation of multiple polypeptide chains through the proteasomal degradation channel.** J Biol. Chem. (2002) 277: 34760-34765

13. Rape M., Jentsch S. **Taking a bite: proteasomal processing**. Nat. Cell. Biol. (2002) 4: E116-116

14. Liu C.W., Corboy M.J., DeMartino G..N., Thomas P.J. **Endoproteolytic activity of the proteasome.** Science (2002) 12: 12

15. Verma R., Deshaies R.J. **A proteasome howdunit: the case of the missing signal.** Cell (2000) 101: 341-344

16. Tongwen Wang**. The 26S proteasome system in the signaling pathways of TGF-β superfamily.** Frontiers in Bioscience (2003) 8:1109-1127

17. Hoyt M.A., Coffino P**. Ubiqutin-free routes into the proteasome**. CMLS Cell. Mol. Life. Sci. (2004) 61: 1-5

18. Sheaff R., Singer J., Swanger J., Smitherman S., Roberts J., Clurman B. **Proteasomal turnover of p21 Cip1 does not require p21Cip1 ubiquitination**. Mol. Cell. (2000) 5: 403-410

19. Thrower J., Hoffman L., Rechsteiner M., Pickart C. **Recognition of the polyubiquitin proteolytic signal.** EMBO J. (2000) 19: 94-102

20. Breitschopf K., Bengal E., Ziv T., Admon A., Ciechanover A. **A novel site for ubiquitination: the N-terminal residue, not internal lysines of MyoD, is essential for conjugation and degradation of the protein.** EMBO J. (1998) 17: 5964-5973

21. Bloom J., Amador V., Bartolini F., DeMartino G., Pagano M. **Proteasome-mediated degradation of p21 via N-terminal ubiquitylation.** Cell (2003) 115: 71-82

22. Ciechanover A., Finley D., Varshavsky A. **Ubiquitin dependence of selective**

protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell (1984) 37: 57-66

23. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., Ichihara, A. **Ornithine decarboxylase is degraded by the 26S Proteasome without ubiquitination.** Nature (1992), 360: 597-599

24. Bercovich Z., Rosenberg-Hasson Y., Ciechanover A., Kahana C. **Degradation of ornithine decarboxylase in reticulocyte lysate is ATP-dependent but ubiquitin-independent.** J. Biol. Chem. (1989) 264:15949-15952

25. Coffino P. **Antizyme, a mediator of ubiquitin-independent proteasomal degradation.** Biochimie (2000) 83: 319-323

26. Jariel-Encontre I., Martin F., Carillo S., Salvat C., Piechaczyk M. **Ubiquitylation is not an absolute requirement for degradation of c-Jun protein by the 26S proteasome.** J. Biol. Chem. (1995) 270: 11623-11627

27. Tarcsa E., Szymanska G., Lecker S., O'Connor C.M., Goldberg A.L. **Ca^{2+}-free calmodulin and calmodulin damaged by in vitro aging are selectively degraded by 26S proteasomes without ubiquitination.** J. Biol. Chem. (2000) 275: 20295-20301

28. Benaroudj N., Tarcsa E., Cascio P., Goldberg A.L. **The unfolding of substrates and ubiquitin-independent protein degradation by proteasomes.** Biochimie (2001) 83: 311-318

29. Asher G.., Lotem J., Sachs L., Kahana C., Shaul Y. **Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1.** Proc. Natl. Acad. Sci. USA (2002) 99: 13125-13130

30. Liu C.W., Corboy M.J., DeMartino G..N., Thomas P.J. **Endoproteolytic Activity of the Proteasome.** Science (2003) 299: 408-411

31. Tanahashi, N., Murakami Y., Minami Y., Shimbara N., Hendil K.B., Tanaka K. A. **Hybrid Proteasomes: Induction by interferon and contribution to ATP-dependent Proteolysis.** J. Biol. Chem. (2000) 275: 14336-14345

32. Rivett J.A. **Purification of a liver alkaline protease which degrades oxidatively**

modified glutamine synthetase. Characterization as a high molecular weight cysteine proteinase. J. Biol. Chem. (1985) 260: 12600-12606

33. Davies K.J.A. **Degradation of oxidized proteins by the 20S proteasome.** Biochimie (2001) 83: 301-310

34. Ferrington D.A., Sun H., Murray K.K., Costa J., Williams T.D., Bigelow D.J., Squier T.C. **Selective Degradation of Oxidized Calmodulin by the 20 S Proteasome.** J. Biol. Chem. (2001) 276: 937-943

35. Ullrich O., Sitte N., Sommerburg O., Sandig V., Davies K.J.A., Grune T. **Influence of DNA binding on the degradation of oxidized histones by the 20S proteasome.** Arch. Biochem. Biophys. (1999) 362: 211-216

36. Grune T., Reinheckel, T., Davies K.J.A. **Degradation of oxidized proteins in mammalian cells.** FASEB J. (1997) 11: 526-529

37. Inai Y., Nishikimi M. **Increased degradation of oxidized proteins in yeast defective in 26 S proteasome assembly.** Arch. Biochem. Biophys. (2002) 404:279-284

38. Shringarpure R., Grune T., Mehlhase J., Davies K.J.A. **Ubiquitin Conjugation Is Not Required for the Degradation of Oxidized Proteins by Proteasome.** J. Biol. Chem. (2003) 278: 311-318

39. Petropoulous L., Hiscott J. **Association between HTLV-1 Tax and I kappa B alpha is dependent on the I kappa B alpha phosphorylation state.** Virology (1998) 252: 189-199

40. Kalejta R.F., Shenk T. **Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein.** Proc. Natl. Acad. Sci. (2002) 100(6): 3263-3268

41. Touitou R., Richardson J., Bose S., Nakanishi M., Rivett J., Allday J. **A degradation signal located in the C-terminus of p21$^{WAF1/CIP1}$ is a binding site for the C8 α-subunit of the 20S proteasome.** EMBO J. (2001) 10: 2367-2375

42. Bochtler M., Hartmann C., Song H.K., Bourenkov G.P. Bartunik H.D. Huber R. **The structures of HsIU and the ATP-dependent protease HsIU-HsIV**. Nature (2000)

403: 800-805

43. Smith C.K., Baker T.A., Sauer R.T. **Lon and Clp family proteases and chaperones share homologous substrate-recognition domains.** Proc. Natl. Acad. Sci. USA (1999) 96: 6678-6682

44. Braun B., Glickman M., Kraft R., Dahlmann B., Kloetzel P., Finley D., Schmidt M. **The base of the proteasome regulatory particle exhibits chaperone-like activity.** Nat. Cell. Biol. (1999) 1: 221-226

45. Orlowski M. **Selective activation of the 20 S proteasome (multicatalytic proteinase complex) by histone h3.** Biochemistry (2001) 40: 15318-15326

46. Fagioli C., Mezghrani A., Sitia R. **Reduction of Interchain Disulfide Bonds Precedes the Dislocation of Ig-μ Chains from the Endoplasmic Reticulum to the Cytosol for Proteasomal Degradation.** J. Biol. Chem. (2001) 276: 40962-40967

47. Yu H., Kaung G., Kobayashi S., Kopito R.R. **Cytosolic Degradation of T-cell Receptor Chains by the Proteasome.** J. Biol. Chem. (1997) 272: 20800-20804

48. Groll M., Bajorek M., Kohler A., Moroder L., Rubin D.M., Huber R., Glickman M.H., Finley, D. **A gated channel into the proteasome core particle**. Nat. Struct. Biol. (2000) 7: 1062-1067

49. Wilk S., Chen W.E. **Synthetic peptide-based activators of the proteasome**. Mol. Biol. Rep. (1997) 24: 119-124

50. Kisselev A.F., Kaganovich D., Goldberg A.L. Binding of Hydrophobic Peptides to **Several Non-catalytic Sites Promotes Peptide Hydrolysis by All Active Sites of 20 S Proteasomes. EVIDENCE FOR PEPTIDE-INDUCED CHANNEL OPENING IN THE α-RINGS.** J. Biol. Chem. (2002) 277: 22260-22270

51. Wilk S., Chen W.E., Magnusson, R.P. **Properties of the nuclear proteasome activator PA28gamma (REGgamma).** Arch. Biochem. Biophys. (2000) 383: 265-271

52. Ustrell V., Hoffman L., Pratt G., Rechstiener M. **PA200, a nuclear proteasome activator involved in DNA repair.** EMBO J. (2002) 21: 3516-3525

53. Lu X., Michaud C., Orlowski M. **Heat shock protein-90 and the catalytic activities of the 20 S proteasome (multicatalytic proteinase complex)**. Arch. Biochem. Biophys. (2001) 387: 163-171

54. Schmidtke G., Emch S., Groettrup M., Holzhutther H.G. **Evidence for the Existence of a Non-catalytic Modifier Site of Peptide Hydrolysis by the 20 S Proteasome**. J. Biol. Chem. (2000) 22056-22063

55. Zachariae W., Nasmyth K. **Whose end is destruction: cell division and the anaphase-promoting complex.** Genes Dev. (1999) 13: 2039-2058

56. Kriwacki R.W, Wu J., Tennant L., Wright P.E., Siuzdak G.. **Probing protein structure using biochemical and biophysical methods. Proteolysis, matrix-assisted laser desorption/ionization mass spectrometry, high performance liquid chromatography and size-exclusion chromatography of p21Waf1/Cip/Sdi1.** J. Chromatogr. A. (1997) 777: 23-30

57. Almrud J.J., Oliveira M.A., Kern A.D., Grishin N.V., Phillips M.A., Hackret M.L. **Crystal structure of human ornithine decarboxylase at 2.1A resolution: structural insights to antizyme binding.** J. Mol. Biol. (2000) 295: 7-16.

58. Seeler JS, Dejean A. **SUMO: of branched proteins and nuclear bodies**. Oncogene. (2001) 20(49):7243-9.

59. Melchior F, Schergaut M, Pichler A. **SUMO: ligases, isopeptidases and nuclear pores.** Trends Biochem Sci. (2003) 28(11):612-8.

60. Johnson ES. **Protein modification by SUMO.** Annu Rev Biochem.(2004) 73:355-82.

61. Schwartz DC, Hochstrasser M. **A superfamily of protein tags: ubiquitin, SUMO and related modifiers.** Trends Biochem Sci. (2003) 28(6):321-8.

62. Muller S, Ledl A, Schmidt D. **SUMO: a regulator of gene expression and genome integrity.** Oncogene. (2004) 23(11):1998-2008.

63. Melchior F, Hengst L. **SUMO-1 and p53**. Cell Cycle. (2002) 1(4):245-9.

(Introduction and Literature Review)

Chapter 4

The Antizyme Family : the Mediator of Ubiquitin-Independent Proteasomal Degradation

4.1. Physiological Role of Antizyme

4.1.1 Polyamine Homeostasis

The polyamines (spermidine, spermine and putrescine) are small abundant multivalent organic cations that are largely bound to negatively charged molecules, such as DNA and RNA [1]. Polyamines are not only essential for cell growth and differentiation, but also are important in cell proliferation and tumour development. Polyamines are found in prokaryotes and eukaryotes. Ornithine Decarboxylase (ODC) is the first rate-limiting enzyme in polyamine biosynthetic pathway, catalyzing the decarboxylation of ornithine to putrescine, which is then converted into spermidine and spermine (Figure 4-1) [1]. Elevated ODC activity or overexpression of ODC can lead to the tumorigenic transformation [2-5] and are found in most human cancers [6].

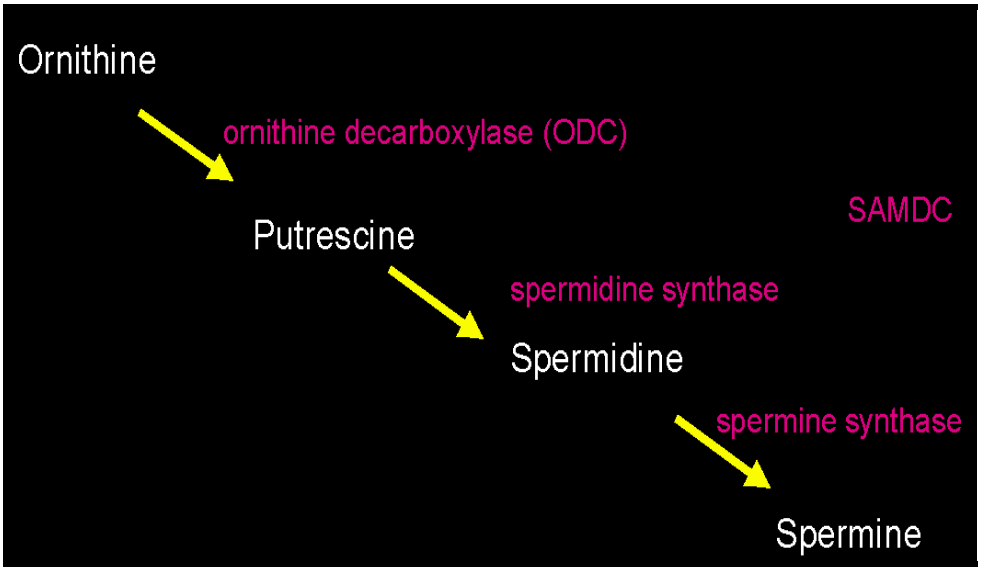


Figure 4-1: Polyamine Biosynthesis

(Diagram adapted from website of Northern IIIinois University-John Mitchell Lab)

4.1.2. Role of Antizyme in Regulatory Feedback Control of Polyamine Pool

Since intracellular polyamine level plays important roles in both normal and neoplastic growth, the polyamine levels, including polyamine uptake [7-8] and biosynthesis [9], are tightly regulated, as shown in Figure 4-2. Antizyme (AZ) and ODC together participate in the feedback regulation of the polyamine homeostasis, restricting the pools of polyamines, the downstream products of ODC enzymatic activity.

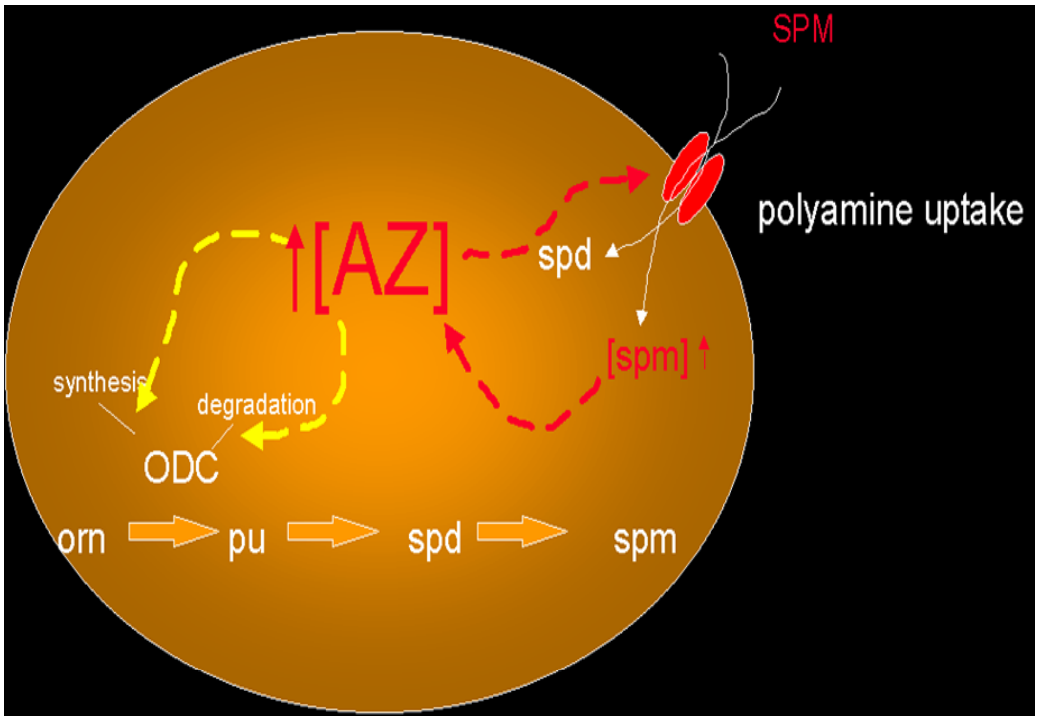


Figure 4-2: Control of Polyamine Pool by Antizyme
(Diagram adapted from website of Northern IIIinois University-John Mitchell Lab)

4.1.2.1. Mechanism of AZ-mediated ODC Degradation

Antizymes bind ODC with very high affinity, therefore stoichiometrically forming the ODC-AZ heterodimers, thereby preventing the formation of the enzymatically active ODC homodimers, which are of the weak self-association. Mutagenesis and structural studies have

identified regions of ODC and AZ required for their interaction and for ODC degradation. A C-terminal ODC domain is required for AZ-dependent degradation. Within AZ, the C-terminal is sufficient for interaction with ODC, but the proteasomal degradation requires additional domain within the AZ N-terminus (Figure 4-3).

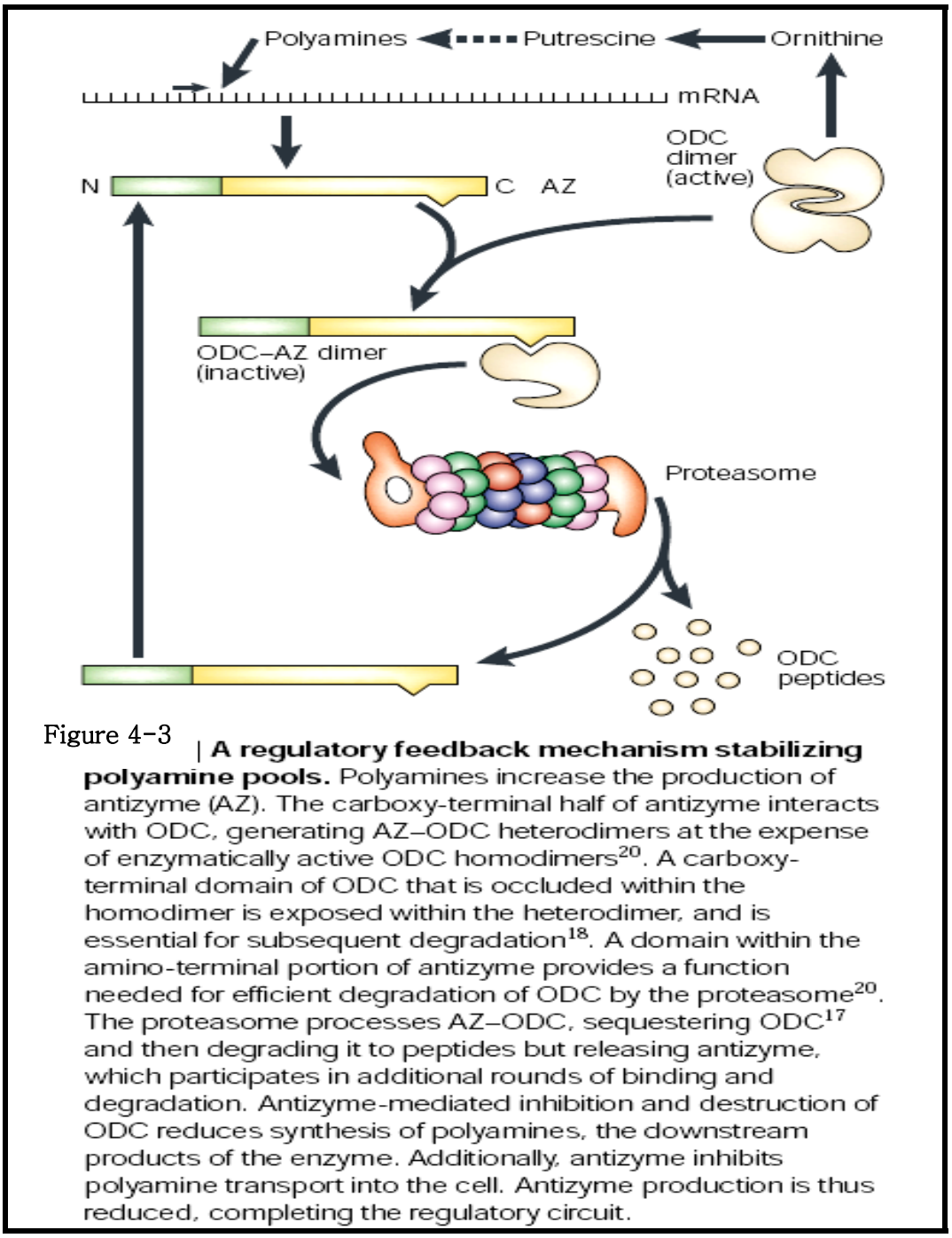


Figure 4-3 | **A regulatory feedback mechanism stabilizing polyamine pools.** Polyamines increase the production of antizyme (AZ). The carboxy-terminal half of antizyme interacts with ODC, generating AZ–ODC heterodimers at the expense of enzymatically active ODC homodimers[20]. A carboxy-terminal domain of ODC that is occluded within the homodimer is exposed within the heterodimer, and is essential for subsequent degradation[18]. A domain within the amino-terminal portion of antizyme provides a function needed for efficient degradation of ODC by the proteasome[20]. The proteasome processes AZ–ODC, sequestering ODC[17] and then degrading it to peptides but releasing antizyme, which participates in additional rounds of binding and degradation. Antizyme-mediated inhibition and destruction of ODC reduces synthesis of polyamines, the downstream products of the enzyme. Additionally, antizyme inhibits polyamine transport into the cell. Antizyme production is thus reduced, completing the regulatory circuit.

(Figure adapted from ref [10])

4.2. Regulation of Antizyme

4.2.1. Synthesis by Polyamine-Induced Translational Frameshift

In response to elevation in cellular polyamine levels, the expression of functional AZ is rapidly induced by a remarkable unique ribosomal frameshift [11] mechanism (Figure 4-4).

Figure 4-4: Antizyme-Induced Translational Frameshifting
(Figure adapted from ref [10])

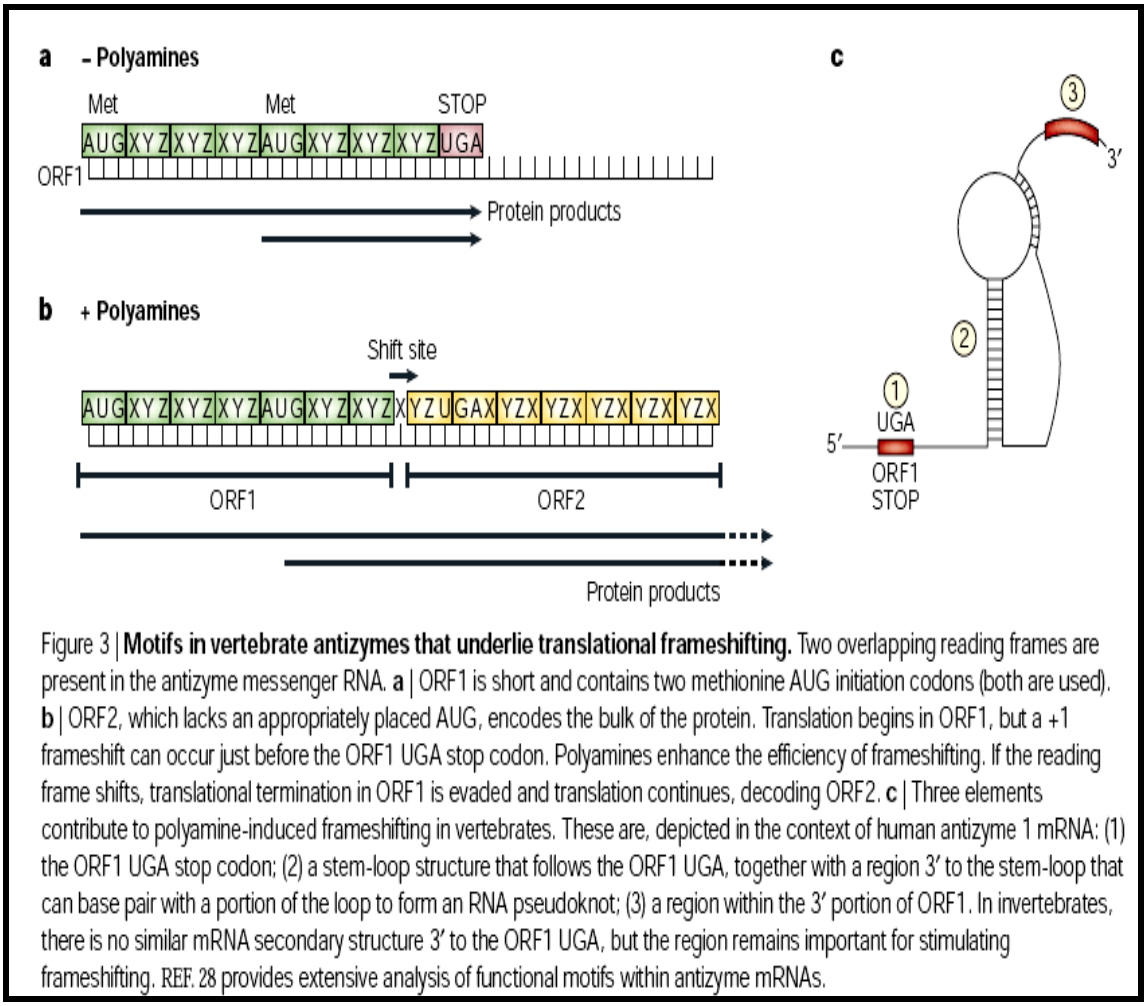


Figure 3 | **Motifs in vertebrate antizymes that underlie translational frameshifting.** Two overlapping reading frames are present in the antizyme messenger RNA. **a** | ORF1 is short and contains two methionine AUG initiation codons (both are used). **b** | ORF2, which lacks an appropriately placed AUG, encodes the bulk of the protein. Translation begins in ORF1, but a +1 frameshift can occur just before the ORF1 UGA stop codon. Polyamines enhance the efficiency of frameshifting. If the reading frame shifts, translational termination in ORF1 is evaded and translation continues, decoding ORF2. **c** | Three elements contribute to polyamine-induced frameshifting in vertebrates. These are, depicted in the context of human antizyme 1 mRNA: (1) the ORF1 UGA stop codon; (2) a stem-loop structure that follows the ORF1 UGA, together with a region 3′ to the stem-loop that can base pair with a portion of the loop to form an RNA pseudoknot; (3) a region within the 3′ portion of ORF1. In invertebrates, there is no similar mRNA secondary structure 3′ to the ORF1 UGA, but the region remains important for stimulating frameshifting. REF. 28 provides extensive analysis of functional motifs within antizyme mRNAs.

The antizyme mRNA has two overlapping open reading frames (ORFs), comprised of a short ORF1 and a long ORF2. The short ORF1 has two potential translational start codons and a stop codon at the frameshift site, but yet does not appear to produce a protein product. The long ORF2 encodes most of the protein but lacks an initiation start site. ORF2 is in the +1

reading frame in relation to ORF1. Therefore, synthesis of a functional, full-length antizyme requires initiation of translation at the start site in ORF1 followed by a translational +1 frameshift at the codon immediately preceding the short ORF1 stop codon. This frameshift is greatly enhanced by the elevation of the intracellular polyamines, the mechanism of which is not completely understood. Two conserved elements that are critical for the polyamine effect, are present in the AZ sequence. One is located 5' to the frameshift site, whereas the other one is situated immediately following the frameshift site, which forms a pseudoknot that stimulates the frameshifting. The induced AZ degrades ODC, thereby impairing further polyamine synthesis [12].

AZ also inhibits polyamine uptake and stimulates polyamine secretion [7-8]. This forms the basis for the feedback control of polyamine levels.

4.2.1.1. The Family of Mammalian Antizymes

In human, antizymes consist of a family of at least four members (AZ1-4) [13]. All the four isoforms share the following characteristics: (1) high structural homology, particularly in the C-terminus; (2) interaction with ODC, which decreases ODC abundance and/or activity; (3) polyamine-induced translational frameshifting that depends on the conserved motifs near the frameshifting site [14]. The best studied member of family is antizyme-1 (AZ1) which consists of 227 amino acids and is widely distributed many cell types [12]. Antizyme-2 has the similar tissue distribution as antizyme-1, but less abundant [15]. Antizyme-3 is testis

specific and is expressed in certain stages of spermatogenesis [16-17]. Antizyme-4 was isolated as an EST clone from a human brain cDNA library and not fully characterized yet [14].

4.2.1.2. Multiple Forms of Antizyme-1

As shown in Figure 4-5, there are multiple forms of AZ1 (29 and 24.5 kDa) due to the presence of two potential start sites and the post-translational modification, such as phosphorylation. The 24.5 kDa form predominates and is synthesized in larger amount whereas the 29 kDa form contains a mitochondrial targeting sequence and is found only in mitochondria [18-20].

Figure 4-5: Multiple forms of Antizyme
(Diagram adapted from website of Northern IIIinois University-John Mitchell Lab)

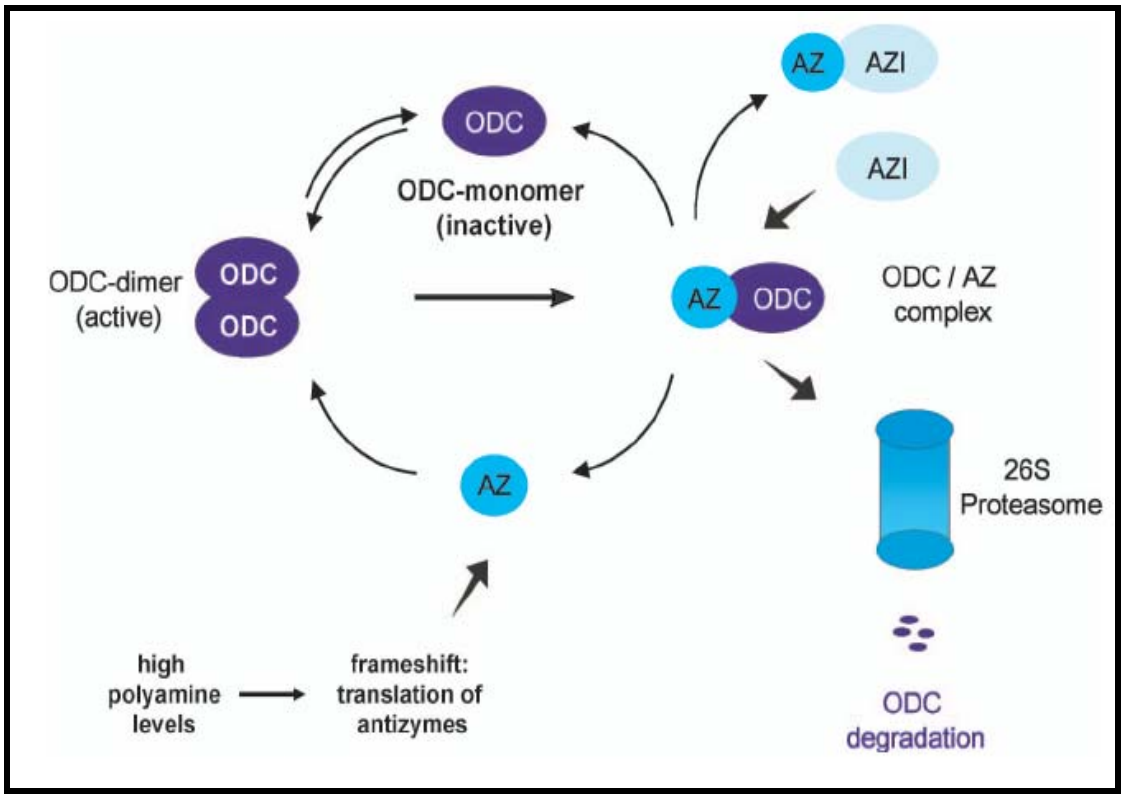
4.2.2. Regulation of Antizyme Protein Stability

Unlike ODC, the degradation of antizyme is mediated through the ubiquitin-dependent pathway. Studies from mammalian and yeast had found that polyamines interfered with the ubiquitination of antizyme and thus blocked the degradation of antizyme [9, 21].

4.2.3. Inhibition of Antizymes by Antizyme Inhibitor (AZI)

In addition to the control of antizyme synthesis by polyamines, all members of the antizyme family are inactivated by a protein, termed the antizyme inhibitor (AZI) [22-23], as shown in Figure 4-6.

Figure 4-6: Schematic diagram showing antizyme (AZ) and antizyme inhibitor (AZI)-mediated regulation of ODC (Diagram adapted from ref [16])



AZI shares substantial homology with ODC but it lacks any enzymatic activity [24]. Antizyme binds more tightly to AZI than they bind to ODC, and thus releasing the ODC from the antizyme:ODC complex [23]. Relief of ODC from antizyme inhibition leads to ODC stabilization. Besides being identified as a physiological player of the polyamine pathway, AZI also plays some possible roles in cell proliferation and cell cycle progression, as shown by the rapid induction of AZI mRNA upon growth stimulation [23] and its upregulation in certain forms of human cancers [25]. Normally AZI has a short half-life (<30 min) and it is ubiquitinated and subsequently degraded by 26S proteasome. However, binding of antizyme stabilizes the AZI by preventing its ubiquitination [26].

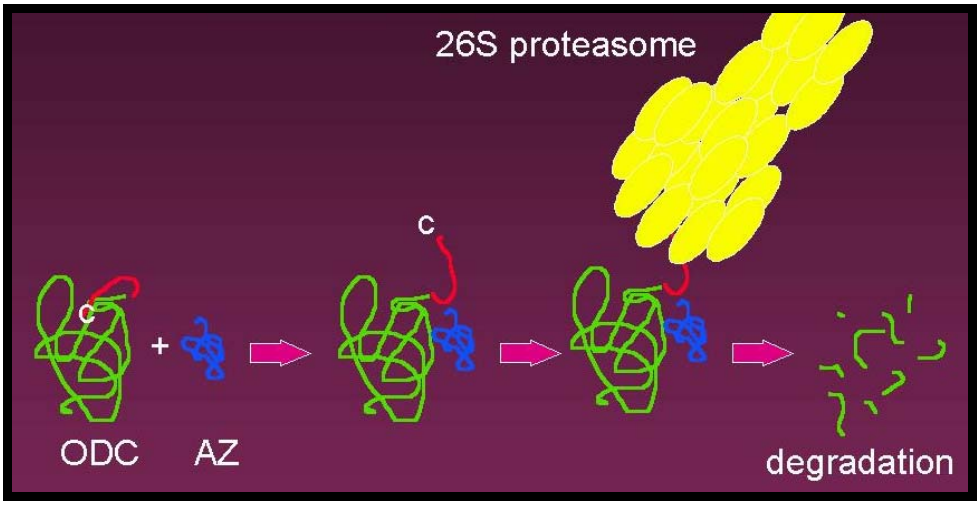
4.3. Antizyme as Alternative Degradation Recognition Signal

Although generally most proteins are ubiquitinated and are therefore recognized and targeted for proteasomal degradation, exceptionally there is a small set of proteasome substrates that are not ubiquitinated and instead use other signals to mark themselves for degradation [27]. Studies on the degradation of ornithine decarboxylase (ODC) demonstrated that poly-ubiquitin tag is no longer the only signal recognized by the 26S proteasome [28-29]. The recognition of ODC by the 26S proteasome is mediated through non-covalent interaction between ODC and the polyamine-induced antizyme (AZ) [28-31].

4.3.1. Mechanism of Antizyme-Mediated Ub-Independent Protein Degradation of ODC

Antizyme promotes the ODC degradation by enhancing ODC association with proteasome, rather than accelerating the rate of proteasomal processing [32]. The attachment of antizyme causes the conformational change in ODC, thereby exposing its C-terminal degradation signal for recognition by 26S proteasome [33], as shown in Figure 4-7. Unlike ubiquitin, AZ is usually spared from destruction and is released from the ODC:AZ complex at the proteasome [29]. Therefore, a single AZ can catalyze multiple rounds of ODC degradation. Interestingly, *in vitro* [34] and *in vivo* [35-36] studies had revealed that ubiquitin did not participate in this AZ-mediated protein degradation process. However, substrate-linked or free polyubiquitin chains could competitively inhibit the AZ-stimulated degradation of ODC [37], suggesting that AZ-ODC and polyubiquitin chains share the same recognition element(s) on the proteasome. In another words, ODC may adapt the molecular mimicry for proteasomal recognition using the antizyme as the functional equivalent of polyubiquitin [38].

Figure 4-7: Antizyme-induced ODC Degradation
(Diagram adapted from website of Northern IIIinois University-John Mitchell Lab)



4.3.2. Other Proteasomal Substrates Targeted by Antizyme

4.3.2.1. Cyclin D1

Although initially thought to accelerate only the degradation of ODC, AZ1 had recently been demonstrated to bind other proteins and facilitate their proteasomal degradation. A study had shown that AZ1 interacted specifically with cyclin D1 and accelerated Ub-independent degradation of cyclin D1 [39]. Stable mutant of cyclin D1 (T286A), which could no longer be targeted for Ub-dependent degradation, was rapidly degraded in the presence of AZ1 overexpression [39].

4.3.2.2. Smad1

Another study had identified Smad1 and proteasomal subunit HsN3 as the interacting proteins of AZ1. HsN3 is one of the seven subunits of the 20S proteasome, the catalytic core of the 26S proteasome. Indeed, AZ1, Smad1 and HsN3 together formed a ternary complex [40-41]. Interestingly, Smad1 also bound ubiquitin and therefore Smad1 ubiquitination, together with AZ and HsN3, played important roles in proteasomal targeting and degradation of Smad1 [40]. Unlike ODC, whose degradation is completely Ub-independent and AZ1-dependent, degradation of cyclin D1 and Smad1 can also be regulated via the Ub-dependent pathway [42].

4.4. Differences Between Antizyme-Mediated vs Ubiquitin-Mediated Protein Degradation

Both Ub-dependent and AZ-mediated degradation target protein to the same 26S proteasome. But, Ub-dependent proteolysis is more complex as several enzyme-catalyzed reactions are required for the covalent attachment of ubiquitins to the target proteins. Instead, AZ-dependent degradation is a very rapid process with no requirement for additional components [38].

They also differ in their ways for tag clearance [38]. Polyubiquitin must be removed by the cleavage of covalent bonds through the intrinsic proteasome enzymatic activity, while the antizyme just dissociates from ODC. With the present limited knowledge, their differences seem to emerge at the earlier steps and they may converge after proteasomal association, given the observation that polyubiquitin and ODC:AZ competitively bind a proteasomal recognition site. However, it may be more complex than what we postulate.

4.5. Conclusion

AZ has evolved recently as an ancient gene family and AZ-mediated, ubiquitin-independent degradation is found to be evolutionarily conserved from *S.cerevisiae* to human [29]. It would be interesting to probe into the role played by AZ-mediated degradation in comparison to Ub-dependent degradation and its specificity.

4.6. References

1. Igarashi K., Kashiwagi K. **Polyamines: mysterious modulators of cellular functions.** Biochem. Biophys. Res. Commun. (2000) 271: 559-564

2. Holtta E., Auvinen M, Andersson L.C. **Polyamines are essential for cell transformation by pp60v-src: Delineation of molecular events relevant for the transformed phenotype.** J. Cell. Biol. (1993) 122: 903-914

3. Shantz L.M., Pegg A.E. **Ornithine decarboxylase induction in transformation by H-Ras and RhoA.** Cancer Res. (1998) 58: 2748-2753

4. Moshier J.A., Dosescu J., Skunca M., Luk G.D**. Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression.** Cancer Res. (1993) 53: 2618-2622

5. Auvinen M., Paasinen A., Andersson L.C., Holtta E. **Ornithine decarboxylase activity is critical for cell transformation.** Nature (1992) 360: 355-358

6. Gerner E.W., Meyskens F.L Jr. **Polyamines and cancer: old molecules, new understanding.** Nat. Rev. Cancer (2004) 4: 781-792

7. Sakata k., Kashiwagi K., Igarashi K. **Properties of a polyamine transporter regulated by antizyme.** Biochem. J. (2000) 347: 297-303

8. Mitchell J.L., Judd G.G., Bareyal-Leyser A., Ling S.Y. **Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells**. Biochem. J. (1994) 299:19-22

9. Palanimurugan R., Scheel H., Hofmann K., Dohmen R.J. **Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme**. EMBO J. (2004) 23: 4857-4867

10. Coffino P. **Regulation of cellular polyamines by antizyme.** Nat. Rev. Mol. Cell. Biol. (2001) 2: 188-194

11. Matsufuji S., Matsufuji T., Miyazaki Y., Murakami Y., Atkins J.F., Gesteland R.F., Hayashi S. **Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme.** Cell (1995) 80: 51-60

12. Ivanov I., gesteland R., Atkins J. **Antizyme expression: a subversion of triplet decoding, which is remarkably conserved by evolution, is a sensor for an autoregulatory circuit.** Nucleic Acids res. (2000) 28: 3185-3196

13. Ivanov I.P. Matsufuji S., Murakami Y., Gesteland R.F, Atkins J.F. **Conservation of polyamine regulation by translational frameshifting from yeast to mammals**. EMBO J. (2000) 19: 1907-1917

14. Coffino P. **Antizyme, a mediator of ubiquitin-independent proteasomal degradation.** Biochimie (2000) 83: 319-323

15. Zhu C., Lang D.W., Coffino P. **Antizyme2 is a negative regulator of ornithine decarboxylase and polyamine transport.** J Biol. Chem. (1999) 274: 26425-26430

16. Ivanov I., Rohrwasser A., Terreros D., Gesteland R., Atkins J. **Discovery of a spermatogenesis, stage specific ornithine decarboxylase antizyme: antizyme 3.** Proc. Natl. Acad. Sci. (2000) 97: 4808-4813

17. Tosaka y., Tanaka H., Yano.Y., Masai K., Nozaki M., Yomogida K, Otani S., Nojima H., Nishimune Y. **Identification and characterization of testis specific ornithine decarboxylase antizyme (OAZ-t) gene: Expression in haploid germ cells and polyamine-induced frameshifting.** Genes Cells (2000) 5: 265-276

18. Feith D.J., Shantz L.M., Pegg A.E. **Targeted antizyme expression in the skin of transgenic mice reduces tumor promoter induction of ornithine decarboxylase and decreases sensitivity to chemical carcinogenesis**. Cancer Res. (2001) 61: 6073-6081

19. Gandre S., Bercovich Z., Kahana C. **Mitochondrial localization of antizyme is determined by context-dependent alternative utilization of two AUG initiation codons**. Mitochondrion (2003) 2: 245-256

20. Hoshino K., Momiyama E., Yoshida K., Nishimura K., Sakai S., Toida T., Kashiwagi K., Igarashi K. **Polyamine Transport by Mammalian Cells and Mitochondria: ROLE OF ANTIZYME AND GLYCOSAMINOGLYCANS.** J. Biol. Chem. (2005) 280: 42801-42808

21. Gandre S., Bercovich Z, Kahana C. **Ornithine decarboxylase-antizyme is rapidly**

degraded through a mechanism that requires functional ubiquitin-dependent proteolytic activity. Eur. J. Biochem. (2002) 269: 1316-1322.

22. Mangold U., Leberer E. **Regulation of all members of the antizyme family by antizyme inhibitor.** Biochem. J. (2005) 385: 21-28

23. Nilsson J., Grahn B., Heby, O. **Antizyme inhibitor is rapidly induced in growth-stimulated mouse fibroblasts and releases ornithine decarboxylase from antizyme suppression.** Biochem. J. (2000) 346: 699-704

24. Murakami Y., Ichiba T., Matsufuji S., Hayashi S. **Cloning of Antizyme Inhibitor, a Highly Homologous Protein to Ornithine Decarboxylase.** J. Biol. Chem. (1996) 271: 3340-3342

25. Jung M.H, Kim S.C., Jeon G.A, Kim S.H., Kim Y., Choi K.S., Park S.I., Joe M.K., Kimm K. **Identification of differentially expressed genes in normal and tumor human gastric tissue.** Genomics (2000) 69: 281-286

26. Bercovich Z., Kahana C. **Degradation of Antizyme Inhibitor, an Ornithine Decarboxylase Homologous Protein, Is Ubiquitin-dependent and Is Inhibited by Antizyme.** J. Biol. Chem. (2004) 279: 54097-5410

27. Verma R., Deshaies R.J. **A proteasome howdunit: the case of missing signal**. Cell (2000) 101: 341-344

28. Coffino, P. **Degradation of ornithine decarboxylase.** in Peters J.M., Finley J.R.H.D.(eds) Ubiquitin and the Biology of the Cell (1998), New York: Plenum Press, 411-427

29. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., Ichihara, A. **Ornithine decarboxylase is degraded by the 26S Proteasome without ubiquitination.** Nature (1992), 360: 597-599

30. Coffino, P. **Antizyme: A family of proteins that modulate cellular polyamines**. Nature Rev Mol Cell Biol (2001) 2:188-194

31. Hayashi S., Murakami Y., Matsufuji S. **Ornithine decarboxylase antizyme: a novel type of regulatory protein.** Trends Biochem. Sci (1996) 21: 27-30

32. Zhang, M., Pickart, C.M., and Coffino, P. **Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate**. EMBO J (2003) 22, 1488-1496

33. Li X., Coffino, P. **Degradation of ornithine decarboxylase: exposure of the C-terminal target by a polyamine-inducible inhibitory protein**. Mol. Cell. Biol (1993) 13: 2377-2383

34. Bercovich Z., Rosenberg-Hasson Y., Ciechanover A., Kahana C. **Degradation of ornithine decarboxylase in reticulocyte lysate is ATP-dependent but ubiquitin-independent.** J. Biol. Chem. (1989) 264:15949-15952

35. Rosenberg-Hasson Y., Bercovich Z. , Ciechanover A., Kahana C. **Degradation of ornithine decarboxylase in mammalian cells is ATP-dependent but ubiquitin-independent.** Eur. J. Biochem. (1989) 185: 469-474

36. Glass J.R., Gerner E.W. **Spermine mediates degradation of ornithine decarboxylase by a non-lysosomal, ubiquitin-independent mechanism.** J. Cell Physiol. (1987) 130:133-141

37. Zhang M., Pickart C.M. and Coffino P. **Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate.** EMBO J. (2003) 22-1488-1496

38. Hoyt M.A., Coffino P. **Ubiquitin-free routes into the proteasome** CMLS, Cell. Mol.Life. Sci. (2004) 61:1-5

39. Newman R.M., Mobascher A., Mangold U., Koike C., Diah S., Schmidt M., Finley D., Zetter B.R. **Antizyme targets cyclin D1 for degradation: A novel mechanism for cell growth repression.** J. Biol. Chem. (2004) 279: 41504-41511

40. Lin Y., Martin J., Gruendler C, Farley J., Meng X., Li B.Y.,Lechleider R., Huff C., Kim R.H., Grasser W.A., Paralkar V., Wang T. **A novel link between the proteasome pathway and the signal transduction pathway of the bone morphohehetic proteins (BMPs).** BMC Cell. Biol. (2002) 3:15

41. Gruendler C., Lin Y., Farley J., Wang T. **Proteasomal degradation of Smad 1 induced by bone morphogenetic proteins.** J. Biol. Chem. (2001) 276: 46533-46543

42. Mangold U. **The antizyme family: Polyamines and beyond.** IUBMB life. (2005) 57(10): 671-676

SECTION 2

Experimental Procedures

(Experimental procedures)

CHAPTER 5

Materials

5.1 Yeast Strain

Yeast Dosage Suppressor Screen

5.1.1. **EGY188** MATa trp1 his3 ura3 leu2::2 LexAop-LEU2

5.2. Bacterial Strain

General

5.2.1. **DH5α** competent cells

GeneEditor *in vitro* Site-Directed Mutagenesis

5.2.2. **BMH 71-18 *mutS*** competent cells

5.2.3. **JM109** competent cells

5.3. Cell Lines

5.3.1. **HeLa, COS7, NIH-3T3, U2OS, MCF7, SW480, K562, 2774** were obtained from the American Type Culture Collections (ATCC).

5.3.2. **HeLa Tet-On** cell line was obtained from Clontech (Cat. No. 630901)

5.3.3. **ts20TG** mouse cells were obtained from Dr. Harvey Ozer, New Jersey, USA.

5.3.4. **ts20-CHO** cells were obtained from Dr. Ger J Strous, Utrecht, Netherlands.

5.3.5. **AT2.1** Dunning rat prostate carcinoma cells were obtained from Dr. John T Isaacs, Baltimore, USA.

5.4. Primers

Primers for Yeast Dosage Suppressor Screen

5.4.1. **Primers for Determination of the Reading Frame and Identity of Positive Clones Isolated from Yeast Dosage Suppressor Screen and PCR Amplification of cDNA Clone**

5' target fusion primer: 5'-CTGATGGGAGATGCCTCC-3'

3' target fusion primer: 5'-GCCGACAACCTTGATTG-3'

5.4.2. **Primers for Sequencing the Junction between LexA and Bait Protein IAK1**

5' bait fusion primer: 5'-CGTCAGCAGAGCTTCACC-3'

5.4.3. **Primers for amplifying or sequencing the cDNA inserts in pCR2.1TOPO**

M13F: 5'-GTAAAACGACGGCCAG-3'

M13R: 5'-CAGGAAACAGCTATGAC-3'

Primers for Northern Blot

5.4.4. **Primers specific for β-actin probe**

β-actin-F: 5'-GTGATGGTGGGCATGGGTCA-3'

β-actin-R: 5'-TTAATGTCACGCACGATTTCCC-3'

Primers for Cloning of AKIP

5.4.5. **Primers for PCR Cloning of full-length AKIP**

AKIP-F: 5'-CGCTGCCGATCGGGGCCGACT-3'

AKIP-R: 5'-ACTACGGATCACAGCAGCAAC-3'

Primers for General Sequencing/PCR Screening

5.4.6. **Primers for Sequencing or PCR Screening of the cDNA Insert in pCEP4**

pCEP-F: 5'-AGAGCTCGTTTAGTGAACCG-3'

EBV-R: 5'-GTGGTTTGTCCAAACTCATC-3'

5.4.7. **Primers for Amplifying or Sequencing the cDNA inserts in pCDNA3**

Sp6: 5'-ATTTAGGTGACACTATAG-3'

T7: 5'-TAATACGACTCACTATAGGG-3'

Primers for Tagging AKIP

5.4.8. **Primers for Generating Flag-tagged AKIP-TR**

1st PCR

AKIP(TR)-F: 5'-CAAGGACGACGATGACAAGGAATTCGGCACG-3'

AKIP-R: 5'- TCATTTGCCCCGCAGGTAGATCTTG -3'

2nd PCR

FLAG: 5'-GCCATGGACTACAAGGACGACGAT-3'

AKIP-R: 5'- TCATTTGCCCCGCAGGTAGATCTTG -3'

5.4.9. **Primers for Generating Flag-tagged Full Length AKIP**

1st PCR

AKIP(FL)-F: 5'-CAAGGACGACGATGACAAGACCCTGCTCCTG-3'

AKIP-R: 5'- TCATTTGCCCCGCAGGTAGATCTTG -3'

2nd PCR

FLAG: 5'-GCCATGGACTACAAGGACGACGAT-3'

AKIP-R: 5'- TCATTTGCCCCGCAGGTAGATCTTG -3'

5.4.10. **Primers for Generating HA-tagged AKIP-TR**

1st PCR

AKIP(TR-HA)-F: 5'-GTCCCAGACTACGCTGATACCGGGACCGCAG -3'

AKIP-R: 5’- TCATTTGCCCCGCAGGTAGATCTTG -3’

2nd PCR

HA: 5’-GCCATGGCATACCCATACGACGTCCCAGACTAC-3’

AKIP-R: 5’- TCATTTGCCCCGCAGGTAGATCTTG -3’

5.4.11. **Primers for Generating HA-tagged Full Length AKIP**

1st PCR

AKIP(FL-HA)-F: 5’-GTCCCAGACTACGCTCTGCTCCTGGGGCGCCTG -3’

AKIP-R: 5’- TCATTTGCCCCGCAGGTAGATCTTG -3’

2nd PCR

HA: 5’-GCCATGGCATACCCATACGACGTCCCAGACTAC-3’

AKIP-R: 5’- TCATTTGCCCCGCAGGTAGATCTTG -3’

Primers for Tagging Aurora Kinase

5.4.12. **Primers for Generating Flag-tagged Human Aurora-A Kinase**

1st PCR

AIK-F: 5’-CAAGAA CGACGATGACAAGCTGGACCGATCTAAAG-3’

AIK-R: 5’-CCTGCACGATTCCTAAGACTGTT-3’

2nd PCR

AIK-FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

AIK-R: 5’-CCTGCACGATTCCTAAGACTGTT-3’

5.4.13. **Primers for Generating HA-tagged Aurora-A Kinase**

1st PCR

AIK-HA-F: 5’-GTCCCAGACTACGCTCTGGACCGATCTAAAG -3’

AIK-R: 5’- CTCCTAAGACTGTTTGCTAGC-3’

2nd PCR

HA: 5’-GCCATGGCATACCCATACGACGTCCCAGACTAC-3’

AIK-R: 5’- CTCCTAAGACTGTTTGCTAGC -3’

5.4.14. **Primers for Generating FLAG-tagged Human Aurora-B Kinase**

1st PCR

AurB-F: 5’-CAAGGACGACGATGACAAGAGGCTGGCCCAGAAGGAG-3’

AurB-R: 5’-GGACCATCAGGCGACAGATTGAAGGGCAG -3’

2nd PCR

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

AurB-R: 5’-GGACCATCAGGCGACAGATTGAAGGGCAG -3’

Primers for Tagging Antizyme and its Regulators

5.4.15. **Primers for Generating His-tagged Antizyme 1 (Wild Type or Mutant)**

1st PCR

AZ1(H)-F: 5’-CATCATAGCCTCGGTGAATTCATGGTGAAATCC-3’

AZ1-R: 5’-GCACTCGAGCTACTCCTCCTCCTCTCCCGAAGACTCTCTC-3’

2nd PCR

HIS:5’GCCATGGGGGGTTCTCATCATCATCATCATCATAGCCTCGGTG 3’

AZ1-R: 5’-GCACTCGAGCTACTCCTCCTCCTCTCCCGAAGACTCTCTC -3’

5.4.16. **Primers for Generating HA-tagged Antizyme 1 (AZ1, Wild Type or Mutant)**

1st PCR

AZ1-HA-F: 5’-GTCCCAGACTACGCTCTGGTGAAATCCTCCCTG-3’

AZ1-R: 5’-GCACTCGAGCTACTCCTCCTCCTCTCCCGAAGACTCTCTC-3’

2nd PCR

HA: 5’-GCCATGGCATACCCATACGACGTCCCAGACTAC-3’

AZ1-R: 5’-GCACTCGAGCTACTCCTCCTCCTCTCCCGAAGACTCTCTC-3’

5.4.17. **Primers for Generating HA-tagged Antizyme Inhibitor (AZI)**

1st PCR

AZI-HA-F: 5'-GTCCCAGACTACGCTCTGAAAGGATTTATTGATG-3'

AZI-R: 5'-CTCGAGTTAAGCTTCAGCGGAAAAGCT -3'

2ndPCR

HA: 5'-GCCATGGCATACCCATACGACGTCCCAGACTAC-3'

AZI-R: 5'-CTCGAGTTAAGCTTCAGCGGAAAAGCT -3'

Primers for Tagging Ubiquitin

5.4.18. **Primers for Generating His-tagged K48R Ubiquitin Mutant**

1st PCR

K48R-F: 5'-CATCATAGCCTCGGTGAATTC-3'

K48R-R: 5'-GAGTTAACCACCACGAAGTCTCAAC-3'

2ndPCR

HIS:5'GCCATGGGGGGTTCTCATCATCATCATCATCATAGCCTCGGTG 3'

K48R-R: 5'- GAGTTAACCACCACGAAGTCTCAAC-3'

Primers for Construction of Deletion Mutants

5.4.19. **Primers for Construction of FLAG-tagged Deletion Mutants of AKIP**

****DNA Template: FLAG-tagged Full Length AKIP**

ΔN99-AKIP

1st PCR

N99-F: 5'-CAAGGACGACGATGACAAGTGCGGGCCCCT-3'

AKIP-R: 5'-TCATTTGCCCCGCAGGTAGATCTTG -3'

2ndPCR

FLAG: 5'-GCCATGGACTACAAGGACGACGAT-3'

AKIP-R: 5’- TCATTTGCCCCGCAGGTAGATCTTG -3’

ΔN198-AKIP

1st PCR

N198-F: 5’-CAAGGACGACGATGACAAGAGGAAGATGTC -3’

AKIP-R: 5’-TCATTTGCCCCGCAGGTAGATCTTG -3’

2ndPCR

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

AKIP-R: 5’- TCATTTGCCCCGCAGGTAGATCTTG -3’

ΔC99-AKIP

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

C99-R: 5’-TCAGATCTGCTTGCGTCTCAGGCGTCC -3’

ΔC198-AKIP

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

C198-R: 5’-TCAGCGGATCTTCAGCACGTTTTTG -3’

5.4.20. **Primers for Construction of FLAG-tagged Deletion Mutants of Aurora-A**

****DNA template: FLAG-tagged Aurora-A Kinase**

ΔN300-AIK

1st PCR

N300-F: 5’-CAAGGACGACGATGACAAGACCCCCCTGCCATCGGCAC-3’

AIK-R: 5’- CTCCTAAGACTGTTTGCTAGC -3’

2ndPCR

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

AIK-R: 5’- CTCCTAAGACTGTTTGCTAGC -3’

ΔN600-AIK

1st PCR

N600-F: 5’-CAAGGACGACGATGACAAGACCCATGATGCTACCAGAGTC-3’

AIK-R: 5’- CTCCTAAGACTGTTTGCTAGC -3’

2nd PCR

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

AIK-R: 5’- CTCCTAAGACTGTTTGCTAGC -3’

ΔC300-AIK

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

C300-R: 5’-GCACTACATTTCAGGGGGCAGGTAGTC -3’

ΔC600-AIK

FLAG: 5’-GCCATGGACTACAAGGACGACGAT-3’

C600-R: 5’-GCACTAGAAATAACCATACAGTCTAAG -3’

Primers for RT-PCR Cloning

5.4.21. **Primers for RT-PCR Cloning of Antizyme 1(AZ1)**

AZ1-F: 5’-GAGGAATTCATGGTGAAATCCTCCCTGCAGCG -3’

AZ1-R: 5’-GCACTCGAGCTACTCCTCCTCCTCTCCCGAAGACTCTCTC-3’

5.4.22. **Primers for RT-PCR Cloning of Antizyme Inhibitor (AZI)**

AZI-F: 5’-GAATTCATGAAAGGATTTATTGATGATGC -3’

AZI-R: 5’-CTCGAGTTAAGCTTCAGCGGAAAAGCT -3’

5.4.23. **Primers for RT-PCR Cloning of Human Aurora-B Kinase**

AurB-F: 5’- CAAGGACGACGATGACAAGAGGCTGGCCCAGAAGGAG-3’

AurB-R: 5’-GGACCATCAGGCGACAGATTGAAGGGCAG -3’

Oligonucleotides for Site-Directed Mutagenesis

5.4.24. **Antizyme 1 (Frameshift Mutation)**

5’-GTGGTGCTCCGATGCCCCTC-3’

5.4.25. **Aurora-A Kinase (A box Stabilizing S51D Mutation)**
5’-CGGGTCTTGTGTCCTGACAATTCTTCCCAGCGG-3’

Primers for Real-Time PCR

5.4.26. **Primers Specific for AKIP**

AKIP(RT)-F: 5’-TTCCTGCCCAGACTGGATAC -3’

AKIP(RT)-R: 5’- CGTCTTCTTCACCAGCTTCC-3’

5.4.27. **Primers Specific for Aurora-A Kinase**

AurA(RT)-F: 5’-ATTACAGCTAGAGGCATCATG -3’

AurA(RT)-R: 5’- GGCGACAGATTGAAGGGC-3’

5.4.28. **Primers Specific for GADPH Housekeeping Gene**

GADPH(RT)-F: 5’-GGTGGTCTCCTCTGACTTCAACA -3’

GADPH(RT)-R: 5’- GTTGCTGTAGCCAAATTCGTTGT-3’

5.5. cDNA Library

Yeast Dosage Suppressor Screen

cDNA Library: HeLa cell from OriGene Technologies, Inc (Cat. No: DLH-103)
Number of Independent Clones: 9.6 x10^6
Size range of Inserts: 0.3-3.3 kb

5.6. Plasmids

5.6.1. **pCR2.1-TOPO** (Invitrogen): TA cloning vector

Yeast Dosage Suppressor Screen

5.6.2. **Bait Plasmids [pEG202]**: HIS3, 2 μm, Amp^R, constitutive ADH promoter expresses LexA-bait fusion protein

5.6.3. **Target Plasmid [pJG4-5]**: TRP1, 2 μm, Amp^R, inducible GAL1 promoter expresses B42-HA tag target fusion protein from HeLa cDNA expression libraries

5.6.4. **Reporter Gene (lacZ) Plasmid [pSH18-34]:** URA3, 2 μm, Amp^R, 8 ops-lacZ (high sensitivity)

Eukaryotic Expression Vector

5.6.5. **pCDNA3** (Invitrogen): Constitutive mammalian expression from CMV promoter

5.6.6. **pIRES** (Clontech): Bicistronic mammalian expression vector for simultaneous translation of two genes of interest from the same mRNA transcript

5.6.7. **pTRE2hyg** (Clontech): Tetracycline or doxycycline-inducible mammalian expression vector, used in conjunction with HeLa Tet-On cell line

5.6.8. **HA-tagged p21** expression construct is a gift from Dr. Michele Pagano.

5.6.9. **His-tagged wild type ubiquitin** and **HA-tagged K48R mutant ubiquitin** expression construct is a gift from Dr. Ivan Dikic.

5.6.10. **Cyclin B1** expression construct is a gift from Dr. Prochownik.

5.7. Antibodies

Custom-synthesized Antibodies

5.7.1. **Anti-AKIP**, pAb, rabbit IgG, 0.75 mg/ml, affinity-purified peptide antibody, BioGenes GmbH
Peptide synthesized for immunization: CQTPKIYLRGK

5.7.2. **Anti-human Aurora-A**, pAb, rabbit IgG, crude serum of peptide antibody
Peptide synthesized for immunization: CQNKESASKQS

Secondary Antibodies for Immunocytochemistry

5.7.3. **Alexa Fluor 488 goat anti-mouse** IgG, 2 mg/ml, Molecular Probes, A-11001

5.7.4. **Alexa Fluor 488 goat anti-rabbit** IgG, 2 mg/ml, Molecular Probes, A-11008

5.7.5. **Alexa Fluor 594 goat anti-rabbit** IgG, 2 mg/ml, Molecular Probes, A-11012

Secondary Antibodies for Western Blot

5.7.6. **Goat Anti-Mouse IgG**, **(H+L) Horseradish Peroxidase Conjugated**, 0.8 mg/ml, Pierce, 31430

5.7.7. **Goat Anti-Rabbit IgG, (H+L) Horseradish Peroxidase Conjugated**, 0.8 mg/ml, Pierce, 31460

5.7.8. **Mouse Anti-Goat IgG, (H+L) Horseradish Peroxidase Conjugated**, 0.8 mg/ml, Pierce, 31400

Aurora Kinases Antibodies

5.7.9. **Anti-Aurora A**, pAb, rabbit IgG, 1 mg/ml, Abcam, ab12324

5.7.10. **Anti-Aurora A/AIK**, pAb, rabbit IgG, Cell Signaling, 3092

5.7.11. **Anti-IAK1/mouse Aurora-A kinase**, mAb, mouse IgG1, 1mg/ml, BD Transduction Laboratories, 610939

5.7.12. **Anti-Aurora-B (H-75)**, pAb, rabbit IgG, 0.2 mg/ml, Santa Cruz Biotech, sc-25426

AKIP Antibody

5.7.13. **Anti-AKIP**, pAb, rabbit IgG, affinity-purified peptide antibody, 0.49 mg/ml, Abcam, ab3883

Antibodies for Loading Control

5.7.14. **Anti-β tubulin**, mAb, mouse IgG1, 2 mg/ml, Sigma, T4026

5.7.15. **Anti-Actin (I-19)**, pAb, goat IgG, 0.2 mg/ml, Santa Cruz Biotech, sc-1616.

Cyclin and CDK Inhibitor Antibodies

5.7.16. **Anti-Cyclin A (H-432)**, pAb, rabbit IgG, 0.2 mg/ml, Santa Cruz Biotech, sc-751

5.7.17. **Anti-Cyclin B1 (H-433)**, pAb, rabbit IgG, 0.2 mg/ml, Santa Cruz Biotech, sc-752

5.7.18. **Anti-Cyclin D1 (DCS-6)**, mAB, mouse IgG2a, 0.2 mg/ml, Santa Cruz Biotech, sc-20044

5.7.19. **Anti-p21 (C-19)**, pAb, rabbit IgG, 0.2 mg/ml, Santa Cruz Biotech, sc-397

Antizyme Antibody

5.7.20. **Anti-Antizyme (AZ)**, pAb, Rabbit IgG, crude serum, was a gift from Dr. John L A Mitchell, Northern Illinois University.

Protein Tag Antibodies

5.7.21. **Anti-FLAG M2**, mAb, mouse IgG1, 2 mg/ml, Stratagene, 200472

5.7.22. **Anti-FLAG**, pAb, rabbit IgG, 0.8 mg/ml, Sigma, F7425

5.7.23. **Anti-HA (Y-11)**, pAb, rabbit IgG, 0.2 mg/ml, Santa Cruz Biotech, sc-805

5.7.24. **Anti-HA**, mAb, mouse IgG1, 3.7 mg/ml, Sigma, H9658

5.7.25. **Anti-HA**, pAb, rabbit IgG, 0.5 mg/ml, Sigma, H6908

5.7.26. **Anti-polyHIS**, mAb, mouse IgG2a, 3mg/ml, Sigma, H1029

Nucleolus Marker Antibody

5.7.27. **Anti-Nucleolin C23 (MS-3)**, mAb, mouse IgG1, 0.2 mg/ml, Santa Cruz Biotech, sc-8031

Ubiquitin Antibody

5.7.28. **Anti-Ubiquitin (P4D1)**, mAb, mouse IgG1, 0.2 mg/ml, Santa Cruz Biotech, sc-8017

5.8. General Buffer Preparation

5.8.1. **Phosphate Buffered Saline (PBS)** [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4]

5.8.2. **TE buffer** (10 mM Tris-HCl, 1 mM EDTA, pH8.0)

Bacterial Transformation

5.8.3. **LB broth** [1% (w/v) Bacto-tryptone. 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl]

5.8.4. **LB Agar** [1% (w/v) Bacto-tryptone. 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl, 2% (w/v) bacto-agar]

5.8.5. **SOC media** [2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose]

Yeast Growth Media

5.8.6. **YPD rich medium**

Peptone	20 g
Yeast Extract	10g
Glucose	20 g
Bacto-Agar	20 g
NaOH	0.1 g

Add 1L of distilled water and sterilize by autoclaving at 121^{o}C for 15 min. For plates, cool to 50^{o}C before pouring.

5.8.7. **Synthetic Complete Drop-Out Selective Medium**

Yeast Nitrogen Base w/o amino acids	1.7 g
Synthetic Complete Drop out Mix	0.6 g
Glucose	20 g
Bacto Agar	20 g

Add 1L of distilled water and sterilize by autoclaving at 121^{o}C for 15 min

For plates, cool to 50^{o}C before pouring.

Competent E.coli Preparation

5.8.8. **TfbI buffer** [30 mM KOAc, 100 mM Rubidium Chloride (RuCl), 10 mM $CaCl_2$. $2H_2O$, 50 mM $MnCl_2.4H_2O$, 15% (v/v) glycerol, adjust pH to 5.8 with diluted acetic acid]

5.8.9. **TfbII buffer** [10 mM MOPS, 75 mM $CaCl_2$. $2H_2O$, 10 mM RuCl, 15% 9v/v] glycerol, adjust pH to 6.5 with diluted NaOH]

Agarose Gel Electrophoresis

5.8.10. **10X TBE** [0.89 M Tris, 0.89 M Boric Acid, 0.02 M EDTA]

5.8.11. **10X Sample Loading Buffer** [0.25% (w/v) Bromophenol Blue, 0.25% (w/v) Xylene Cyanol, 50% (v/v) Glycerol]

Southern Blotting

5.8.12. **20X SSPE** [3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, pH 7.4]

5.8.13. **20X SSC** [3M NaCl, 0.3M trisodium citrate, pH 7.0]

5.8.14. **Denaturation Buffer** [1.5 M NaCl, 0.5 M NaOH]

5.8.15. **Neutralization Buffer** [1.5 M NaCl, 0.5 M Tris.Cl, pH 7.0]

5.8.16. **50X Denhardt's Solution** [1% (w/v) Ficoll 400, 1% (w/v) Polyvinylpyrrolidone, 1% (w/v) BSA]

5.8.17. **Hybridization Buffer** [5X SSPE, 10X Denhardt's Solution, 100 μg/ml ssDNA, 2% SDS]

5.8.18. **Stripping Buffer** [0.1X SSC, 0.1% (w/v) SDS, 0.2 M Tris-HCl, pH 7.5]

Northern Blotting

5.8.19. **Hybridization Buffer** [1% (w/v) BSA, 1 mM EDTA, 0.5 M PO_4 Buffer, pH 7.2, 7% (w/v) SDS]

5.8.20. **Wash Buffer A** [0.5% BSA, 1 mM EDTA, 40 mM PO_4 Buffer, 5% SDS]

5.8.21. **Wash Buffer B** [1 mM EDTA, 40 mM PO_4 Buffer, 1% SDS]

5.8.22. **20X SSC** [3M NaCl, 0.3M trisodium citrate, pH 7.0]

5.8.23. **Stripping Buffer** [0.5% (w/v) SDS]

5.8.24. **10X MOPS** [0.2 M MOPS, 0.05 M Na Acetate, 0.01 M EDTA]

5.8.25. **DEPC-treated water** [0.1% DEPC (Sigma, P/N D5758) in dH_2O, stir overnight and autoclave]

5.8.26. **Sample Loading Buffer** [49.2% (v/v) Formamide, 16.4% (v/v) Formaldehyde, 9.8% (v/v) 10X MOPS, 24.6% of 0.1% Bromophenol Blue/50% Glycerol]

SDS-PAGE and Western Blotting

5.8.27. **Lower Tris**, **pH 8.8** [1.5 M Tris base, 0.4% (w/v) SDS, adjust pH to 8.8 with conc. HCl]

5.8.28. **Upper Tris**, **pH 6.8** [0.5 M Tris base, 0.4% (w/v) SDS, adjust pH to 6.8 with conc. HCl]

5.8.29. **10X Laemli Running Buffer** [0.25 M Tris Base, 1.92 M Glycine, 1% (w/v) SDS]

5.8.30. **Blotting Buffer** [10% (v/v) 10X Laemli Buffer, 20% (v/v) Methanol]

5.8.31. **10X TBS** [1.5 M NaCl, 0.2 M Tris base]

5.8.32. **5X Sample Buffer** [0.6 M Tris, pH 6.8, 6.25% (w/v) SDS, 25% (v/v) Glycerol, 20% (v/v) Mercaptoethanol, 0.063% (w/v) Bromophenol Blue]

5.8.33. **Blocking Buffer** [5% non-fat milk powder in 1X TBS]

5.8.34. **Diluent Buffer for 1^o Antibody** [2% BSA in 1X TBS, 0.02% sodium azide]

5.8.35. **Wash Buffer** [1X TBS, 0.05% Tween 20]

Immunocytochemistry

5.8.36. **Blocking Buffer** [1X TBS, 1% (w/v) BSA, 0.1% (v/v) Triton X-100, 10% (v/v) goat serum, 0.02% (w/v) sodium azide]

Flow Cytometry (FACS)

5.8.37. **PI Staining Buffer**[1X PBS, 0.1%(v/v)Triton-X100, 50µg/ml PI, 100µg/ml Rnase A]

5.9. Buffers for *In vivo* Ubiquitination Assay

5.9.1. **Lysis Buffer G** [6 M Guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 10 mM imidazole]

5.9.2. **Wash Buffer I** [Buffer G diluted in 25 mM Tris-HCl at 1:4 ratio, pH 6.8, 20 mM imidazole]

5.9.3. **Wash Buffer II** [25 mM Tris-HCl, pH 6.8, 20 mM imidazole]

5.9.4. **Elution Buffer (2X SDS Sample Buffer)** [90 mM Tris.Cl, pH 6.8, 20%(v/v) Glycerol, 2% (w/v) SDS, 0.1 M EDTA, 0.1 M DTT, 0.02% (w/v)Bromophenol Blue]

5.10. Buffers for Immunoprecipitation

General Immunoprecipitation

5.10.1. **Lysis Buffer** [1X TBS, 10% (v/v) glycerol, 1% (v/v) NP-40, 1X protease inhibitor cocktail]

5.10.2. **Wash Buffer I** [1X TBS, 10% (v/v) glycerol, 0.5% (v/v) NP-40, 1% BSA]

5.10.3. **Wash Buffer II** [1X TBS, 10% (v/v) glycerol, 0.5% (v/v) NP-40]

Flag Immunoprecipitation-- EzView Red Anti-FLAG M2 Affinity Gel

5.10.4. **Bead Equilibration Buffer** [50 mM Tris HCl, pH 7.4, 150 mM NaCl]

5.10.5. **Lysis Buffer** [50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1X Protease Inhibitor Cocktail, 1% (v/v) Triton X-100]

5.10.6. **Wash Buffer** [50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100]

5.10.7. **Elution Buffer (2X sample buffer)** [125 mM Tris HCl, pH 6.8, 4% (w/v) SDS, 20% Glycerol (v/v), 0.004% (w/v) Bromophenol Blue, 100 mM 2-Mercaptoethanol]

(Experimental Procedures)

CHAPTER 6

Methods

6.1. Methods for Yeast Dosage Suppressor Screen

6.1.1. Yeast Transformation—LiAc Method

The yeast strain was inoculated in 10 ml of YEPD or appropriate drop-out medium and incubated at 30°C with shaking at 250 rpm overnight to stationary phase ($O.D._{600}$ >1.5). $O.D._{600}$ of the overnight culture was measured and the culture was diluted to final $O.D_{600}$=0.1 in a final volume of 50 ml. The diluted culture was grown at 30°C with shaking for 4-6 hours until $O.D_{600}$ =0.4-0.45 (log phase). The culture was then harvested at 3000 rpm, 5 min, RT. The cell pellet was washed once in 5 ml of sterile dH_2O and resuspended in 0.25 ml of 1x TE / 0.1M LiAc, which was sufficient for 5 transformation reactions. Fifty μl of cell suspension was aliquoted into 1.5 ml microcentrifuge tube. One hundred μg of ssDNA (boiled for 5 min and quick chilled on ice for 5 min) and 1 μg (linearized) or 0.1 μg (circular) DNA were added to the cell suspension. Three hundreds and ninety five μl of transformation reaction mix (1X TE, 0.1M LiAc, 40% PEG) was then added. The mixture was vortexed vigorously and incubated at 30°C for 30 min. Prior to the heat shock, DMSO was added to a final conc of 10% and mixed by gentle inversion. The mixture was heat shocked in the 42°C water bath for 20 min and later chilled on ice for 2 min. The heat-shocked cell mixture was transferred to 15 ml drop-out medium and allowed to recover for 1 hour at 30°C with shaking. The transformed cells were harvested at 3000 rpm, 5 min, RT and resuspended in dH_2O before plating on the drop-out medium plate. The plate was incubated at 30°C for 3-4 days.

6.1.2. Yeast Dosage Suppressor Large-scale cDNA Library Screening

For cDNA library screening, EGY188 cells were grown to log phase in YPD and co-transformed with plasmids containing 150 μg of Aurora-A-pEG202 and 150 μg HeLa cell cDNAs in pJG4-5 using the LiAc method. The resulting transformants were selected on galactose containing synthetic dropout media lacking histidine and tryptophan (SD-His-Trp). Yeast clones, which survived the Aurora-A mediated cytotoxicity, were reconfirmed by streaking onto glucose- and galactose-containing synthetic dropout media, and the clones which grew only on the galactose-containing plates were characterized further by sequencing.

6.1.3. Yeast Colony PCR

Single yeast colony was inoculated into 20 μl of dH_2O. The cell suspension was then boiled for 5 min. Five μl of the boiled cell suspension was used for setting up the PCR reaction.

6.1.4. Rapid Yeast Plasmid Isolation

Two ml of yeast culture was grown to saturation. The cells were collected by centrifugation. The cell pellet was resuspended in 0.2 ml Buffer A (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCL, pH 8.0, 1 mM EDTA, pH 8.0), 0.2 ml phenol : chloroform : isoamyl alcohol (25:24:1) and 0.2 ml glass beads. The mixture was vortexed for 2 min and spinned for 5 min at RT. One to two μl of the aqueous layer was used to transform the competent *E.coli.*

6.1.5. Rapid Yeast Protein Isolation

One point five ml of overnight grown cells were first pelleted by centrifugation and resuspended in 1 ml of 0.25 M NaOH / 1% 2-mercaptoethanol, followed by incubation on ice for 10 min. Fifty percent of trichloroacetic acid was then added to the mixture and incubated on ice for another 10 min before pelleted by centrifugation at 13K rpm for 10 min. The cell pellet was resuspended in 1 ml of ice-cold acetone and recentrifuged at 14K rpm, 10 min. The pellet was air dried and resuspended in 200-500 μl of SDS sample buffer.

6.2. Molecular Biology Methods

6.2.1. TOPO TA Cloning

The TOPO TA cloning kit (Invitrogen, P/N K4500-01) was used for subcloning PCR product into a pCR2.1-TOPO vector. For PCR products generated by Taq polymerase, PCR products were directly used in the TOPO cloning reaction. Four μl of PCR product was mixed gently with 1 μl of salt solution and 1 μl of pCR2.1-TOPO vector, incubated for 30 min at RT and chilled on ice. Two μl of the reaction mixture was mixed gently with a vial of One Shot Chemical Competent *E.coli* and incubated on ice for 25 min. After heat shock at 42^{o}C for 30 sec, the cells were immediately cooled on ice for 2 min, and 250 μl of SOC media was then added to the cells. After shaking at 200 rpm for 60 min at 37^{o}C, 10-50 μl of the transformation mixture was spread on a prewarmed 100 μg/ml ampicillin containing LB plate with X-gal and IPTG and incubated overnight at 37^{o}C.

6.2.2. Subcloning

To clone the PCR product containing gene of interest into an expression vector or subclone the gene of interest from one expression vector to another, 5 μg of plasmid vector(s) were independently digested with appropriate restriction enzymes at their optimal temperature (37^{o}C / 25^{o}C / 55^{o}C) for overnight and dephosphorylated. After agarose gel electrophoresis, the desired bands of DNA fragment were excised and purified by the QIAquick Gel Extraction Kit (Qiagen, 28704). The concentration of eluted DNA fragments were determined by agarose gel electrophoresis using a quantitative DNA marker (Fermentas, SM0243S & SM0313S). For ligation reactions, the molar ratio of vector : insert (gene of interest) was at least 1:5. 20 μl of ligation reaction mixture containing vector, insert, 1X ligation buffer and 1 μl T4 DNA ligase (Roche) was incubated for 5 min. The ligation reaction mixture could then be used directly for the transformation of competent *E.coli*.

Use of Pfu DNA polymerase for DNA amplification resulted in blunt-ended PCR product, therefore subsequent phosphate modification of the PCR product by the T4 polynucleotide kinase (NEB) was necessary for PCR cloning.

6.2.3. Competent Cells Preparation (Rubidium Chloride Method)

A single colony of *DH5α* was inoculated in 5 ml of LB broth and cultured overnight at 37^{o}C. One ml of the overnight culture was inoculated into 100 ml of LB broth and incubated at 37^{o}C with shaking at 240 rpm. The growth of bacteria culture was monitored using a

spectrophotometer. When the absorbance reading at 600 nm reached 0.48, the bacteria culture was then kept on ice for 15 min and centrifuged at 5,000 rpm for 5 min at 4^{o}C without applying brake. The bacterial cell pellet was resuspended in 40 ml of TfbI buffer and incubated on ice for 15 min. After centrifugation at 5,000 rpm for 5 min at 4^{o}C, the cell pellet was then resuspended in 4 ml of TfbII buffer. The competent cells were transferred to pre-chilled 1.5 ml tubes in 200 µl aliquots and snap-frozen in liquid nitrogen before transferring to -80^{o}C for long term storage.

6.2.4. Bacterial Transformation

Ten ng of plasmid DNA or 10 µl of ligation mixture was mixed with 100 µl of competent *E.coli* and incubated on ice for 1 hr. This transformation mixture was heat shocked at 42^{o}C for 1 min and incubated again on ice for 2 min. Nine hundreds µl of pre-warmed SOC medium was added and the transformation mixture was incubated at 37^{o}C for 1 hr with shaking. After 1 hr, the transformation mixture was plated on LB agar plate with appropriate selective antibiotic and incubated overnight at 37^{o}C for colony formation.

6.2.5. Bacterial Colony PCR Screening

The bacterial colonies were individually picked using toothpick and resuspended in 20 µl dH_2O. The resuspended bacterial colonies were boiled for 5 min before using 5 µl of the boiled cells for PCR set up.

6.2.6. Polymerase Chain Reaction (PCR)

For normal PCR screening, Taq DNA polymerase (Promega) without the proof-reading (3'→5' exonuclease) activity was used. In the PCR set-up, 10-20 ng of plasmid DNA was used as the template and mixed with final concentration of 1X reaction buffer, 0.2 mM for each dNTP, 2 mM $MgCl_2$, 1 μM of forward primer, 1 μM of reverse primer and 2.5 U of Taq polymerase in a thin-walled reaction tube. The tubes were then placed in a thermal cycler and PCR reaction was run with selected thermal cycling profile (1 min extension time for every 1 kb of amplicon).

For PCR cloning, Pfu DNA polymerase (Promega) with proof-reading activity was used instead. In the PCR set up, 50 ng of plasmid DNA was used as the template and mixed with final concentration of 1X reaction buffer, 0.2 mM for each dNTP, 2 mM $MgSO_4$, 1 μM of forward primer, 1 μM of reverse primer and 2.5 U of Pfu DNA polymerase. In this case, 2 min extension time was required for every 1 kb of amplicon.

6.2.7. Plasmid Isolation

Plasmid was isolated from 5 ml of overnight *E.coli* cultures in selective LB broth using QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's instructions. For large scale of plasmid isolation, a single colony was inoculated into 200-400 ml (copy number-dependent) selective LB broth and grown at 37^{o}C for 16 hrs with vigorous shaking (~240 rpm). Plasmid isolation was then performed using the QIAGEN Plasmid Endofree Maxi Kit according to

manufacturer's instructions.

6.2.8. DNA Sequencing

DNA sequencing was performed using the ABI PRISM BigDye Terminator v1.1 cycle sequencing Ready Reaction Kit (Applied Biosystem). Thirty to ninety ng of PCR products or 200-500 ng of plasmid DNA in 5 μl was mixed with 4 μl of BigDye reaction mixture, 1 μl of 10 μM primer and incubated for 25 cycles of 96^{o}C for 10 sec, 50^{o}C for 5 sec and 60^{o}C for 4 min. The reaction mixture was precipitated with 10 μl dH_2O, 2 μl of 3 M sodium acetate and 50 μl of 95% ethanol at RT for 15 min and centrifugation at 13,000 rpm for 20 min at RT. The pellet was washed with 250 μl of 70% ethanol and vacuum dried. DNA was resuspended in 15 μl of ABI loading solution and denatured at 95^{o}C for 2-5 min before running on ABI prism 377XL automated DNA sequencer.

6.2.9. Total RNA Isolation

Total RNA isolation from cell lines was carried out with Trizol Reagent (Gibco) according the manufacturer's instruction. Briefly, 2 x 10^7 cells were harvested and lysed in 1.5 ml Trizol reagent. The lysates were homogenized and incubated at room temperature for 5 min. 0.3 ml of chloroform (Sigma) was added, followed by vigorous shaking and incubation at room temperature for 5 min. The samples were centrifuged at 12,000 g for 15 min at 4^{o}C. The upper aqueous phase was transferred to a fresh tube and 0.7 ml isopropyl alcohol (Sigma) was added to precipitate RNA from this aqueous phase. The samples were incubated at room

temperature for 10 min, followed by centrifugation at 12,000g for 10 min at 4°C. The RNA precipitates were washed with 75% ethanol and re-centrifuged at 7,500 g for 5 min at 4°C. The air-dried RNA pellets were eventually resuspended in DEPC-treated dH_2O.

6.2.10. Reverse Transcription

The reverse transcription from total RNA template was carried out using the ImProm-II Reverse Transcription System (Promega), according to the manufacturer's instruction. Briefly, 1 μg of total RNA was mixed with 0.5 μg $Oligo(dT)_{15}$ primer or 10-20 pmole gene-specific primer and heat denatured at 70°C for 5 min, followed by quick chill on ice water for 5 min. This denatured RNA template was added to the reverse transcription reaction mix containing 1X reaction buffer, 6 mM $MgCl_2$, 0.5 mM dNTP mix, 20U recombinant RNasin ribonuclease inhibitor and 1 μl ImProm-II reverse transcriptase. The annealing reaction was carried out at 25°C for 5 min and subsequently extension reaction was carried out at 42°C for 1 hr. At the end of incubation, the reverse transcriptase was thermally inactivated at 70°C for 15 min. The heat-inactivated reverse transcription reaction products could be used directly for PCR amplification of cDNA (1 μl for 100 μl PCR reaction).

6.2.11. Southern Blot Analysis

DNA fragments in the agarose gel were pre-rinsed with 0.25 M HCl, followed by denaturation with the Denaturation Buffer and subsequent neutralization with the Neutralization Buffer. The denatured DNA fragments were transferred to Hybond N+

membrane by capillary transfer. The DNA blot was fixed by UV cross-linking and baked at 80^{o}C for 1 hr. The blot was then pre-hybridized for 3 hr at 65^{o}C before subjected to overnight hybridization in the presence of denatured labeled cDNA probe. The hybridized blot was washed twice at 65^{o}C with 2X SSC/0.1% SDS first, followed by two washes with 0.2X SSC/0.1% SDS and last two washes with 0.1X SSC/0.1% SDS. The blot could then be exposed to X-ray film at –80^{o}C using the intensifying screen. For stripping of blot signal, the blot was incubated at 45^{o}C for 30 min in 0.4 M NaOH and transferred to incubation with the membrane stripping buffer for 15 min.

6.2.12. Northern Blot Analysis

Pre-made blots containing poly(A) RNA isolated from adult human tissues and a human cancer cell line panel were purchased from Clontech and used for hybridization with AKIP-specific probe. Blots were hybridized according to Church and Gilbert method [1] with a 477-bp AKIP 3'-end fragment labeled using a High Prime DNA labeling kit (Roche), according to the manufacturer's instruction. Blots were then stripped and reprobed with β-actin to quantitate RNA loading.

AKIP cDNA Probe Labeling

Two hundreds ng of AKIP cDNA probe was boiled for 10 min and quick chilled on ice. To the denatured cDNA probe, 4 μl High Prime reaction mixture, 3 μl of dATP, dGTP, dTTP

mixture and 5 μl of [α^{32}P] dCTP (3000 Ci/mmol) were added and incubated for 37^{o}C for 10 min. The labeling reaction was stopped by heating to 65^{o}C for 10 min and the unincorporated nucleotides were removed by NucTrap probe purification columns (Stratagene). The probes were boiled for 5 min and quick chilled on ice before use.

Church & Gilbert Hybridization [1]

The membrane was pre-hybridized with the hybridization buffer for 1 hr before the overnight hybridization with the ^{32}P-labeled AKIP cDNA probe at 42^{o}C. The hybridized membrane was washed two times with Buffer A at 65^{o}C, 30 min for each wash, followed by three 30 min washes with Buffer B at 65^{o}C. The membrane was exposed using screen for PhosphorImager (BioRad). Stripping of blot signal was done by incubating the blot with boiled stripping buffer and allowed to cool down to the room temperature.

6.2.13. Real-Time PCR

The QuantiTech^{TM} SYBR Green PCR kit (Qiagen, P/N 204143) was used according to the manufacturer's instructions. One to two μl of cDNA was mixed with 2 μl of 10 μM primer mix, 10 μl of QuantiTech^{TM} SYBR Green PCR master mix and dH_2O in a final volume of 20 μl. Real time PCR was performed using a Rotor-Gene real-time PCR machine (Corbett Research, Australia). The cycle profile of PCR included an initial hot start at 95^{o}C for 15 min, followed by 40 cycles of 95^{o}C for 15 sec, 50-60^{o}C for 30 sec and 72^{o}C for 30 sec. All PCR

reactions were performed in duplicate. Standard curves of GADPH, Aurora-A and AKIP were generated independently by 10X serial dilution of template DNA (diluted PCR product) in each run. Melting curves were analyzed for each sample to check for product specificity. The relative copy number of each sample was calculated according to the corresponding standard curve using RotorGene v4.6 software. Normalization was performed in each sample by dividing the copy number of Aurora-A and AKIP to that of GADPH. The relative expression levels were calculated by arbitrarily designating the lowest normalized value to 1.

6.1.14. Site-Directed Mutagenesis

The site-directed mutagenesis was carried out using the GeneEditor *in vitro* Site-Directed Mutagenesis System from Promega, according to the manufacturer's instruction. The overview of GeneEditor *in vitro* Site-Directed Mutagenesis System is schematically diagramed in Figure 6-1.

To summarize, the GeneEditor system provides a selection oligonucleotide encoding mutation that alters the amplicillin resistance gene in the plasmid vector, generating a new additional resistance to the GeneEditor Antibiotic Selection Mix. This selection oligonucleotide is annealed to the double-stranded DNA template together with the mutagenic oligonucleotide that introduces the desired mutation. Subsequent synthesis and ligation of the mutant strand link the two oligonucleotides. The resistance to GeneEditor Antibiotic Selection Mix encoded by this mutant DNA strand facilitates high efficiency selection of the desired

mutation. The drawback is that only the cloning vector with ampicillin resistant selectable marker can be used for this system. Mutants generated by this system retain ampicillin resistance in the meantime also gain resistance to the GeneEditor Antibiotic Selection Mix.

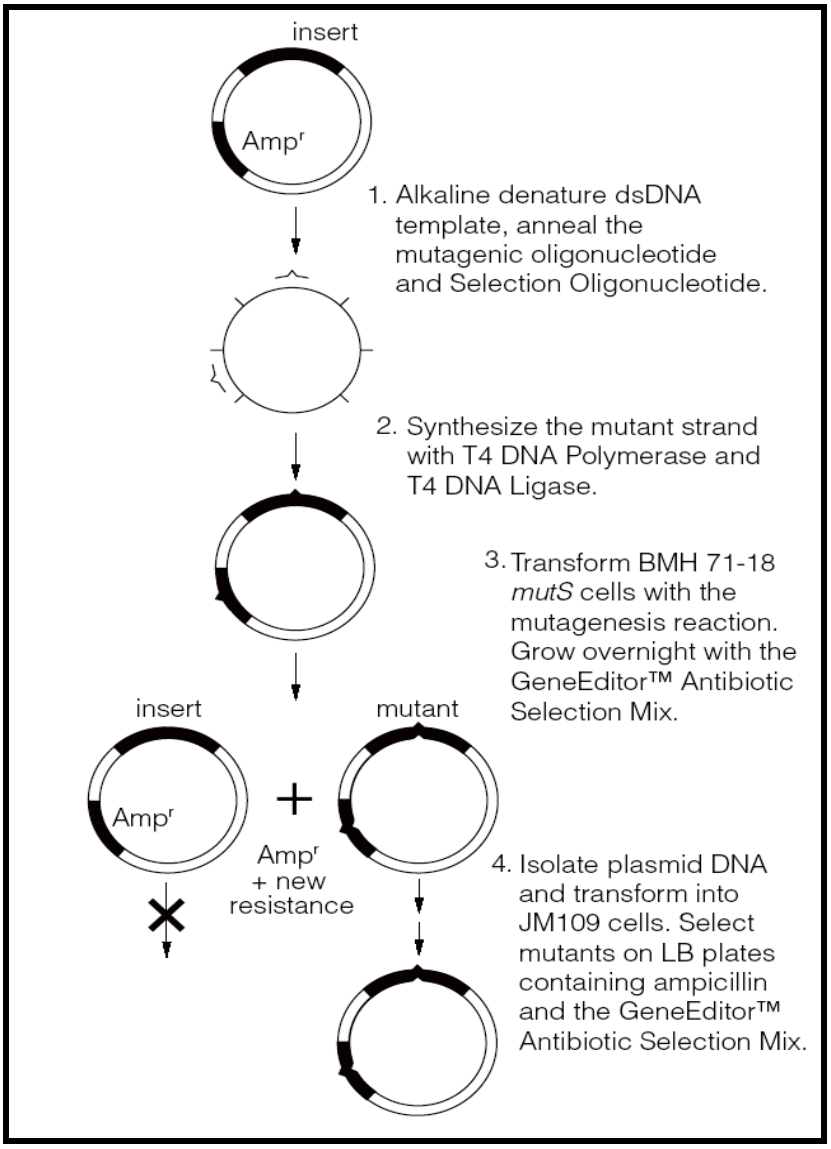


Figure 6-1: Schematic Diagram of the GeneEditor *In vitro* Mutagenesis Procedure (Adapted from Promega Technical Manual No.047)

In brief for the protocol, the dsDNA template was first alkaline denatured by treating 0.5 pmol dsDNA template with 2 μl 2 M NaOH/2 mM EDTA in 20 μl reaction for 5 min at room temperature. The reaction was neutralized by adding 2 μl of 2M ammonium acetate, pH 4.6

and the DNA was precipitated with 75 μl 100% ethanol at –70°C for 30 min, followed by washing of DNA pellet with 70% ethanol. The pellet was resuspended in TE buffer, pH 8.0 and was ready for the next annealing reaction. For hybridization of both selection and mutagenic oligonucleotides to the denatured DNA template, 0.05 pmol of template DNA together with 0.25 pmol selection oligonucleotide and 1.25 pmol mutagenic oligonucleotide, were diluted in 1X annealing buffer and heated to 75°C for 5 min, followed by slow cooling down to 37°C. Once the annealing reaction mixture had cooled down, 1X synthesis buffer containing T4 DNA polymerase and T4 DNA ligase was added to the mixture and incubated at 37°C for 90 min to perform mutant strand synthesis and ligation. Once completed, an aliquot of the mutagenesis reaction mixture was used to transform the DNA repair defective BMH 71-18 *mutS* competent cells and cells were grown in the selective media containing the GeneEditor Antibiotic Selection Mix to select for clones containing the mutant plasmid. Plasmids resistant to the novel GeneEditor Antibiotic Selection Mix were then isolated and re-transformed into the final host strain, JM109, under the same selection conditions. As this system generally produces 60-90% mutants, so colonies were further screened by direct DNA sequencing.

6.3. Cell Biology Methods

6.3.1. Cell Culture and Synchronization

ts20-TG mouse cell, U2OS, 2774, NIH-3T3 and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) in 37^{o}C humidified incubator with 5% CO_2. HeLa, MCF7, SW480, K562 cells were maintained in RPMI 1640 medium (Sigma) at 37^{o}C. AT2.1 cells were maintained in RPMI 1640 medium supplemented with 250 nM dexamethasone (Sigma). ts20 Chinese Hamster lung cell line, which harbors the temperature-sensitive mutation in E1 ubiquitin-activating enzyme, was maintained at 30^{o}C in α-MEM medium (Sigma) supplemented with 4.5g Glucose/L. Inactivation of E1 ubiquitin-activating enzyme had been carried out by incubating the cells at 40^{o}C for 16hrs. All mediums were supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO), 2 mM L-glutamine (GIBCO), 0.1 mM non-essential amino acid (GIBCO) and 1 mM sodium pyruvate (GIBCO). For long term storage of cell stock, ~10^6 cells were resuspended in cryovials in 0.8 ml FBS containing 8% DMSO (Sigma) and 2% glycerol (USB), frozen overnight at -80^{o}C and then transferred to liquid nitrogen.

For synchronization of the cells at G1/S phase border by double thymidine block, the subconfluent growing cells were first treated with 2 mM thymidine, an inhibitor of DNA synthesis for 12 hr, followed by the wash and release from G1/S or S phase into thymidine-free medium for 8 hr and re-incubated with 2 mM thymidine-containing medium

for 12-14 hr. For release from G1/S arrest, the cells were washed 3 times in serum-free medium and replaced with thymidine-free medium. Alternatively, the G1/S synchronization could also be achieved by treatment with 1 μg/ml Aphidicolin for 24 hr.

For enrichment of mitotic cells by nocodazole arrest, the subconfluent growing cells were treated with 0.1 μg/ml nocodazole, a microtubule depolymerizing drug, for 16 hr. For subsequent release from the M phase arrest, the round mitotic cells were collected by the mechanical mitotic shake-off, washed 3 times in serum-free medium, and replaced with nocodazole-free medium.

6.3.2. Transfection

Cells were plated and grown in their respective culture medium one day before the transfection. Eighty to ninety percent of cell confluency should be reached on the day of transfection. Transfection had been carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended protocol. Briefly, for a 60 mm dish transfection, 3 μg of plasmid DNA and optimal volume of Lipofectamine 2000 (7.5 μl-12 μl) were diluted separately in 250 μl OptiMEM I reduced serum medium (GIBCO) and incubated at RT for 5 min. The two were mixed in a final volume of 500 μl and incubated at RT for another 20 min. During the incubation, the seeded cells were washed twice with and resuspended in 1.5 ml of their respective medium without antibiotics. The DNA-Lipofectamine complexes were added onto the pre-rinsed cells. After incubation for

5-16 hours, the medium was discarded and replenished with 5 ml fresh medium without antibiotics.

6.3.3. Cell Lysis

For normal western blot analysis, the cells were lysed with 1X Laemli Buffer, followed by pulsed sonication (3 x 5 sec) on ice and subsequently cleared by centrifugation at 13,000 rpm for 5 min at 4^{o}C.

For immunoprecipitation, the cells were lysed for 30 min on ice in a different lysis buffer described in chapter 5. The lysates were then cleared by centrifugation at 13,000 rpm for 20 min at 4^{o}C.

The protein concentrations of the lysates were assayed using Coomassie Plus Protein Assay Reagent (Pierce).

6.3.4. Flow Cytometry

Cells were collected by trypsinization, pelleted and resuspended in phosphate-buffered saline (PBS) to a final volume of about 1 x 10^{6} cells/ml. Two volumes of cold, absolute ethanol were added and the samples were stored at -20^{o}C until the day of analysis. At that time, the cell were pelleted, washed twice with PBS + 0.1% (v/v) Triton X-100 and finally resuspended in PI (Sigma) staining solution. Samples were stored at 4^{o}C for at least 1 hr in the dark prior to analysis.

Flow cytometry analysis for PI fluorescence was performed using a FACScan machine

from Becton Dickinson and the CellQuest software with doublet discrimination. For each analysis, at least 20000 gated events were collected to permit accurate and reliable cell cycle analysis of the cell population. The PI fluorescence signal was collected at the 572/26 bandpass (FL2). Data analysis was performed using CellQuest and ModfitLT. The PI fluorescence was collected on a linear scale and all mean fluorescence intensity values were determined as linear values from the CellQuest software.

6.3.5. Immunocytochemistry

For immunofluorescence staining, the dividing cells were grown on glass coverslips in 35-mm culture dish. The cells were fixed in –20^{o}C methanol (MERCK) for 5 min or 4% paraformaldehyde (Sigma) for 30 min at room temperature. After fixation, the cells were washed 3 times in TBS and then incubated in blocking buffer for 30 min at room temperature. Incubation with the primary antibodies diluted in blocking buffer was carried out for 1 hr at room temperature, and the coverslips were then washed 3 times in TBS. The cells were then incubated with respective Alexa Fluor-conjugated secondary antibodies for 1 hr at room temperature, washed 3 times in TBS and mounted on slides. For propidium iodide (PI) staining, cells were incubated with 0.05 μg/ml PI (Sigma). For DAPI staining, the cells were counterstained with the mounting medium containing DAPI. The cells on slides were analyzed by using a Leica epifluorescence microscope equipped with a multiband filter set.

6.4. Protein Biology Methods

6.4.1. SDS-PAGE and Western Blot Analysis

SDS-PAGE

12 % Resolving Gel

40% Acrylamide/Bis-acrylamide (37.5:1)	3 ml
Lower Tris Buffer	2.5 ml
10% APS	0.2 ml
TEMED	10 µl
dH_2O	4.5 ml
Total Volume	10 ml

4% Stacking Gel

40% Acrylamide/Bis-acrylamide (37.5:1)	1.3 ml
Upper Tris Buffer	2.5 ml
10% APS	0.25 ml
TEMED	17 µl
dH_2O	6.2 ml
Total Volume	10 ml

The SDS-PAGE was run with 1X Laemli running buffer at 160V.

Western Blot

The resolved proteins on SDS-PAGE were transferred to nitrocellulose membrane using the Western blotting apparatus. The Western Blot was carried out using the transfer buffer and run with cooling ice at 320 mA for at least 75 min. At the end of protein transfer, the membrane was incubated at RT with blocking buffer (5% Non-Fat Milk) for at least 1 hr before probing with primary antibody for overnight at 4^oC. After washing the blot 3 times (10 min each) with the

wash buffer, secondary HRP-conjugated antibody was added and incubated at RT for 1 hr. Again, the blot was washed 3 times with wash buffer before proceeding to chemiluminescence detection using Pico or Dura Enhanced Chemiluminescence Reagent (Pierce).

6.4.2. *In vivo* Ubiquitination Assay [2]

The transfected cells were treated with 20 μM MG132 for 12 hours prior to harvest. The collected cells were lysed in 1 ml of Buffer G per 60 mm dish. The lysate was sonicated in pulses (3 x 5sec) to shear the genomic DNA and incubated with 100 μl of 50% slurry of nickel-NTA-agarose (Qiagen) with rotation for 3 hours at room temperature. The beads were washed twice with 1 ml Buffer G, twice with 1 ml of Wash Buffer I, and twice with Wash Buffer II, 5 min for each wash with rotation. Precipitated ubiquitinated proteins were eluted by boiling beads in 2X SDS sample buffer and analyzed by immunoblotting.

6.4.3. General Immunoprecipitation

Prior to the immunoprecipitation, 1 mg of total protein lysate was precleared by incubation with 80 μl of 50% slurry of protein-A or -G agarose (Sigma) for 1 hour at 4^{o}C. For antibody coupling to the protein-A or –G agarose, 20 μl of rabbit anti-human Aurora-A crude serum or 6 μg of FLAG M2 mouse monoclonal antibody were incubated with 80 μl of 50% slurry of protein-A or -G agarose for 1 hr at room temperature. For immunoprecipitation, the pre-cleared lysate and the antibody-coupled protein-A or –G agarose were mixed and rotated for 2 hr at 4^{o}C. Immune complexes were washed twice with wash buffer I and twice with

wash buffer II, 5 min each with rotation. The immune complexes were solubilized by boiling with SDS sample buffer and resolved by SDS-PAGE.

6.4.4. Immunoprecipitation with EzView Red ANTI-FLAG M2 Affinity Gel

EzView Red ANTI-FLAG M2 Affinity Gel (Sigma) is a highly visible, red colored ANTI-FLAG M2 agarose affinity gel (ANTI-FLAG M2 mAb covalently coupled to agarose CL-4B beaded particles) for use in immunoprecipitation of the FLAG-tagged proteins from cell lysates.

Forty μl of the 50% slurry EzView Red ANTI-FLAG M2 Affinity Gel beads were used for each immunoprecipitation reaction. Before immunoprecipitation, the beads were washed and equilibrated with the bead equilibration buffer. Five hundreds μg of cell lysates were then incubated with the equilibrated beads and they were rotated for overnight binding at 4^{o}C. At the end of binding, the beads were washed 6 times with wash buffer, 5 min for each wash with rotation. The immunoprecipitates were eventually eluted with 2X sample buffer without any reducing agent, which was only added after boiling and transfer of supernatants to fresh tubes.

6.5. References

1. Church, GM, Gilbert W. **Genomic Sequencing**. Proc. Natl. Acad. Sci. USA. (1984) 81, 1991-1995.

2. Honda K, Mihara H, Kato Y, Yamaguchi A, Tanaka H, Yasuda H, Furukawa K, Urano T. **Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway.** Oncogene. (2000) 19(24):2812-9.

Section 3

Results and Discussions

(Results and Discussions)

CHAPTER 7

Identification of Aurora-A Kinase Interacting Protein (AKIP) and Characterization of its Role in Negative Regulation of Aurora-A Kinase

7.1. Summary

Overexpression of Aurora-A plays an important role in tumorigenesis. The present work aims to identify the negative regulators of human Aurora-A. Exploiting the lethal phenotype associated with the overexpression of Aurora-A in yeast, we performed a dosage suppressor human cDNA library screen in yeast and reported the identification of a novel negative regulator of Aurora-A, named as AKIP (Aurora-A Kinase Interacting Protein). The full-length AKIP cDNA contains a 597-bp open reading frame, which encodes a 199-amino acid polypeptide. AKIP amino acid sequence shares high homology to its orthologs in rat and mouse. It is ubiquitously expressed in various tissues. Distinct from Aurora-A, AKIP mRNA expression is cell cycle-independent and its protein expression is regulated at the post-translational manner. AKIP is normally an unstable protein and is degraded by the proteasome-dependent pathway. AKIP is a nuclear protein, with specific localization to the nucleolus in the nucleus during interphase and to mitotic spindle and post-mitotic bridge in mitosis. AKIP colocalizes with Aurora-A in mitosis. Interestingly, AKIP interacts specifically with Aurora-A *in vivo*. Isolated as the negative regulator of Aurora-A, AKIP down-regulates Aurora-A at the level of its protein stability. This AKIP-mediated Aurora-A destabilization is dose- and time-dependent as well as highly specific. Furthermore, AKIP:Aurora-A interaction is essential for the AKIP-mediated Aurora-A degradation, which is mediated through the proteasome.

7.2. Results

7.2.1. Yeast Dosage Suppressor cDNA Library Screen for Potential Negative Regulator(s) of Human Aurora-A Kinase

Overexpression of Aurora-A kinase is lethal in yeast. By exploiting this lethal phenotype resulted from Aurora-A kinase overexpression, we performed the dosage suppressor cDNA library screen, aiming to isolate the mammalian proteins that could suppress the lethal phenotype and rescued the yeast when co-transformed and expressed, as shown in Figure 7-1.

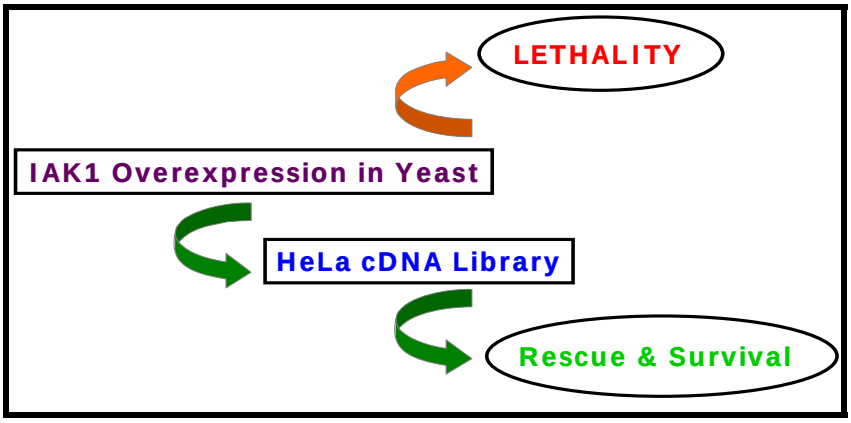


Figure 7-1: Yeast Dosage Suppressor Screen

For this purpose, a plasmid construct was made in yeast expression vector pEG202 where constitutive expression of the full-length mouse Aurora-A kinase in yeast was under the control of alcohol dehydrogenase (ADH) promoter. Yeast strain EGY188 was co-transformed with this Aurora-A plasmid and a HeLa cDNA library in yeast expression vector pJG4-5, where cDNAs were expressed under the galactose-inducible GAL1 promoter. A total of 0.5 x 10^6 co-transformants were screened, and the resulting positive clones were subjected to secondary selection for the galactose-dependent reversal of Aurora-A-mediated cell death and characterized further by DNA sequencing, as summarized in Figure 7-2.

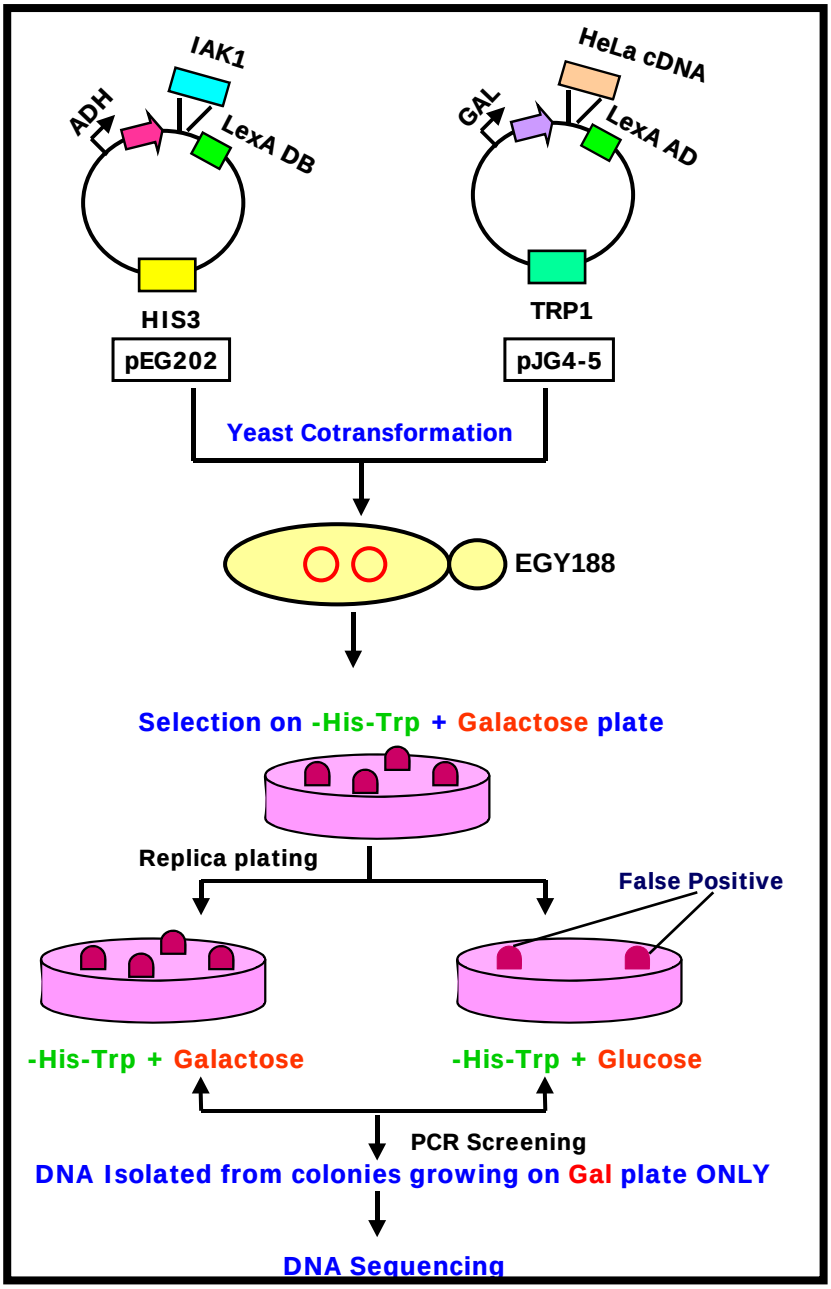


Figure 7-2: Schematic Diagram Depicting the Yeast Dosage Suppressor Screen for Isolation of Potential Aurora-A Negative Regulator(s)

7.2.2. Isolation of the Novel Full Length AKIP

Interestingly, sequence analysis of a total of 141 positive clones revealed a 477-bp partial cDNA fragment, which we designated as AKIP (Aurora-A Kinase Interacting Protein), containing the 3'-end of the mRNA, was represented 24 times, as shown in Table 7-1.

Table 7-1: List of Candidate Suppressor Proteins Isolated from Yeast Dosage Suppressor Screen	
Candidate Protein	**Frequency**
AKIP	**24**
SLM-1	**9**
KIA0905	**1**
Ribosomal Protein	**4**
Ferritin H chain	**1**
Mitochondria Protein	**2**
Chromosome 20, P1 clone	**1**
Putative Glioblastoma Protein	**1**
Cytokeratin 18	**1**
BRAC2	**1**
ARP2/3 Complex	**1**
KIAA0108	**1**
23kDa Highly Basic Protein	**2**
Integrin β4 Binding Protein	**1**
CGI-98	**1**
G Protein β Subunit	**1**
Guanine Nucleotide Binding Protein	**1**
Tumourous Imaginal Discs	**1**
KIAA1082	**1**
Ca^{2+} Binding Protein	**1**
Other candidate clones were either non-coding or had very short homology	

The authenticity of these 24 clones was verified by the galactose-dependent rescue from Aurora-A mediated cell death, as shown in Figure 7-3. The high frequency (17% of the total positive clones) and the reproducible rescue from the Aurora-A-mediated lethality by AKIP prompted us to characterize this cDNA fragment further.

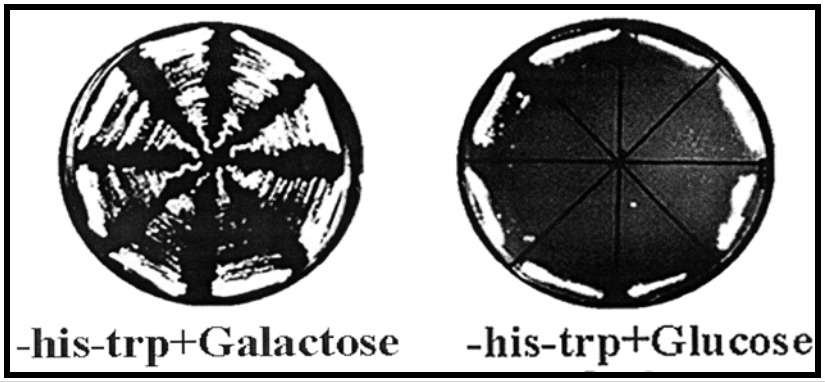


Figure 7-3: AKIP Suppresses Aurora-A-Induced Yeast Cell Death

Eight representative yeast clones expressing both Aurora-A and AKIP were streaked on both galactose and glucose containing synthetic medium lacking histidine and tryptophan. Photographs were taken after 4 days at 30^{o}C

The predicted translation product of the cDNA fragment isolated from the dosage suppressor screen in yeast is presented in Figure 7-4. Comparison of the protein and nucleotide sequence of AKIP with the sequences in the GenBank database revealed that it was identical to the sequence corresponding to many of the EST clones and full-length clones, encoding a protein with uncharacterized function.

```
DTGTAGTVAP PQSYQCPPSQ IGEGAEQGDE GVADAPQIQC KNVLKIRRRK  50
MNHHKYRKLV KKTRFLRRKV QEGRLRRKQI KFEKDLRRIW LKAGLKEAPE 100
GWQTPKIYLR GK                                           112
```

Figure 7-4: AKIP Amino Acid Sequence

The deduced amino acid sequence of AKIP isolated by dosage suppressor screen in yeast is presented. This sequence lacks the 87 amino acids from the N-terminus of the full-length AKIP protein.

We went on to clone the putative full-length AKIP cDNA by 5'-Rapid Amplification of cDNA Ends (5'-RACE). The full-length AKIP cDNA contains a 597-bp open reading frame that encodes a 199-amino acid polypeptide with a predicted molecular mass of 22 kDa.

Figure 7-5 compares the deduced amino acid sequence of AKIP with the homologous sequences available in databases. Human AKIP shares 72 and 73% identity at the amino acid level over its entire length with the mouse and rat AKIP, respectively. However, AKIP-related sequences are not found in the genomes of the lower eukaryotes, such as yeast, Drosophila and *C.elegans*. Based on the above information, we conclude that AKIP is a novel gene.

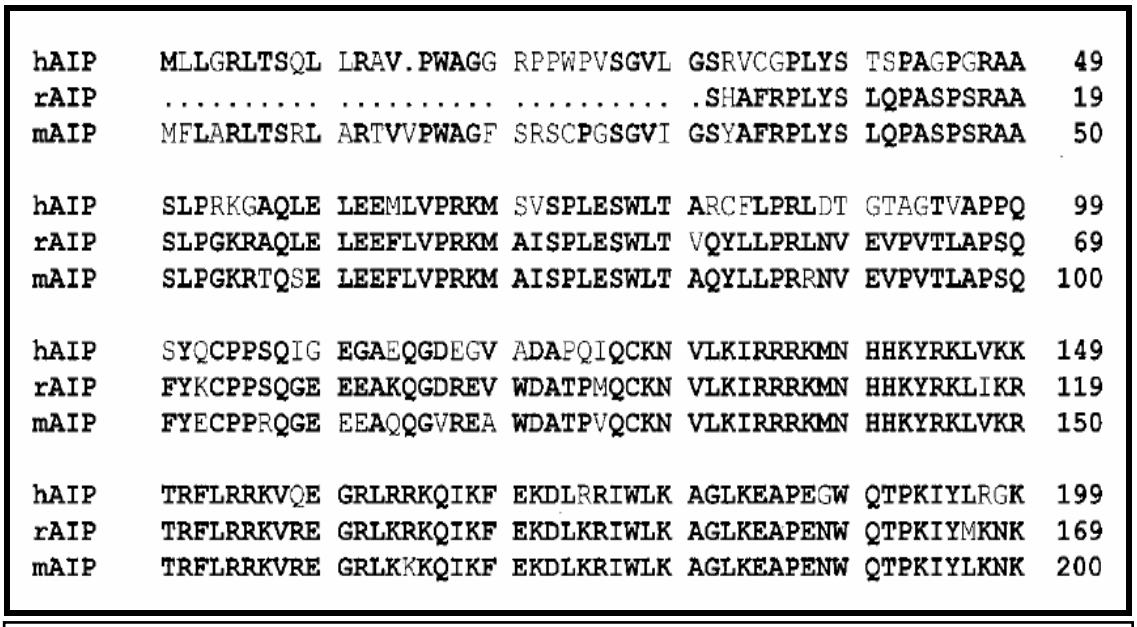


Figure 7-5: Amino Acid Sequence Alignment of Human, Mouse and Rat AKIP

Amino acid sequence alignment of human AKIP (hAIP) with those of mouse (mAIP) and rat (rAIP). The mouse and rat sequences were derived from the EST database. The mouse AKIP sequence was derived from EST clones AI425574 and AA545527, and the corresponding rat AKIP sequence was derived from EST clone AI104388. Identical amino acids in the sequences are presented in *bold face*. AKIP was previously published as AIP.

7.2.3. Characterization of AKIP

7.2.3.1. Tissue Distribution

Northern Blot analysis of human tissues and cancer cell lines indicated that AKIP is ubiquitously expressed in a wide variety of tissues, especially high in heart and testis, as shown in Figure 7-6.

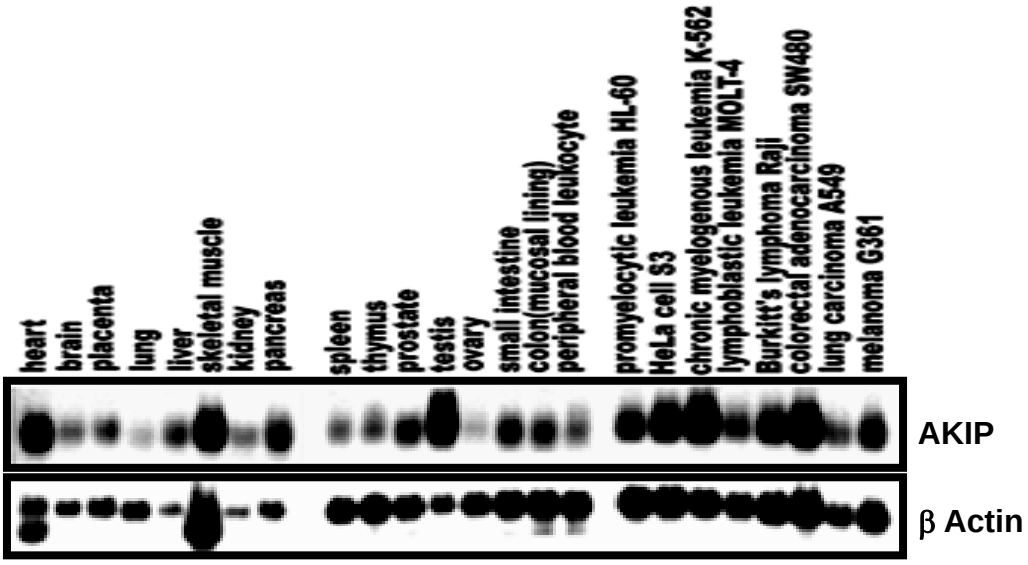


Figure 7-6: AKIP mRNA Expression in Various Human Tissues and Cancer Cell Lines
Northern blot analysis of AKIP mRNA in adult human tissues was carried out with pre-made Northern blots purchased from Clontech. The blots were hybridized with a 477-bp AKIP cDNA fragment derived from the yeast dosage suppressor screen. The blot was stripped and reprobed with β actin.

7.2.3.2. Endogenous mRNA and Protein Expression of AKIP

7.2.3.2.1. mRNA Expression in Cell Cycle

Since AKIP was isolated from the screen as the potential suppressor for Aurora-A kinase, and Aurora-A mRNA expression is regulated in the cell cycle-dependent manner, therefore it would be interesting to investigate if AKIP mRNA expression is cell cycle regulated as well. For the current study, we collected cells from different points of cell cycle after release from

the G1/S arrest and analyzed the AKIP, Aurora-A and GADPH mRNA expression by real-time PCR. Aurora-A kinase was used as the positive control gene with the cell cycle regulated mRNA expression profile, whereas the housekeeping gene GADPH was used as the normalization control.

As shown in Figure 7-7, Aurora-A displayed the expected cell cycle expression pattern, low in G1 phase (0 or 12 hr post G1/S release) and high in M phase (6 hr post G1/S release). However, in contrast, there was no fluctuation in the AKIP mRNA expression during the cell cycle progression, suggesting that AKIP is not cell cycle–regulated at the transcriptional level.

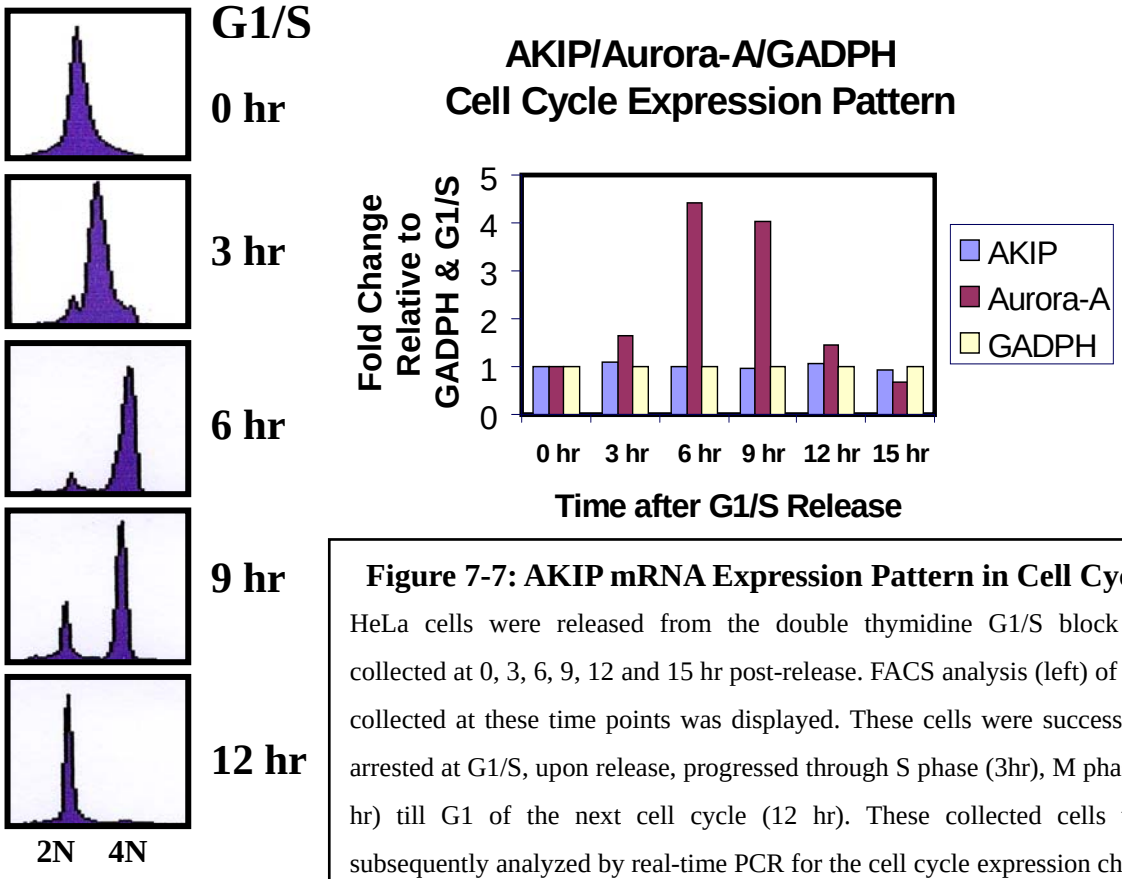


Figure 7-7: AKIP mRNA Expression Pattern in Cell Cycle

HeLa cells were released from the double thymidine G1/S block and collected at 0, 3, 6, 9, 12 and 15 hr post-release. FACS analysis (left) of cells collected at these time points was displayed. These cells were successfully arrested at G1/S, upon release, progressed through S phase (3hr), M phase (6 hr) till G1 of the next cell cycle (12 hr). These collected cells were subsequently analyzed by real-time PCR for the cell cycle expression change of AKIP and Aurora-A. GADPH was used as the normalization control.

7.2.3.2.2. Endogenous Protein of AKIP

To detect the endogenous AKIP protein, we had custom synthesized a small immunogenic peptide from the C-terminus of AKIP and used it for rabbit immunization to generate the rabbit anti-AKIP polyclonal peptide antibody. The anti-AKIP peptide antibody was subsequently affinity purifed from the crude serum. This affinity-purified anti-AKIP peptide antibody was assessed for its reactivity against both the exogenously and endogenously expressed AKIP. Our study had shown that it could efficiently detect the exogenously expressed AKIP, as shown in Figure 7-8.

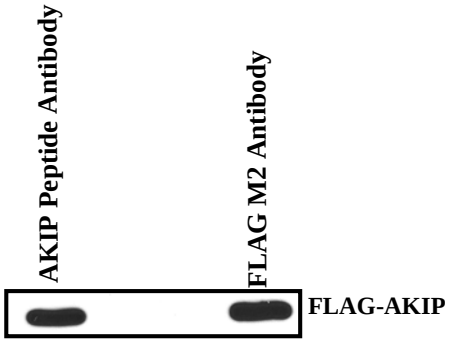


Figure 7-8: Functional Testing of AKIP Peptide Antibody

HeLa cell lysate expressing the FLAG-tagged AKIP were analyzed for AKIP protein expression using anti-AKIP peptide polyclonal antibody (left panel, against C-terminus of AKIP) and anti-FLAG monoclonal antibody (right panel, against N-terminus of AKIP).

Surprisingly, it could not detect any endogenous protein of AKIP in all the cancer cell lines tested, which were under their basal state. However, when we delivered the proteasomal stress to these cells by treatment with MG132, we could start to observe the appearance of AKIP protein with the predicted molecular weight of 22 kDa. The observed increase in the protein stability of AKIP upon proteasomal stress was time-dependent, as shown in Figure 7-9.

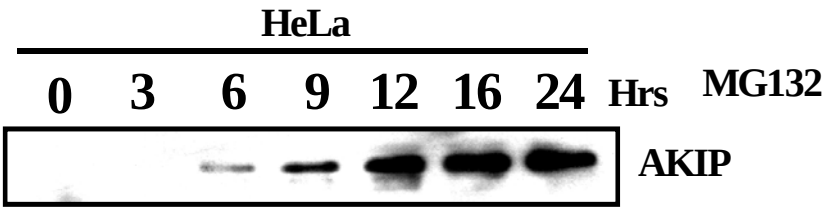


Figure 7-9: Time-dependent Stabilization of Endogenous AKIP Protein Upon Proteasomal Inhibition

HeLa cells were treated with 20 μM Mg132 for the indicated duration and harvested for Western Blot analysis of the endogenous AKIP using the affinity-purified anti-AKIP peptide antibody.

Similar MG132 treatment of other cancer cell lines indicated that AKIP was an ubiquitous protein and it was only detectable upon inhibition of proteasomal degradation process, as shown in Figure 7-10. This implied that AKIP is normally an unstable protein and its protein degradation is mediated through the proteasomal pathway.

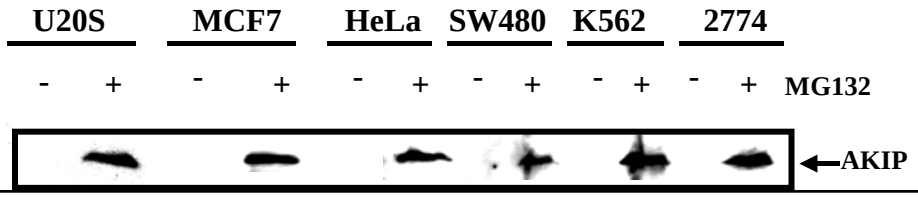


Figure 7-10: Endogenous AKIP Protein in Various Cancer Cell Lines

U2OS (osteosarcoma cell), MCF7 (breast cancer cell), HeLa (cervical cancer cell), SW480 (colon cancer cell), K562 (chronic myelogenous leukemia) and 2774 (ovarian cancer cell) were treated with 20 μM Mg132 for 24 hrs and harvested for Western Blot analysis of the endogenous AKIP using the affinity-purified anti-AKIP peptide antibody.

7.2.3.3. Domain Organization and Subcellular Localization

7.2.3.3.1. Nuclear Localization Signal

Computer-assisted search for the motifs presented in AKIP protein found a tandem bipartite nuclear localization signal at the C-terminus of AKIP (Figure 7-11), suggesting AKIP could be a nuclear protein.

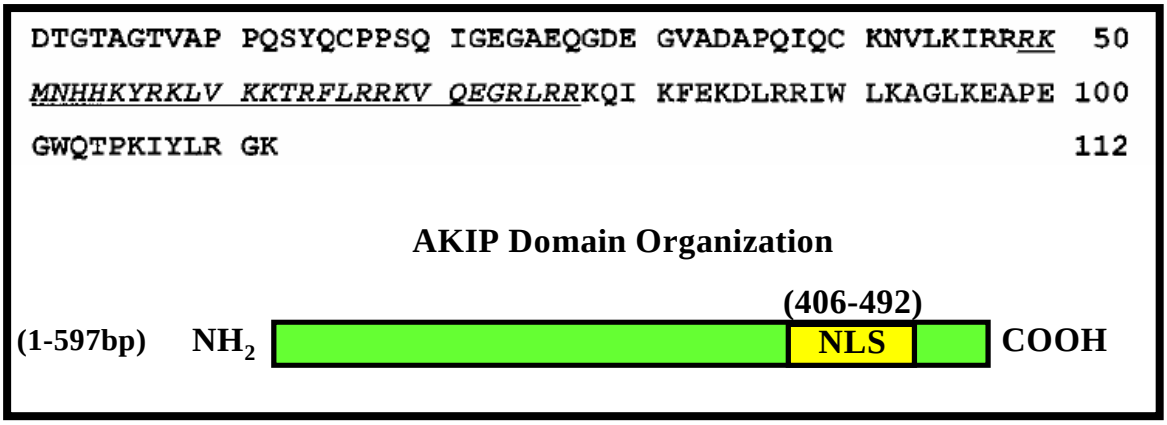


Figure 7-11: Nuclear Localization Signal (NLS) of AKIP

The location of the tandem bipartite nuclear localization signal (NLS) is highlighted with *italics* and underlining in the truncated form of AKIP (AKIP-TR) and schematically in the full-length AKIP.

7.2.3.3.2. Subcellular Localization

Indeed, ectopically expressed FLAG epitope-tagged AKIP was localized to the nuclear compartment of the cell, as shown in Figure 7-12.

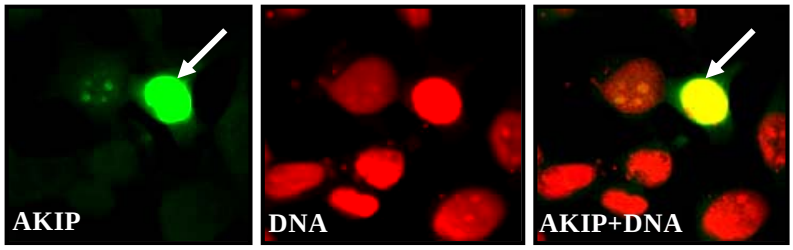


Figure 7-12: Nuclear Localization of AKIP

HeLa cells were transiently transfected with a FLAG-tagged AKIP cDNA, and the subcellular localization of the transfected AKIP protein (left panel) was detected by staining with FLAG M2 monoclonal antibody followed by fluorescence microscopy. Counterstaining of DNA (middle panel) was carried out with propidium iodide. Right panel represents the merged image to show the nuclear localization of the transfected AKIP protein.

A doxycycline-inducible HeLa Tet-On cell line, stably expressing the AKIP, was also constructed subsequently. Upon doxycycline induction, the selected positive clones displayed very tight control of the AKIP protein expression with no background leaky expression, as shown in Figure 7-13.

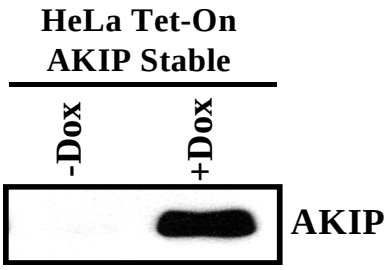


Figure 7-13: Doxycycline-Inducible AKIP-Expressing HeLa Tet-On Stable Cell Line
HeLa Tet-On cells were transfected with AKIP-pTRE2hyg and positive AKIP expressing clones were selected by hygromycin. One of the positive clones, showing high inducibility and low background expression, was shown, where AKIP expression was induced by doxycycline for 24 hr.

Interestingly, in some of the selected positive clones that expressed lower level of AKIP protein upon doxycycline induction, we observed a nucleolar-like subcellular localization for the AKIP protein in the interphase cells, as shown in the Figure 7-14.

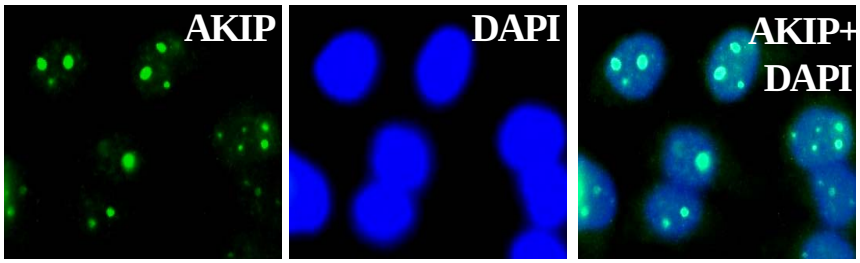


Figure 7-14: Nucleolar-like Localization Pattern of AKIP
FLAG-tagged AKIP inducible stable HeLa cells were induced with doxycycline for 24 hrs, and the subcellular localization of the AKIP protein (left panel) was detected by staining with FLAG M2 monoclonal antibody followed by fluorescence microscopy. Counterstaining of DNA (middle panel) was carried out with DAPI. Right panel represents the merged image to show the nuclear localization of the AKIP protein.

To investigate whether or not AKIP protein was localized to the nucleolus, we immunostained the cells with the nucleolar marker protein, C23 and looked for the co-localization of AKIP with the C23. Indeed, Figure 7-15 had clearly demonstrated that AKIP was indeed localized to nucleolus during interphase.

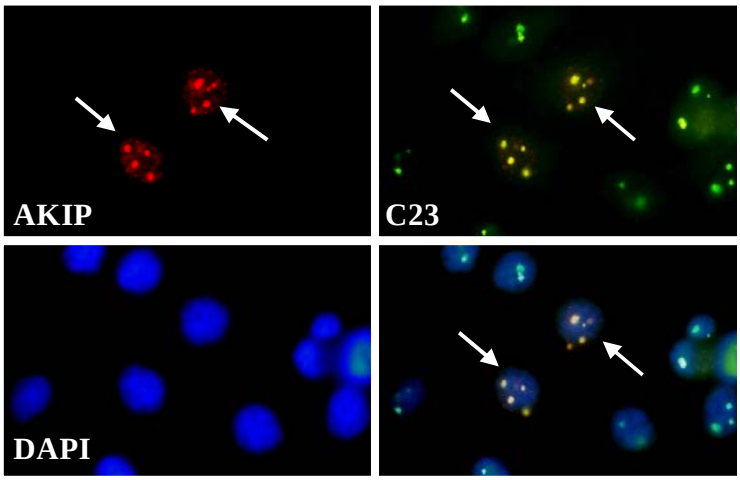


Figure 7-15: Localization of AKIP in Interphase- Nucleolus

FLAG-tagged AKIP inducible stable HeLa cells were induced with doxycycline for 24 hrs, and the subcellular localization of the AKIP (top left panel) and C23 (top right panel) were detected by staining with FLAG rabbit polyclonal antibody (red) and C23 mouse monoclonal antibody (green), respectively, followed by fluorescence microscopy. Counterstaining of DNA (bottom left panel) was carried out with DAPI (blue). Bottom right panel represents the merged image to show the co-localization of AKIP and C23 in the nucleolus of the nucleus. Due to fluorescence bleach-through between channels, two AKIP expressing cells in the top right panel displayed the colocalization of the AKIP and C23 (yellow) whereas other surrounding non-expressing cells were stained with C23 (green) only.

Besides, we also attempted to detect AKIP localization in mitotic cells. As shown in Figure 7-16 and 7-17, AKIP could localize to the mitotic spindles in metaphase and post-mitotic bridge in telophase.

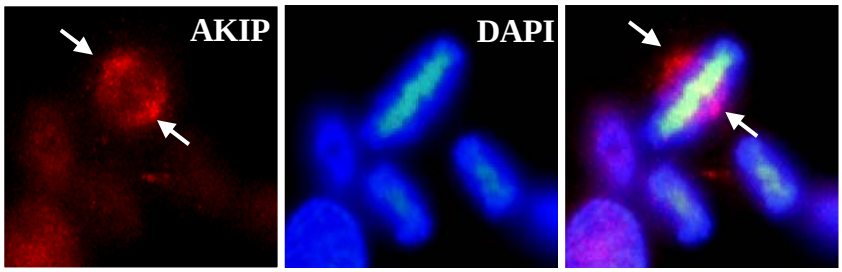


Figure 7-16: Localization of AKIP in Mitosis-Mitotic Spindle in Metaphase

FLAG-tagged AKIP inducible stable HeLa cells were induced with doxycycline for 24 hrs, and the subcellular localization of the AKIP protein (left panel) was detected by staining with FLAG polyclonal antibody followed by fluorescence microscopy. Counterstaining of DNA (middle panel) was carried out with DAPI. Right panel represents the merged image to show the mitotic spindle localization of AKIP in metaphase.

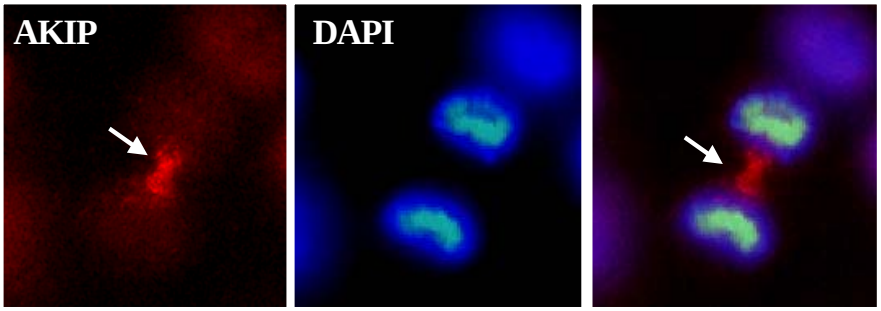


Figure 7-17: Localization of AKIP in Mitosis-Post-Mitotic Bridge in Telophase

FLAG-tagged AKIP inducible stable HeLa cells were induced with doxycycline for 24 hrs, and the subcellular localization of the AKIP protein (left panel) was detected by staining with FLAG polyclonal antibody followed by fluorescence microscopy. Counterstaining of DNA (middle panel) was carried out with DAPI. Right panel represents the merged image to show the post-mitotic bridge localization of AKIP in telophase.

Since AKIP could localize to the mitotic structure and Aurora-A is known to be a mitotic kinase, they might colocalize together during mitosis. Indeed, as shown in Figure 7-18, we could detect colocalization of AKIP and Aurora-A during mitosis.

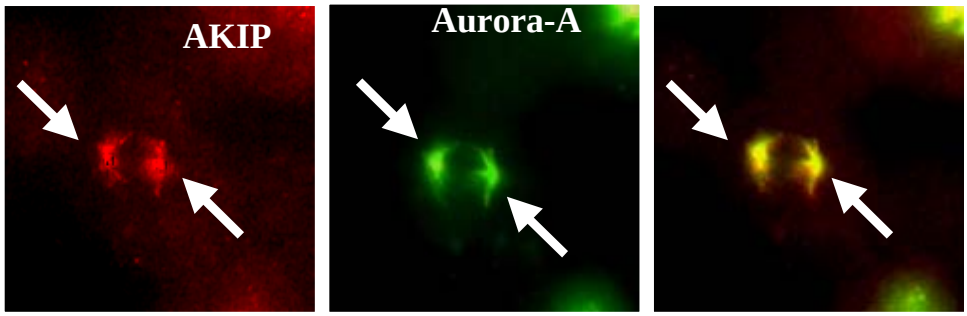


Figure 7-18: Colocalization of AKIP and Aurora-A in Mitosis

FLAG-tagged AKIP inducible stable HeLa cells were induced with doxycycline for 24 hrs, and the subcellular localization of the AKIP protein (left panel) and Aurora-A (middle panel) was detected by staining with FLAG polyclonal antibody and IAK1 monoclonal antibody, respectively, followed by fluorescence microscopy. Right panel represents the merged image to show the colocalization (yellow) of the AKIP and Aurora-A protein.

7.2.4. *In vivo* Interaction of Aurora-A Kinase and AKIP

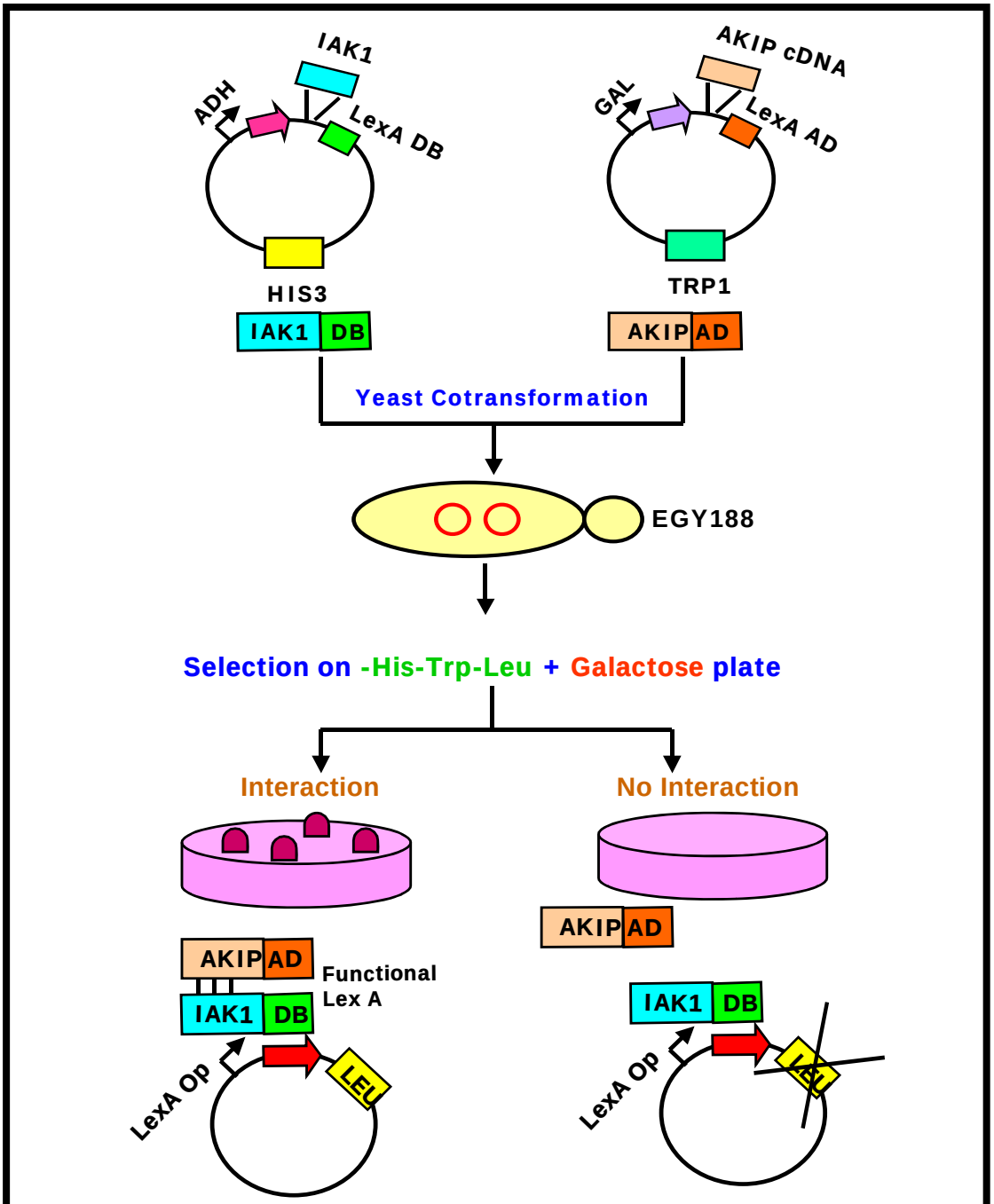
7.2.4.1. Yeast Two-Hybrid Assay

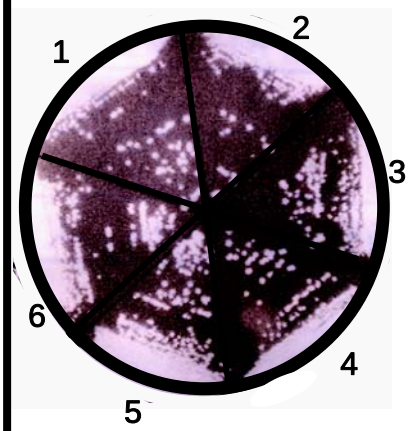
Through the yeast dosage suppressor screen, we had isolated AKIP as a potential negative regulator for Aurora-A kinase. However, the dosage suppressor screen was capable of identifying both the direct and indirect regulator(s) of Aurora-A kinase.

To distinguish whether AKIP regulates Aurora-A kinase directly or indirectly, we performed the yeast two-hybrid *in vivo* interaction assay to assess whether or not the AKIP cDNA ("prey" protein, fused to LexA activation domain) interacted with Aurora-A kinase ("bait" protein, fused to LexA DNA binding domain). Their *in vivo* interaction in yeast thereby reconstituted the functional full length LexA transcriptional factor, which could then bind to the LexA operator to activate the LEU reporter gene expression, and thus the survival of yeast on the –His-Trp-Leu + Galactose selective plate, as shown in Figure 7-19. Indeed, the yeast co-transformants of AKIP and Aurora-A formed colonies on the –His-Trp-Leu selective plate. This gave us some preliminary information that AKIP might interact directly with Aurora-A *in vivo*.

Figure 7-19: Overview of Yeast Two Hybrid Assay

IAK1 cDNA is fused to the DNA binding domain (DB) of LexA whereas AKIP cDNA is fused to the activation domain (AD) of LexA, forming the IAK1-DB (Bait) and AKIP-AD (Prey) fusion protein respectively. Only when the "prey" protein interacts with "bait" protein, this brings closer the AD to DB, thereby reconstituting functional LexA transcriptional factor which can then transactivates the LEU reporter transcriptional expression, allowing yeast to survive and grow as Leu+ colonies on the –Leu selective plate. In the absence of bait-prey interaction, only the bait protein with the DB domain can bind to the LexA operator, but LexA DB alone is not functional.





Aurora-A: AKIP *In vivo* Interaction in Yeast

Six representative yeast clones expressing both Aurora-A and AKIP were streaked on galactose containing synthetic medium lacking histidine, tryptophan and Leucine. Photographs were taken after 4 days at 30°C

7.2.4.2. *In vivo* Interaction between Exogenous Aurora-A and Exogenous AKIP

To verify a similar *in vivo* interaction between AKIP and Aurora-A occurs in the mammalian cell context, HeLa cells were co-transfected with Aurora-A together with FLAG-tagged AKIP (truncated or full length), and the cell lysate were subjected to coimmunoprecipitation followed by Western blot analysis.

As shown in Figure 7-20, the transfected Aurora-A was immunoprecipitated using the Aurora-A specific antibody. As expected from the yeast data, both AKIP-TR and AKIP were found to be co-immunoprecipitated with the Aurora-A.

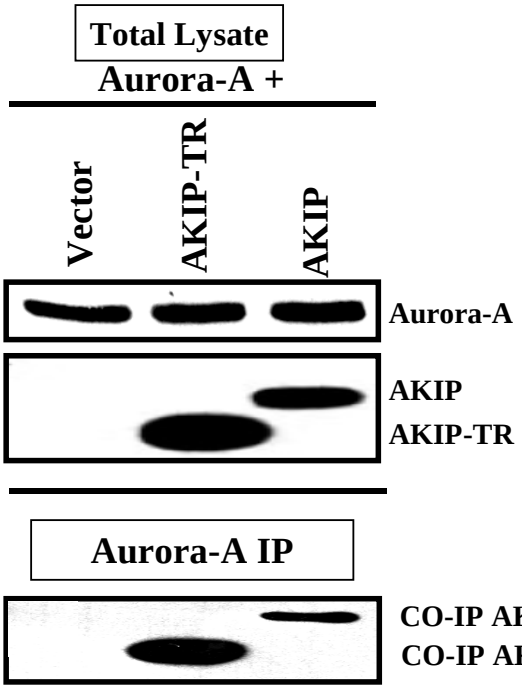


Figure 7-20: *In vivo* Interaction of Exogenous Aurora-A vs Exogenous AKIP: Aurora-A Immunoprecipitation

HeLa cells were co-transfected with Aurora-A and either empty vector or FLAG-tagged AKIP-TR or FLAG-tagged AKIP at 1:1 ratio. Cell lysates were used for immunoprecipitation with rabbit antiserum against Aurora-A. The immunoprecipitates were probed for the interacting AKIP, using the anti-FLAG mouse monoclonal antibody. The corresponding lysates were probed for Aurora-A and AKIP exogenous expression using anti-Aurora-A rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively.

On the other hand, the reverse immunoprecipitation of the AKIP also pulled down the Aurora-A specifically, as shown in Figure 7-21.

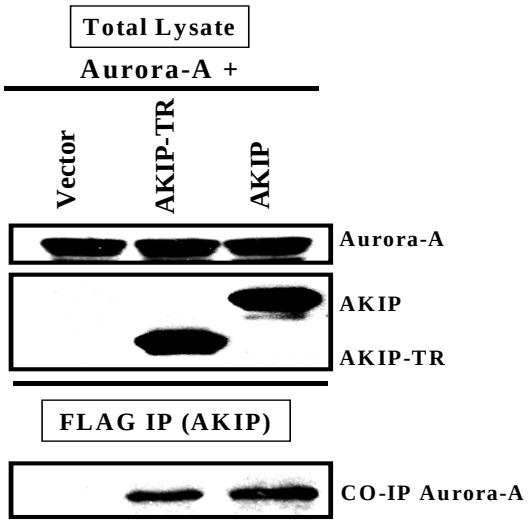


Figure 7-21: *In vivo* Interaction of Exogenous Aurora-A vs Exogenous AKIP: AKIP Immunoprecipitation

HeLa cells were co-transfected with Aurora-A and either empty vector or FLAG-tagged AKIP-TR or FLAG-tagged AKIP at 1:1 ratio. Cell lysates were used for immunoprecipitation with FLAG mouse monoclonal antibody. The immunoprecipitates were probed for the interacting Aurora-A, using the anti-Aurora-A rabbit polyclonal antibody. The corresponding lysates were probed for Aurora-A and AKIP exogenous expression using anti-Aurora-A rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively.

7.2.4.3. *In vivo* Interaction between Endogenous Aurora-A and Exogenous AKIP

To explore the interaction further, we overexpressed the FLAG-tagged AKIP cDNA into HeLa cells and attempted to coimmunoprecipitate the endogenous Aurora-A with the transfected AKIP protein.

The immunoprecipitation of the transfected AKIP pulled down the endogenous Aurora-A, as shown in Figure 7-22. Conversely, the reverse immunoprecipitation of endogenous Aurora-A also co-immunoprecipitated the transfected AKIP, as shown in Figure 7-23.

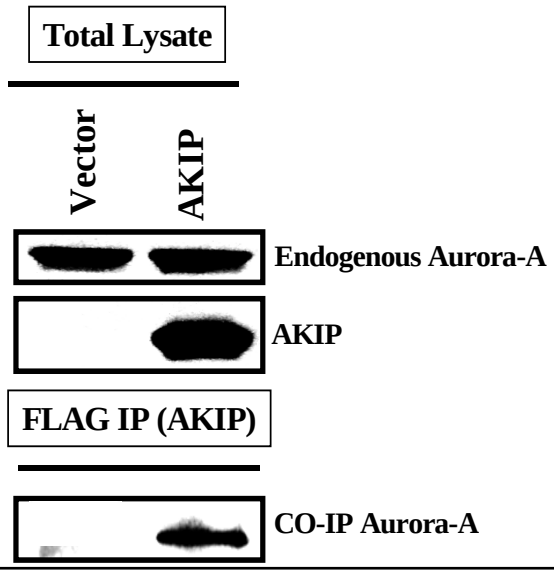


Figure 7-22: *In vivo* Interaction of Endogenous Aurora-A vs Exogenous AKIP: AKIP Immunoprecipitation

HeLa cells were transfected with FLAG-tagged AKIP. Cell lysates were used for AKIP immunoprecipitation with FLAG mouse monoclonal antibody. The immunoprecipitates were probed for the interacting endogenous Aurora-A, using the anti-Aurora-A rabbit polyclonal antibody. The corresponding lysates were probed for Aurora-A and AKIP expression using anti-Aurora-A rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively.

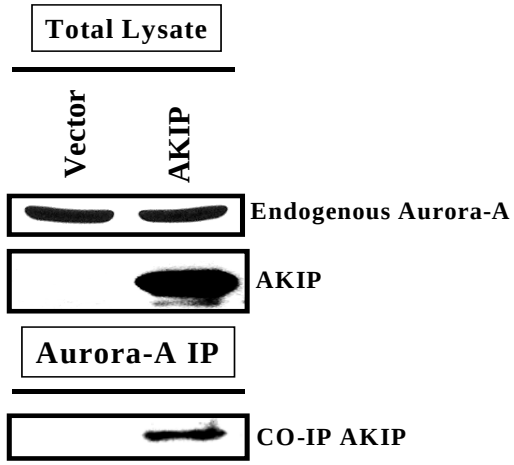


Figure 7-23: *In vivo* Interaction of Endogenous Aurora-A vs Exogenous AKIP: Aurora-A Immunoprecipitation

HeLa cells were transfected with FLAG-tagged AKIP. Cell lysates were used for endogenous Aurora-A immunoprecipitation with anti-Aurora-A rabbit polyclonal antibody. The immunoprecipitates were probed for the interacting AKIP, using the anti-FLAG mouse monoclonal antibody. The corresponding lysates were probed for Aurora-A and AKIP expression using anti-Aurora-A rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively.

7.2.5. AKIP Negatively Regulates Protein Stability of Aurora-A Kinase

Since AKIP has been isolated as the negative regulator of Aurora-A kinase, we presumed that the direct interaction of AKIP and Aurora-A kinase should result in the down-regulation of either the stability and/or the activity of Aurora-A kinase.

To study the impact of AKIP-Aurora-A interaction on Aurora-A protein stability, the endogenous protein level of Aurora-A in the AKIP-transfected cells was investigated. However, the initial attempts to study the effect of AKIP overexpression on the endogenous protein level of Aurora-A were unsuccessful probably because of the lower transfection efficiency.

Instead, we employed the alternative strategy where COS7 cells were co-transfected with Aurora-A and FLAG-tagged AKIP or AKIP-TR expression constructs at 1:9, and the effect of AKIP or AKIP-TR overexpression on the protein stability of exogenous Aurora-A was followed. As presented in Figure 7-24, overexpression of both the full length AKIP or N-terminally truncated form of AKIP (AKIP-TR) could down-regulate the Aurora-A protein, however, the AKIP-TR showed a better efficiency in that it could completely deplete the ectopically expressed Aurora-A protein in COS7 cells. This was probably due to higher levels of AKIP-TR protein accumulated inside the cells or better binding of AKIP-TR to Aurora-A kinase. AKIP appeared to be less stable compared to its truncated form. Hence, AKIP-TR would be used for all subsequent studies related to the AKIP-mediated Aurora-A degradation.

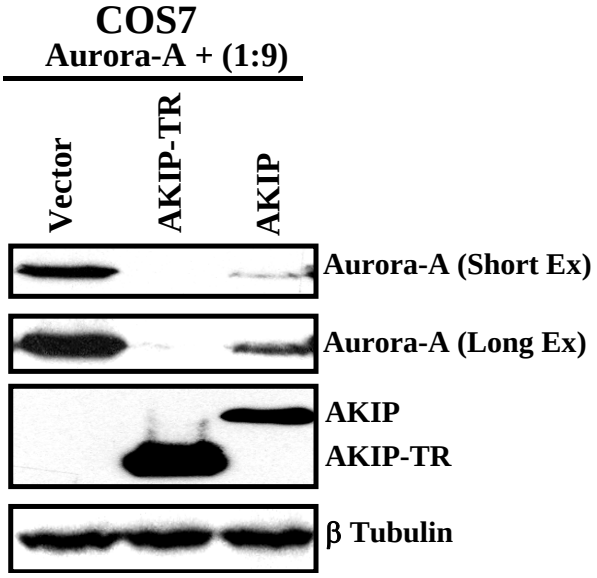


Figure 7-24: Effect of AKIP Overexpression on Exogenous Aurora-A

COS7 cells were co-transfected with Aurora-A and either FLAG-tagged AKIP-TR or AKIP or empty pCDNA3 vector at 1:9 ratio for 36 hrs before harvested for Western blot analysis of Aurora-A and AKIP using anti-AIK1 rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively. β tubulin was used as the loading control.

To further characterize the nature of AKIP-mediated Aurora-A degradation, NIH-3T3 or COS7 cells were co-transfected with Aurora-A and FLAG-tagged AKIP-TR expression constructs at different ratios, starting from 1:0 to 1:9, and the effect of differential AKIP-TR expression on the protein stability of exogenous Aurora-A was followed and compared, as shown in Figure 7-25. On the other hand, the co-transfection ratio was fixed at 1:9 for Aurora-A: AKIP-TR and the effect of different time frame of AKIP-TR expression on protein stability of exogenous Aurora-A was analyzed and compared, as shown in Figure 7-26. As presented in Figure 7-25 and Figure 7-26, overexpression of AKIP-TR could down-regulate the Aurora-A protein in both dose- and time-dependent manner.

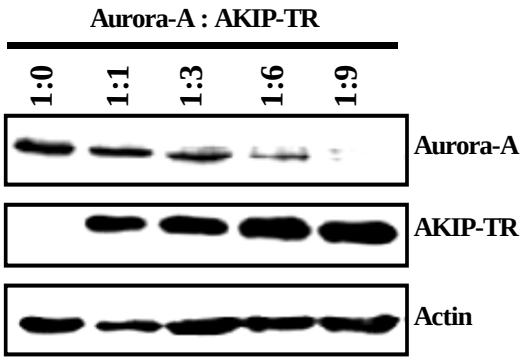


Figure 7-25: Dose-Dependence of AKIP-mediated Down-regulation of Aurora-A

NIH-3T3 cells were co-transfected with Aurora-A and FLAG-tagged AKIP-TR at different ratios starting from 1:0 to 1:9, for 36 hrs before harvested for Western Blot analysis of Aurora-A and AKIP using anti-AIK1 rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively. Actin was used as the loading control.

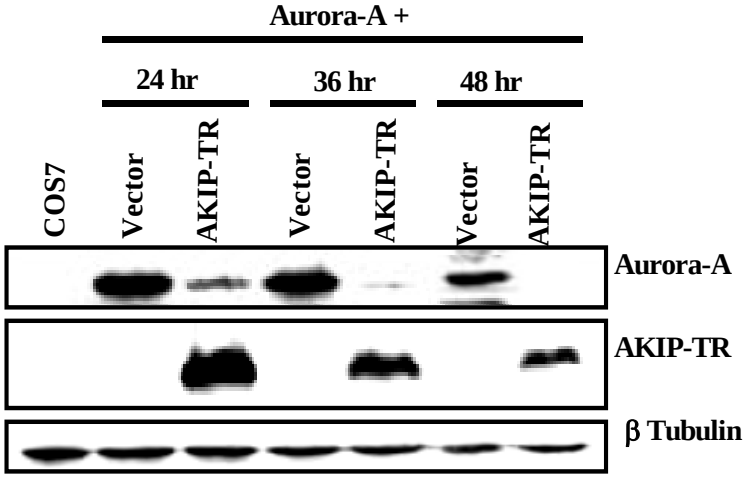


Figure 7-26: Time-Dependence of AKIP-mediated Down-regulation of Aurora-A Protein

COS 7 cells were co-transfected with Aurora-A and FLAG-tagged AKIP-TR at 1:9, for different time points, up to 48 hrs before harvested for Western Blot analysis of Aurora-A and AKIP using anti-IAK1 and anti-FLAG mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

7.2.5.1. Role of AKIP:Aurora-A Kinase Interaction

7.2.5.1.1. Construction of Deletion Mutants of AKIP

To address the question of whether the interaction between AKIP and Aurora-A is a necessary step for the down-regulation of Aurora-A, we attempted to isolate a deletion mutant of AKIP protein, which does not interact with the Aurora-A protein. A total of four deletion mutants (ΔN99-AKIP, ΔN198-AKIP, ΔC99-AKIP, ΔC198-AKIP) lacking regions from either the N or C terminus of AKIP were constructed and used for *in vivo* Aurora-A interaction studies. The size and location of these deletions in the different AKIP deletion mutants in relation to the wild type AKIP are indicated in Figure 7-27.

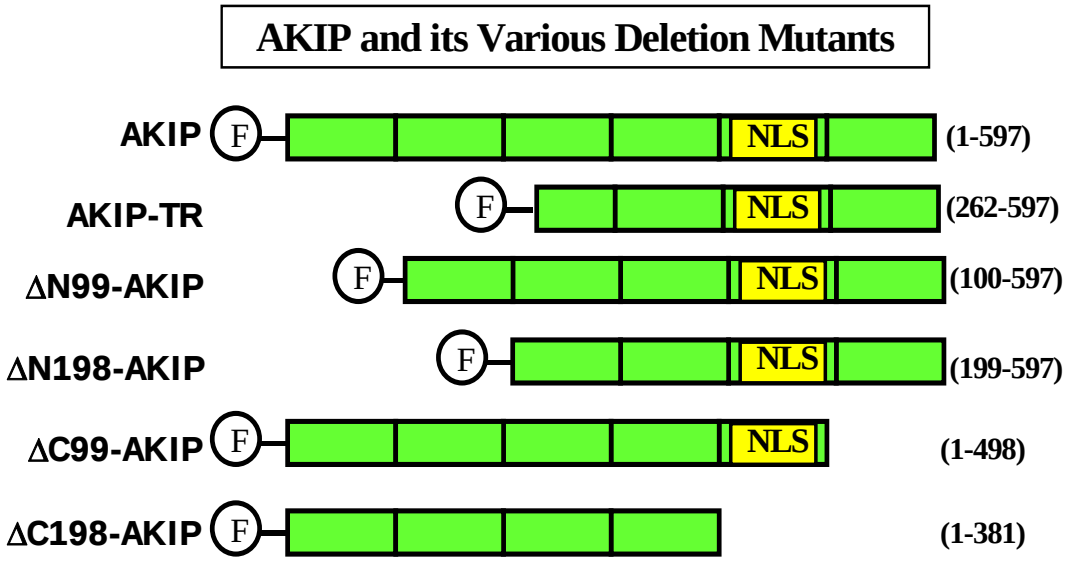


Figure 7-27: AKIP and its Various Deletion Mutants

Comparisons of the size and location of the deletions of all the AKIP mutant proteins with full-length AKIP protein. All of the AKIP variants contain a FLAG tag at the N-terminus. The numbers within parentheses denote the nucleotides of AKIP cDNA, and number 1 corresponds to the nucleotide A of the translational start ATG.

Expression of these deletion mutants in HeLa as well as COS7 cells showed that these mutant proteins had comparable stability except the ΔC198-AKIP mutant, which displayed relatively lower protein stability. As shown in Figure 7-27, this mutant lacked the bipartite nuclear localization signal (NLS). It is unclear whether the lack of nuclear localization signal is responsible for the lower stability and function of this mutant. However, all of the deletion mutants could be expressed successfully in both HeLa and COS7 cells.

7.2.5.1.2. Identification of Aurora-A Non-Interacting Mutant of AKIP

To compensate for the lower stability of the ΔC198-AKIP mutant, a higher ratio of ΔC198-AKIP to Aurora-A (9:1) was used instead of the usual 1:1 in the interaction assay without compromising the levels of Aurora-A protein. Under these conditions, the expression of ΔC198-AKIP was comparable to other AKIP mutant proteins. The data presented in Figure 7-28 suggested that the ΔN99-AKIP and ΔN198-AKIP mutants could interact with Aurora-A protein efficiently, like the wild type AKIP protein. The ΔC99-AKIP mutant could also interact with Aurora-A protein, albeit at the lower efficiency. However, the ΔC198-AKIP mutant did not show any interaction with Aurora-A protein. ΔC198-AKIP was thereby identified as the Aurora-A non-interacting mutant of AKIP. This suggests that the amino acids 127-166 of AKIP contain elements that are necessary for the interaction with Aurora-A protein.

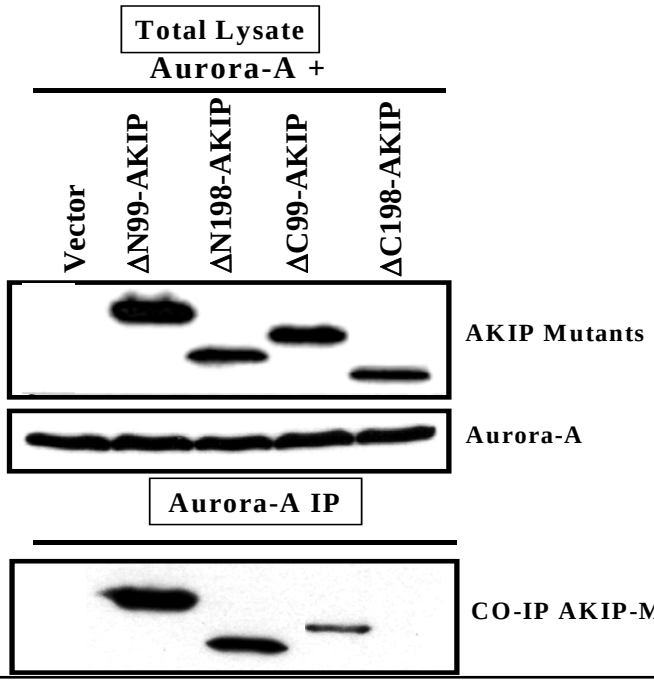


Figure 7-28: *In vivo* Interaction between Aurora-A and AKIP Deletion Mutants: Aurora-A Immunoprecipitation

HeLa cells were co-transfected with Aurora-A and FLAG-tagged AKIP mutant constructs at 1:1 ratio, except for the ΔC198-AKIP was co-transfected at a higher ratio of 1:9. 24 hrs post-transfection, the transfected cells were harvested for Aurora-A immunoprecipitation using the anti-AIK1 rabbit polyclonal antibody. The immunoprecipitates were probed for the interacting AKIP mutant(s), using the anti-FLAG mouse monoclonal antibody. The corresponding lysates were probed for Aurora-A and AKIP mutants expression using anti-Aurora-A rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively.

7.2.5.1.3. Aurora-A:AKIP Interaction is Essential for AKIP-mediated Aurora-A Degradation

To further investigate the efficacy of the non-interacting ΔC198-AKIP mutant in degrading the Aurora-A protein, an *in vivo* Aurora-A degradation assays was performed using the wild type AKIP and these AKIP mutants. The results presented in Figure 7-29 demonstrated that the non-interacting ΔC198-AKIP mutant was less efficient in degrading Aurora-A protein compared with the wild type and other deletion mutants. This suggested that the AKIP-Aurora-A interaction is important for the degradation of Aurora-A protein.

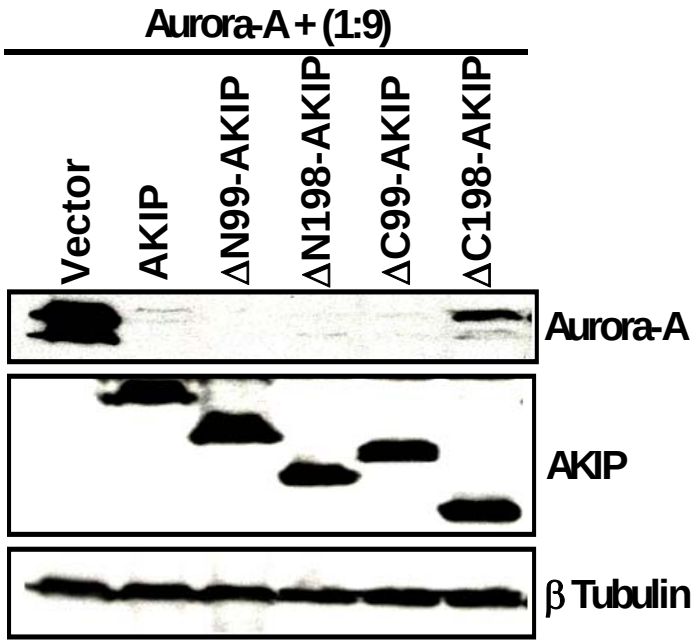


Figure 7-29: AKIP Mutants-mediated Aurora-A Degradation

COS7 cells were co-transfected with Aurora-A and either empty vector or the individual AKIP mutant (AKIP, ΔN99-AKIP, ΔN198-AKIP, ΔC99-AKIP, ΔC198-AKIP) constructs at a 1:9 ratio. 36 hrs post-transfection, the cells were harvested for Western Blot analysis of the Aurora-A and AKIP using anti-AIK1 rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody. β tubulin was used as the loading control.

7.2.5.2. Specificity

To verify the specificity for the effect of AKIP overexpression on down-regulation of Aurora-A protein, the effect of AKIP overexpression on mouse (Figure 7-30) or human (Figure 7-31) Aurora-B, another closely related member of the Aurora kinase family, as well as human cyclin B1 (Figure 7-32) was investigated. The rationale for selecting cyclin B1 is that, similar to Aurora-A, it is also degraded through the proteasome-dependent pathway.

As shown Figure 7-30 to 7-32, the overexpression of AKIP-TR did not affect the protein stability of either Aurora-B or cyclin B1. This implied that AKIP targeted Aurora-A more specifically than Aurora-B for degradation. Also, the failure of AKIP to down-regulate cyclin

B1 suggested that the effect of AKIP on Aurora-A protein stability is not mediated through the generalized activation of the proteolytic machinery.

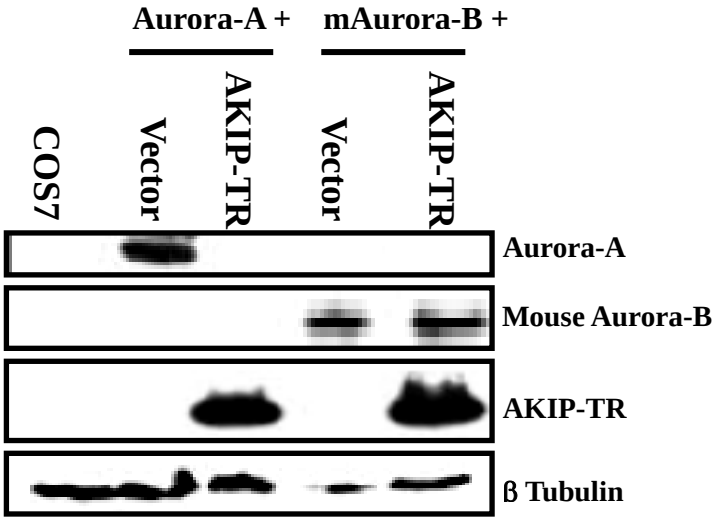


Figure 7-30: Effect of AKIP Overexpression on Mouse Aurora-B Stability

COS7 cells were co-transfected with either Aurora-A or mouse Aurora-B and AKIP-TR constructs at a 1:9 ratio. 36 hrs post-transfection, the cells were harvested for Western Blot analysis of the effect of AKIP-TR overexpression on the levels of Aurora-A or mouse Aurora-B.

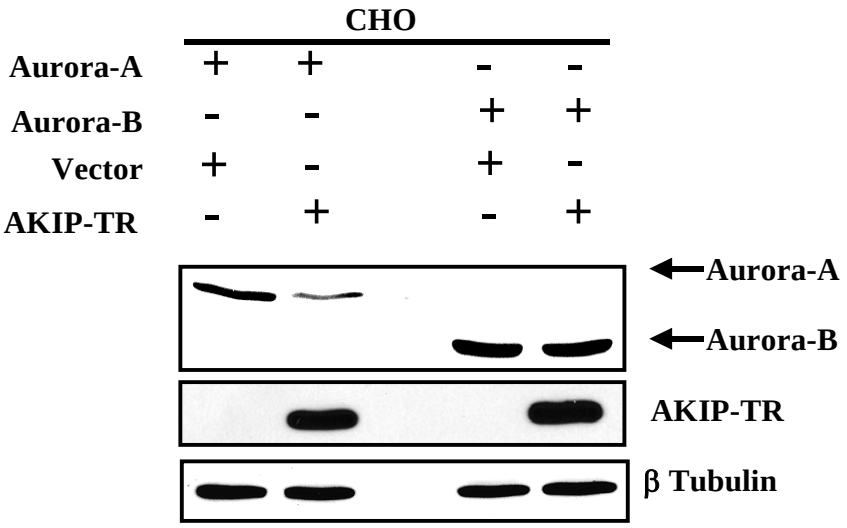


Figure 7-31: Effect of AKIP Overexpression on Human Aurora-B Stability

CHO cells were co-transfected with either human Aurora-A or human Aurora-B and AKIP-TR constructs at a 1:5 ratio. 36 hrs post-transfection, the cells were harvested for Western Blot analysis of the effect of AKIP-TR overexpression on the levels of Aurora-A or Aurora-B.

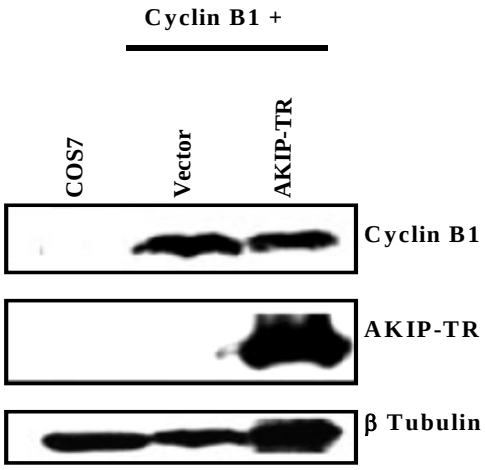


Figure 7-32: Effect of AKIP Overexpression on Human Cyclin B1 Stability

COS7 cells were co-transfected with human cyclin B1 and either empty vector or AKIP-TR constructs at a 1:9 ratio. 36 hrs post-transfection, the cells were harvested for Western Blot analysis of the effect of AKIP-TR overexpression on the levels of cyclin B1.

7.2.5.3. Proteasome Dependency

It has been shown that the proteasome plays a major role in the regulation of Aurora-A stability. Hence, it is possible that the effect of AKIP overexpression on the down-regulation of Aurora-A could be mediated through the potentiation of proteasome-dependent degradation of Aurora-A. To address this question, we followed the AKIP-TR-mediated down-regulation of Aurora-A in the presence and absence of specific proteasome inhibitors, such as MG132, ALLN, and clasto-lactacystin β-lactone.

As shown in Figure 7-33, these proteasome inhibitors could specifically reverse the AKIP-mediated down-regulation of Aurora-A protein to different levels depending on their potencies to inhibit the proteasome machinery. Calpain inhibitor ALLM could not reverse the AKIP-mediated degradation of Aurora-A protein, suggesting that the cystein protease calpain

is unlikely to play a role in the AKIP-mediated down-regulation of Aurora-A. Taken together, these results indicated that the proteasome plays a major role in the AKIP-mediated down-regulation of Aurora-A protein.

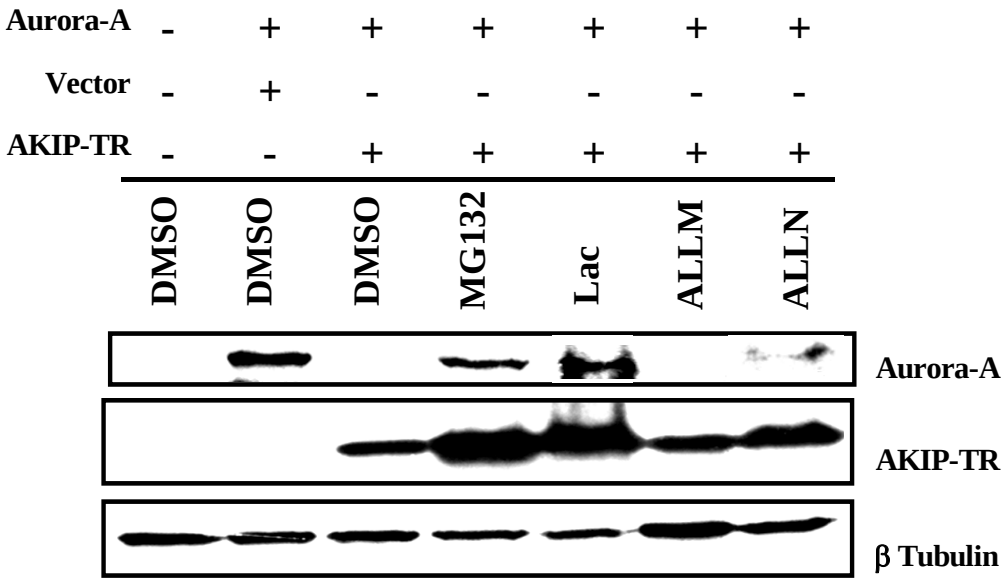


Figure 7-33: Proteasome-Dependence of AKIP-mediated Aurora-A Degradation

COS7 cells were co-transfected with Aurora-A in combination with either empty vector or FLAG-tagged AKIP-TR. Co-transfection with AKIP-TR construct was carried out in 5 sets. 4 sets were treated with the proteasome inhibitors: MG132 (20 μM), ALLN (150 μM), Lactacystin β-lactone (25 μM) and the calpain inhibitor ALLM (25 μM), the last set were treated with the vehicle diethyl suffixed (DMSO) for 12 hrs. 36 hrs post-transfection, the cells were harvested for Western Blot analysis of Aurora-A and AKIP protein. β tubulin was used as the loading control.

7.3. Discussions

The serine/threonine Aurora-A kinase plays multiple crucial roles during cell division, including mitotic entry, bipolar spindle formation, chromosome segregation and cytokinesis [1-6]. Ectopic overexpression of Aurora-A kinase in somatic cells could lead to oncogenic transformation and tumor formation in nude mice [7-11]. Overexpression of Aurora-A protein occurs in a remarkably high proportion of human cancers [7-11]. Regulation of Aurora-A kinase expression and activity is of utmost importance and this can occur at multiple levels, such as gene amplification, transcription, phosphorylation and degradation through the proteasome-dependent pathway [10-15]. Currently, in an attempt to understand further the negative regulation of Aurora-A kinase at molecular level, we had employed the dosage suppressor screen in yeast and had isolated the AKIP, a novel negative regulator of Aurora-A kinase. We had shown that AKIP interacted specifically with Aurora-A and down-regulated Aurora-A kinase by potentiating its degradation through the proteasome-dependent pathway.

To date, other negative regulators of Aurora-A, which target protein stability of Aurora-A, had been identified and they include Cdh1 [14-15], Chfr [16] and hCDC4 [17]. Cdh1 is a well-known APC/C activator that targets many mitotic proteins for ubiqutin-dependent proteolysis during late mitosis and G1 in somatic cell cycle. Similarly, Cdh1 regulates the cell cycle-dependent destruction of Aurora-A kinase during the mitotic exit and G1. Interestingly, this Cdh1-mediated Aurora-A degradation requires a D box in the C-terminal catalytic domain

and a novel A box in the N-terminal non-catalytic domain. Moreover, phosphorylation adds another level of complexity in the Cdh1-mediated Aurora-A degradation. Phosphorylation of Ser51 in the A box negatively regulates the Aurora-A degradation and its dephosphorylation during mitotic exit enables the recognition of both the A box and D box by Cdh1-activated APC/C. Surprisingly, the N-terminal KEN sequence, previously identified as a Cdh1 recognition signal in other proteins, is not essential for this Cdh1-mediated Aurora-A degradation. However, it seems unlikely that AKIP-mediated Aurora-A degradation is mediated through the Cdh1-mediated cell cycle-dependent pathway as the transcriptional expression of AKIP is cell cycle-independent as well as the highly unstable nature of AKIP protein. The A box mutant or D box mutant, which is defective in the Cdh1-dependent degradation, will be useful to address the role of the Cdh1 in AKIP-mediated Aurora-A degradation. Cdc20, another APC/C activator, was previously shown to interact with human Aurora-A [18] but it remains to be answered whether Cdc20 targets Aurora-A for degradation.

Though AKIP seems to be an unstable protein, we cannot exclude the possibility that AKIP may be regulated in the post-translational level in the cell cycle-dependent manner, where its protein stability increases at a specific phase of cell cycle, which we might have overlooked in our study. A more detailed cell cycle study of endogenous AKIP protein will give us some clues. Besides, our study had shown that ectopically expressed AKIP was localized to the sub-nuclear compartment—nucleolus during interphase and some mitotic structures in mitosis.

However, when anti-AKIP antibody was used to study the localization pattern of the endogenous AKIP, we could only detect the AKIP localization on the mitotic structure, but not in nucleolus (data not shown). This discrepancy might result from different adapted protein conformations of AKIP, which are regulated by its subcellular localization. It is possible that AKIP interacts with different anchoring proteins when present in different subcellular compartment.

Localization to these specialized subcellular structures may pose difficulties for us to detect the endogenous AKIP protein. Cell fractionation to specifically isolate and enrich these subcellular components may be more appropriate. Therefore, it is yet to confirm whether AKIP is truly an unstable protein. Studies on the half-life of the exogenous AKIP protein should give us some hints. It should be noted that an antibody against a small peptide of AKIP may not be useful for immunodetection of the endogenous AKIP in its native conformation. The use of a polyclonal antibody against the whole molecule of AKIP may be the solution for detection of endogenous protein by immunocytochemistry or western blot. If AKIP protein is really unstable in the cellular context, it will be interesting to search for the normal physiological trigger or specific cellular stress for the induction of its expression.

On the other hand, Chfr constitutes another regulator of Aurora-A protein stability but its regulation is not cell cycle-dependent. Chfr is an E3 ubiquitin ligase and a newly identified mitotic checkpoint protein, which responds to mitotic stress. Chfr binds Aurora-A and

ubiquitinates Aurora-A *in vivo* [16]. Sequence analysis of AKIP does not reveal any similarity to either F box proteins [19] or U box proteins [20], which plays crucial role in targeting and ubiquitination. Therefore, we exclude the possibility that AKIP may act as the ubiquitin ligase. However, even if AKIP cannot play any direct role in ubiquitination of Aurora-A, AKIP may play an indirect role by enhancing the interaction of Aurora-A with its specific E3 ligase, thereby promoting ubiquitination of Aurora-A and facilitating its proteasomal degradation. So, it will be interesting to probe into the role of AKIP on potentiation of Aurora-A ubiquitination in the future.

We had clearly shown that AKIP down-regulated Aurora-A kinase in the proteasome-dependent manner, either through the 20S or 26S proteasome. Our study had observed the failure of AKIP-dependent cyclin B1 degradation and this confirms the notion that the AKIP does not activate the proteasome machinery in a generic way. The specific interaction of AKIP with Aurora-A kinase as well as the essential nature of the AKIP:Aurora-A interaction for the degradation of Aurora-A raise interesting possibilities, such as AKIP may directly modify and/or target Aurora-A kinase for destabilization or AKIP:Aurora-A interaction evolves as the rate-limiting step in the Aurora-A kinase degradation.

Analysis of the deletion mutants of AKIP for their interaction with and degradation of Aurora-A had demonstrated that the ΔC198-AKIP mutant lacked the elements essential for

the AKIP:Aurora-A interaction, and this interaction was important for the degradation of the Aurora-A kinase. Interestingly, this mutant lacks the nuclear localization signal and therefore was excluded from nucleus and localized to cytoplasm (data not shown). This suggests that the targeting of AKIP to the nucleus may be necessary for the interaction and degradation of Aurora-A kinase. The study of AKIP-mediated Aurora-A degradation in the presence of the nuclear export inhibitor LMB may help further addressing the absolute requirement of nuclear localization for the AKIP-mediated Aurora-A degradation.

Since AKIP was isolated as the negative regulator of Aurora-A oncogene, its potential as a candidate tumour suppressor is definitely worth to be explored in the future. What is the role of AKIP in cancer? Is there any inverse correlation between Aurora-A and AKIP expression in cancer? Surprisingly, our data on AKIP mRNA expression in a panel of cancer cell lines from different tissue origins had demonstrated that AKIP was overexpressed in most of them. This finding is irreconcilable with our hypothesis that AKIP could be a tumour suppressor gene. One rare example of tumour suppressor gene having significantly higher mRNA expression in tumours than normal tissues is WT1 (Wilms' Tumor) [21]. It is likely that these highly expressed WT1 or AKIP mRNA are produced from the mutated genes, which encode for the non-functional protein. Whether or not this increased AKIP mRNA expression directly correlates with its protein expression is not known yet and need to be further verified. If AKIP is a specific tumour suppressor for Aurora-A oncogene, its wild type mRNA and protein

expression should be down-regulated in those tumours overexpressing Aurora-A kinase.

A systematic comparative analysis of AKIP versus Aurora-A mRNA and/or protein expression in Aurora-A overexpressing primary tumours versus their normal counterparts should be able to clarify the tumour suppressor property of AKIP. This can be further supported by the study of the effect of siRNA down-regulation of AKIP expression in normal cells. If AKIP does play the anti-tumour role, long term inhibition or down-regulation of AKIP expression may lead to increase in endogenous Aurora-A expression, which could be potentially oncogenic and may eventually transform the normal cells.

7.4. Conclusion

In summary, we had successfully identified a novel negative regulator of Aurora-A kinase. Further understanding of the normal function of AKIP as well as the characterization of the molecular mechanisms involved in the AKIP-mediated destabilization of Aurora-A are necessary for the future studies. Excitingly, the targeted degradation of Aurora-A by AKIP provides us an alternative strategy to manipulate the endogenous level of the oncogenic Aurora-A kinase. Hence, AKIP could therefore be a potential target gene for anti-cancer drugs in the future.

7.5. References

1. Andrews PD, Knatko E, Moore WJ, Swedlow JR. **Mitotic mechanics: the auroras come into view.** Curr Opin Cell Biol. (2003) 15(6):672-83.

2. Crane R, Gadea B, Littlepage L, Wu H, Ruderman JV. **Aurora A, meiosis and mitosis.** Biol Cell. 2004 Apr;96(3):215-29.

3. Ducat D, Zheng Y. **Aurora kinases in spindle assembly and chromosome segregation.** Exp Cell Res. (2004) 301(1):60-7.

4. Marumoto T, Zhang D, Saya H. **Aurora-A - a guardian of poles**. Nat Rev Cancer. (2005) 5(1):42-50.

5. Bolanos-Garcia VM. **Aurora kinases.** Int J Biochem Cell Biol. (2005) 37(8):1572-7.

6. Dutertre S, Descamps S, Prigent C. **On the role of aurora-A in centrosome function**. Oncogene. (2002) 21(40):6175-83.

7. Giet R, Petretti C, Prigent C. **Aurora kinases, aneuploidy and cancer, a coincidence or a real link?** Trends Cell Biol. (2005) 15(5):241-50.

8. Meraldi P, Honda R, Nigg EA. **Aurora kinases link chromosome segregation and cell division to cancer susceptibility.** Curr Opin Genet Dev. 2004 Feb;14(1):29-36.

9. Katayama H, Brinkley WR, Sen S. **The Aurora kinases: role in cell transformation and tumorigenesis.** Cancer Metastasis Rev. (2003) 22(4):451-64.

10. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR, Sen S. **Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation.** Nat Genet. (1998) 20(2):189-93.

11. Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B, Schryver B, Flanagan P, Clairvoyant F, Ginther C, Chan CS, Novotny M, Slamon DJ, Plowman GD. **A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers.** EMBO J. (1998) 17(11):3052-65.

12. Honda K, Mihara H, Kato Y, Yamaguchi A, Tanaka H, Yasuda H, Furukawa K, Urano T. **Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway**. Oncogene. (2000) 19(24):2812-9.

13. Walter AO, Seghezzi W, Korver W, Sheung J, Lees E. **The mitotic serine/threonine kinase Aurora2/AIK is regulated by phosphorylation and degradation**. Oncogene. 2000 Oct 5;19(42):4906-16.

14. Littlepage LE, Ruderman JV. **Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit.** Genes Dev. (2002) 16(17):2274-85.

15. Taguchi S, Honda K, Sugiura K, Yamaguchi A, Furukawa K, Urano T. **Degradation of human Aurora-A protein kinase is mediated by hCdh1.** FEBS Lett. (2002) 519(1-3):59-65.

16. Yu X, Minter-Dykhouse K, Malureanu L, Zhao WM, Zhang D, Merkle CJ, Ward IM, Saya H, Fang G, van Deursen J, Chen J. **Chfr is required for tumor suppression and Aurora A regulation.** Nat Genet. (2005) 37(4):401-6.

17. Mao JH, Perez-Losada J, Wu D, Delrosario R, Tsunematsu R, Nakayama KI, Brown K, Bryson S, Balmain A. **Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene.** Nature. (2004) 432(7018):775-9.

18. Farruggio DC, Townsley FM, Ruderman JV. **Cdc20 associates with the kinase aurora2/Aik.** Proc Natl Acad Sci USA. (1999) 96(13) 7306-11.

19. Farruggio DC, Townsley FM, Ruderman JV. **The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction.** Prog Biophys Mol Biol. (1999) 72(3):299-328.

20. Hatakeyama S, Yada M, Matsumoto M, Ishida N, Nakayama KI. **U box proteins as a new family of ubiquitin-protein ligases.** J Biol Chem. (2001) 276(35):33111-20.

21. Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, Sugiyama H, Noguchi S. **High Expression of Wilms' Tumor Suppressor Gene predicts Poor Prognosis in Breast Cancer Patients.** Clin Cancer Res. (2002) 8(5):1167-1171.

(Results and Discussions)

CHAPTER 8

AKIP-mediated Aurora-A Kinase Protein Degradation Through Alternative Ubiquitin-Independent, Proteasome-Dependent Pathway

8.1. Summary

Aurora-A normally undergoes cell cycle-dependent regulation of both its synthesis and protein degradation in mitosis. The cell cycle-dependent turnover of Aurora-A is mediated by Cdh1 through the APC/C-ubiquitin-proteasome-dependent pathway. AKIP, previously isolated as the negative regulator of Aurora-A kinase, also destabilizes the Aurora-A through the proteasome-dependent pathway. The present work aims to further investigate the mechanism of the AKIP-mediated Aurora-A degradation. Interestingly, AKIP degrades Aurora-A through the proteasome-dependent but Ub-independent pathway. AKIP-mediated Aurora-A degradation is cell cycle-independent, which is distinctly different from the Cdh1-mediated Aurora-A degradation. A Box mutant, which cannot be targeted for proteasome-dependent degradation by Cdh1, can still be degraded by AKIP. It is also clearly shown that AKIP inhibits the polyubiquitination of Aurora-A and this requires the binding of AKIP to Aurora-A. We have identified a domain in Aurora-A protein, which is important for the ubiquitination of Aurora-A. Interestingly, AKIP targets and binds to this ubiquitination domain, which may probably lead to inhibition of Aurora-A ubiquitination. Inhibition of cellular polyubiquitination either by expression of the K48R dominant negative ubiquitin mutant or temperature-sensitive inactivation of the E1 ubiquitin-activating enzyme, did not abolish the AKIP-mediated Aurora-A degradation. Indeed, the Ub-independent degradation pathway does exist for Aurora-A degradation. Therefore, Aurora-A can be targeted for both Ub-dependent

and Ub-independent protein degradation. This AKIP-mediated Ub-independent degradation of Aurora-A is very specific and mediated through the proteasome. Taken together, there exists an Ub-independent alternative pathway for Aurora-A degradation and AKIP potentiates Aurora-A degradation through this Ub-independent, yet proteasome-dependent pathway.

8.2. Results

8.2.1. AKIP-TR-mediated Aurora-A Degradation Pathway is Distinctively Different from the known "Cdh1-Ubiquitin-Proteasome- Cell Cycle"-Dependent Pathway

8.2.1.1. Cell Cycle-Independence

As mentioned earlier, multiple regulators of Aurora-A kinase protein stability, like cdh1, hCDC4 and Chfr had been isolated recently. All of them target Aurora-A through the ubiquitin-dependent proteasome-dependent degradation pathway, in particular, Cdh1 regulates the degradation of Aurora-A in the cell-cycle dependent manner. As a further step to understand the role of AKIP in Aurora-A degradation, we sought to investigate the mechanism by which AKIP destabilizes Aurora-A.

To address the possible role of AKIP in cell-cycle dependent turnover of Aurora-A, we carried out the *in vivo* AKIP-TR-mediated Aurora-A degradation assays in cells synchronized at different phases of the cell cycle. Cells were initially co-transfected with Aurora-A and either AKIP-TR or empty vector and subsequently synchronized with cell cycle phase-specific inhibitors before being harvested for analysis. The cell synchronization worked well, as shown in Figure 8-1, that under the identical transfection conditions, the ectopically expressed Aurora-A displayed the normal cell cycle-dependent protein stability, low in G1/S phase and high in M phase. Interestingly, the results presented in Figure 8-1 demonstrated that AKIP-TR degraded Aurora-A independently of the cell cycle. This was in contrast to the Cdh1, where Cdh1 showed a cell cycle-specific differential effect on steady-state levels of

Aurora-A, with no effect in M phase cells but significant decrease in log or G1/S phase cells [6]. Thus, this provided the first evidence that AKIP functions differently from Cdh1 in the regulation of Aurora-A protein stability.

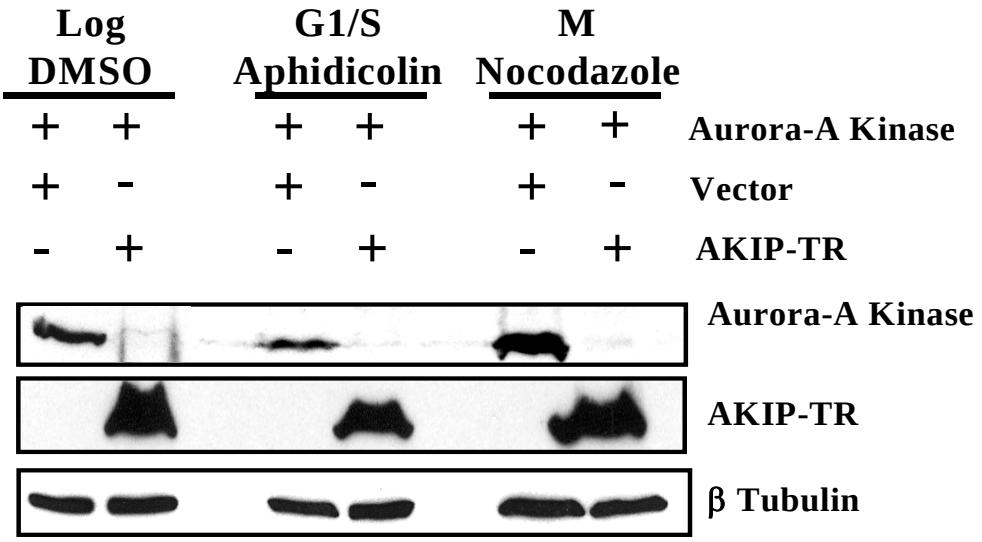


Figure 8-1: Cell Cycle-Independence of AKIP-TR-mediated Aurora-A Degradation

COS7 cells were co-transfected with HA-tagged human Aurora-A and either empty vector or FLAG-tagged AKIP-TR at 1:9 ratio respectively. 24 hrs post-transfection, the transfected cells were collected at different phases of cell cycle by another 16-hr treatment with either DMSO (Log), Aphidicolin (G1/S) and Nocodazole (M). Cell extracts were analyzed for Aurora-A and AKIP-TR proteins using the anti-HA and anti-FLAG mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

8.2.1.2. AKIP-TR can Degrade Ubiquitination-Defective A-Box Stabilizing Mutant of Aurora-A

As mentioned earlier, the Cdh1-ubiquitin-dependent proteasomal degradation of Aurora-A involves two degradation boxes, namely N-terminal A Box and C-terminal D3 Box. The point mutation S51D in the A Box blocks the Cdh1-mediated destabilization of Aurora-A, probably through the inhibition of Aurora-A ubiquitination. To further explore whether polyubiquitination is necessary for AKIP-TR-dependent Aurora-A degradation, the effect of

AKIP-TR overexpression on the protein stability of A-box mutant was studied.

For the present study, we had constructed the A Box mutant by the site-directed mutagenesis of Ser51 of Aurora-A. Before using the A Box mutant for the AKIP-TR-mediated degradation assay, we verified its functionality that whether it was resistant to the normal cell cycle-dependent degradation. The change in the protein stability of A Box mutant during the transition from M to G1 phase was monitored and compared to that of its wild type counterpart. Cells expressing the wild type Aurora-A or A Box mutant were initially arrested in M phase by nocodazole and subsequently released into G1 in the presence of cycloheximide, which inhibited further protein synthesis. The change in the protein level of endogenous cyclin B1 served as the positive control for verifying the success of M to G1 transition.

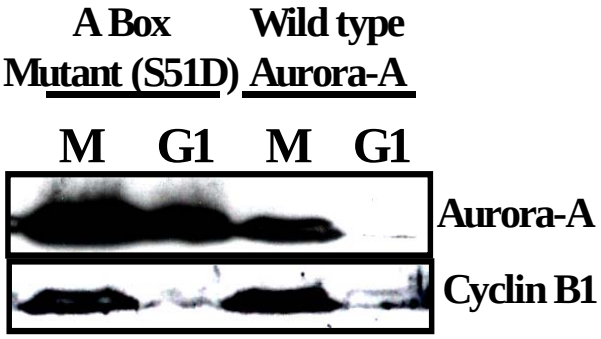


Figure 8-2: Stability of Wild-type and A Box Stabilizing Mutant of Aurora-A from M to G1 Transition

HeLa cells were transfected with either HA-tagged wild-type or A-Box mutant of Aurora-A. 24 hrs post-transfection, the cells were treated with 0.1 μg/ml Nocodazole for 16 hrs to arrest them at M phase. The floating mitotic cells were collected by mitotic shake-off and released in fresh medium containing 50 μg/ml cycloheximide. The cells were harvested for analysis 4 hrs post-mitotic release. Stability of wild-type and A-Box mutant of Aurora-A at the M-G1 transition was detected using the anti-HA mouse monoclonal antibody. Endogenous cyclin B1 levels were used as the positive control to verify the M-G1 transition.

As shown in Figure 8-2, wild-type Aurora-A was degraded rapidly upon exit from mitosis into G1 phase, whereas in contrast, the A-box mutant of Aurora-A remained stabilized throughout the M-G1 transition, indicating the A Box mutant of Aurora-A was functionally defective in the cell cycle-dependent degradation.

When this A-box mutant of Aurora-A, which no longer could be targeted for ubiquitin-dependent degradation, was used for the *in vivo* AKIP-TR-mediated degradation assay, the results showed that the A-box mutant of Aurora-A could still be degraded by AKIP-TR at the same efficiency as the wild-type Aurora-A in the presence of AKIP-TR, as shown in Figure 8-3. These results suggested that AKIP-TR could target even a non-ubiquitylatable form of Aurora-A, further supporting the cell cycle-independent nature of AKIP-TR-mediated Aurora-A degradation.

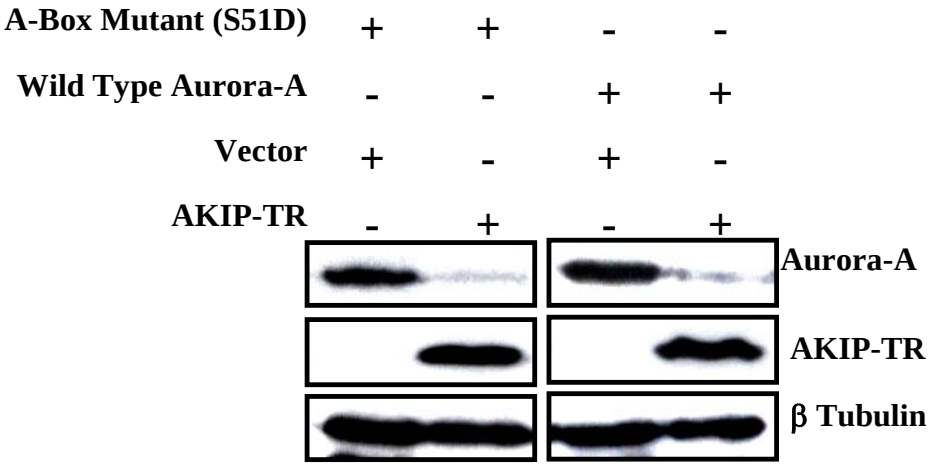


Figure 8-3: Effect of AKIP Overexpression on Stability of A-Box Mutant of Aurora-A

COS7 cells were co-transfected with HA-tagged Aurora-A (wild-type or A-Box mutant) and either empty vector or FLAG-tagged AKIP-TR at 1:9 ratio. The cells were harvested for Western Blot analysis 36 hrs post-transfection. Aurora-A and AKIP-TR were detected by anti-HA and anti-FLAG mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

8.2.2. Role of AKIP-TR on Ubiquitination of Aurora-A

8.2.2.1. AKIP Inhibits Polyubiquitination of Aurora-A

Ubiquitination represents one of the essential modifications to target the proteins for recognition by 26S proteasome and subsequent degradation. It was previously shown that Aurora-A was poly-ubiquitinated before cell cycle-dependent degradation by APC/C. In order to understand the mechanism of AKIP-mediated degradation, we asked whether AKIP played any role in the ubiquitination of Aurora-A.

To determine the possibility that AKIP might potentiate the poly-ubiquitination of Aurora-A and therefore enhance its degradation similar to cdh1 and chfr, we employed the *in vivo* Aurora-A ubiquitination assays to monitor any change in the level of Aurora-A ubiquitination in the absence and presence of AKIP overexpression.

HeLa cells were co-transfected with wild-type Aurora-A, wild-type ubiquitin and either empty vector or AKIP expression constructs. ΔC198-AKIP mutant, an Aurora-A non-interacting mutant of AKIP, was also used as a control to verify whether the interaction between AKIP and Aurora-A was essential for the AKIP-mediated Aurora-A ubiquitination.

As shown in Figure 8-4, Aurora-A could be ubiquitinated readily at the basal state. However, to our surprise, in the presence of AKIP-TR or AKIP co-expression, polyubiquitination of Aurora-A was drastically diminished. This AKIP-mediated inhibition of polyubiquitination was specific to Aurora-A kinase as the total cellular polyubiquitination was unaffected by the AKIP.

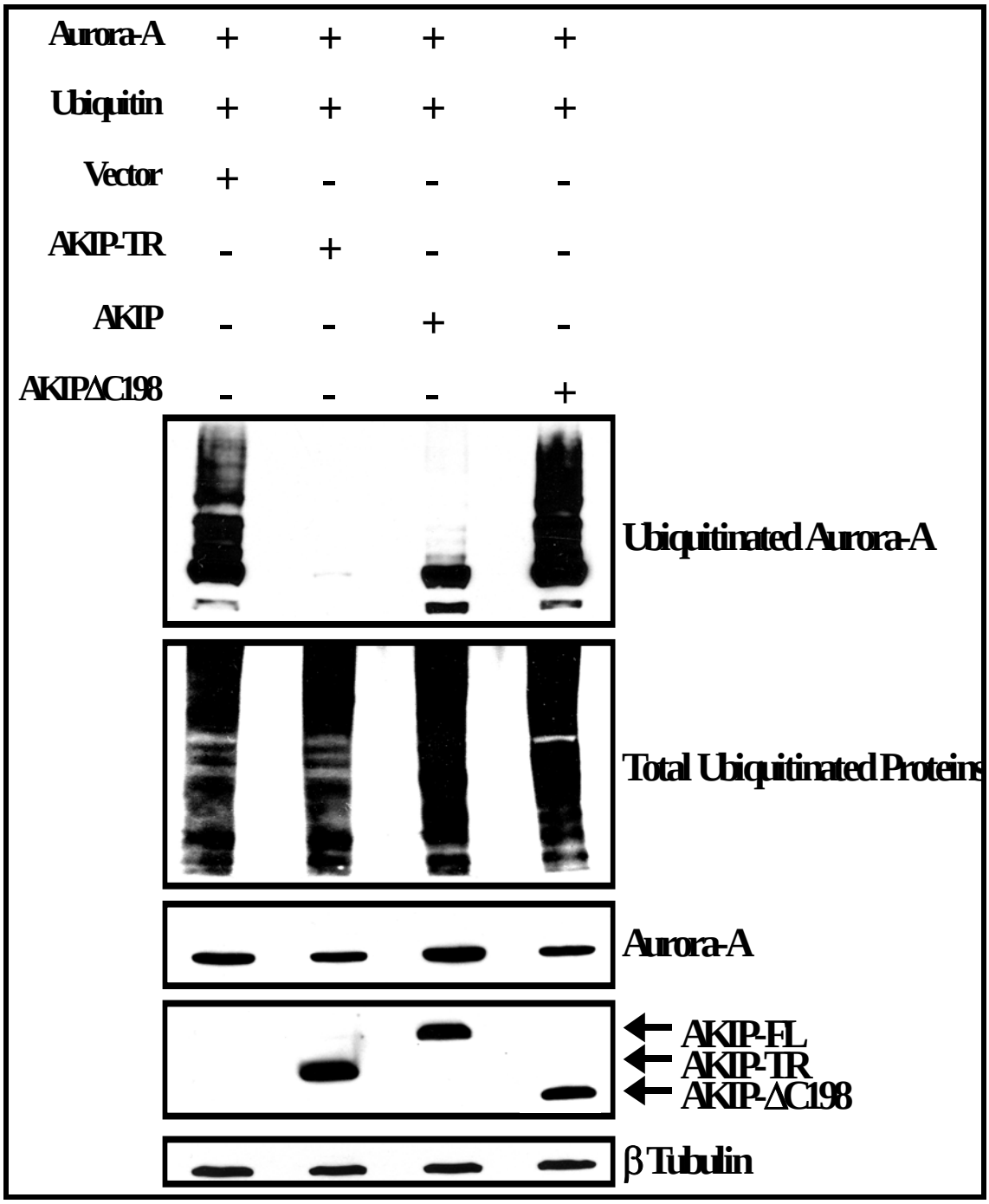


Figure 8-4: Aurora-A Polyubiquitination in the Presence of AKIP

HeLa cells were transiently transfected with HA-tagged Aurora-A in combination with either empty vector pCDNA3 or AKIP constructs (AKIP-TR, AKIP and ΔC198-AKIP mutant) at 1:9 ratio, respectively in the presence of an expression construct encoding His-tagged wild-type ubiquitin (His-Ub). All the His-Ub-tagged proteins were pulled down and subsequently analyzed specifically for the ubiquitinated species of Aurora-A. Total cellular polyubiquitination is detected using the anti-ubiquitin mouse monoclonal antibody. Aurora-A and AKIP proteins in the total cell lysate were probed with anti-HA and anti-FLAG mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

Moreover, we also ruled out that the drop in polyubiquitination level of Aurora-A was due to the concomitant decrease in the Aurora-A protein stability in the presence of AKIP as the Aurora-A protein levels were maintained with proteasome inhibitor MG132 in all cell lysates. MG132 blocks the protein degradation but not the protein modification by ubiquitin. In another word, AKIP indeed directly inhibited the polyubiquitination of Aurora-A.

Interestingly, ΔC198-AKIP mutant, that did not interact with Aurora-A and was less efficient in targeting Aurora-A for degradation, restored similar level of Aurora-A polyubiquitination to the basal state as the empty vector control. These observations suggested that AKIP inhibited polyubiquitination of Aurora-A, and this inhibition required interaction between AKIP and Aurora-A. This supported the previous findings that the AKIP-mediated Aurora-A degradation was not through the more common Ub-dependent mechanism.

8.2.2.2. Identification of the Ubiquitination Domain of Aurora-A

We had demonstrated that the *in vivo* interaction between AKIP-TR and Aurora-A was essential for the AKIP-TR-mediated Ub-independent degradation of Aurora-A and binding of the AKIP-TR to Aurora-A inhibited the *in vivo* ubiquitination of Aurora-A very efficiently. So, how did AKIP-TR interaction with Aurora-A affect the ubiquitination of Aurora-A? To address this question, we would like to identify and narrow down the region(s) of Aurora-A, which contains the potential ubiquitination sites and assess whether AKIP-TR specifically

inhibits the ubiquitination by directly binding in the vicinity of this ubiquitination region and overriding the Ub-dependent degradation mode.

To search for the potential ubiquitination region(s) in Aurora-A, we had performed systematic deletion of Aurora-A from both N- and C-terminus and constructed four deletion mutants of Aurora-A, two of which were N-terminal deletion mutants, named as ΔN300-AIK1 (deletion of 309 bp from N-terminus) and ΔN600-AIK1 (deletion of 609 bp from N-terminus), whereas the other two were C-terminal deletion mutants, named as ΔC300-AIK1 (deletion of 312 bp from C-terminus) and ΔC600-AIK1 (deletion of 612 bp from C-terminus), as shown in Figure 8-5.

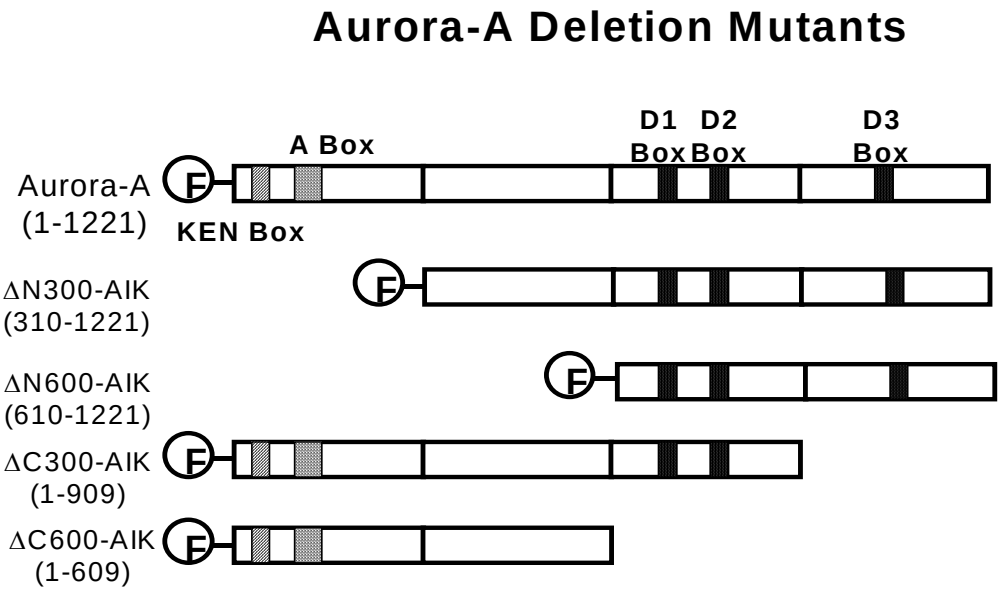


Figure 8-5: Aurora-A Kinase and Its Various Deletion Mutants

Comparison of the size and location of the deletions of all the Aurora-A deletion mutant proteins with full length Aurora-A protein. All of the Aurora-A variants contain a FLAG tag at the N-terminus. The *numbers* within the *parentheses* denote the nucleotides of Aurora-A cDNA, and number 1 corresponds to the nucleotide A of the translational start ATG. The locations of KEN, A, and D (D1,D2, D3) boxes are indicated.

All the Aurora-A deletion mutants were tagged with FLAG epitope at their N-terminus. Various degradation motifs had been identified in Aurora-A, which include KEN Box and A Box at the N-terminus and D Boxes (D1-3) at the C-terminus. Both ΔN300-AIK1 and ΔN600-AIK1 mutants lacked the KEN box and A Box, ΔC300-AIK1 lacked D3 Box, however, Δ600-AIK1 had all the three D Boxes deleted.

To identify the region of Aurora-A that is important in regulating the ubiquitination, we had carried out the *in vivo* ubiquitination assay using these Aurora-A deletion mutants and attempted to detect their *in vivo* ubiquitination level. The Aurora-A mutant, which carried the deletion of ubiquitination region, should have markedly reduced or totally abolished ubiquitination. As shown in Figure 8-6, ubiquitination of ΔC600-AIK1 was totally abolished, whereas the ubiquitination of the other three mutants like ΔN300-AIK1, ΔN600-AIK1 and ΔC300-AIK1 were unaffected as demonstrated by their ubiquitination ladders. However, in comparison with the normal wild type Aurora-A counterpart, ΔN300-AIK1 and ΔN600-AIK1 both had reduced level of ubiquitination, whereas interestingly, the ΔC300-AIK1 had markedly increased level of ubiquitination, suggesting the probable presence of negative element for ubiquitination in the region of Aurora-A from a.a. 910 to 1221. From these results, we could conclude that the region of Aurora-A from a.a. 610 to 909 should carry either the critical functional ubiquitination sites or the recognition domain for ubiquitination enzymes.

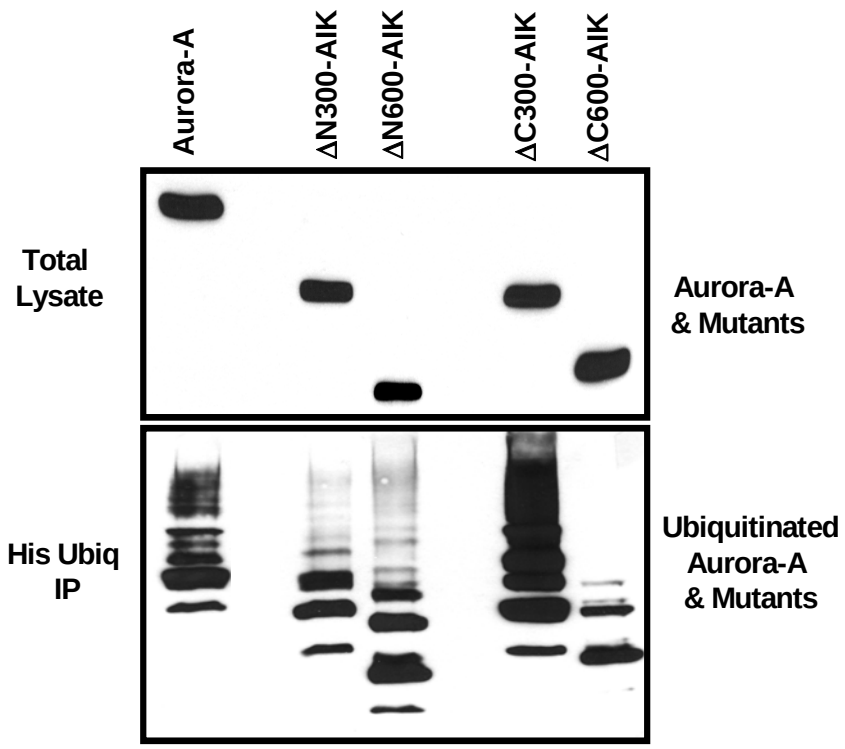


Figure 8-6: Mapping for Ubiquitination Domain in Aurora-A

HeLa cells were transfected with His-tagged wild-type ubiquitin and FLAG-tagged Aurora-A or its various deletion mutants. 24 hours post-transfection, the transfected cells were treated with 20 μM MG132 for 16 hours before harvested for His immunoprecipitation. The ubiquitinated Aurora-A and the deletion mutants were specifically detected using anti-FLAG mouse monoclonal antibody.

8.2.2.3. *In vivo* Binding of AKIP-TR to Ubiquitination Domain of Aurora-A

As shown previously, the [AKIP-TR]-[Aurora-A] interaction was necessary to inhibit the ubiquitination of Aurora-A. With our identification of the ubiquitination region of Aurora-A, we could now examine the possibility whether or not AKIP-TR might bind to this critical region of Aurora-A and therefore block its ubiquitination. *In vivo* interaction between AKIP-TR and the ubiquitination-defective mutant, ΔC600-AIK1, would be studied and compared to the wild-type and other deletion mutants of Aurora-A. As shown in Figure 8-7, no interaction could be detected between AKIP-TR and ΔC600-AIK1 *in vivo,* whereas the *in*

vivo interaction between AKIP-TR and the wild-type or other deletion mutants (ΔN300-AIK1, ΔN600-AIK1, ΔC300-AIK1) of Aurora-A were readily detected. In conclusion, AKIP-TR targets the ubiquitination region of Aurora-A and masks subsequent recognition and ubiqutin conjugation. This probably could be one of the mechanisms on how the [AKIP-TR]-[Aurora-A] interaction inhibits the Aurora-A ubiquitination.

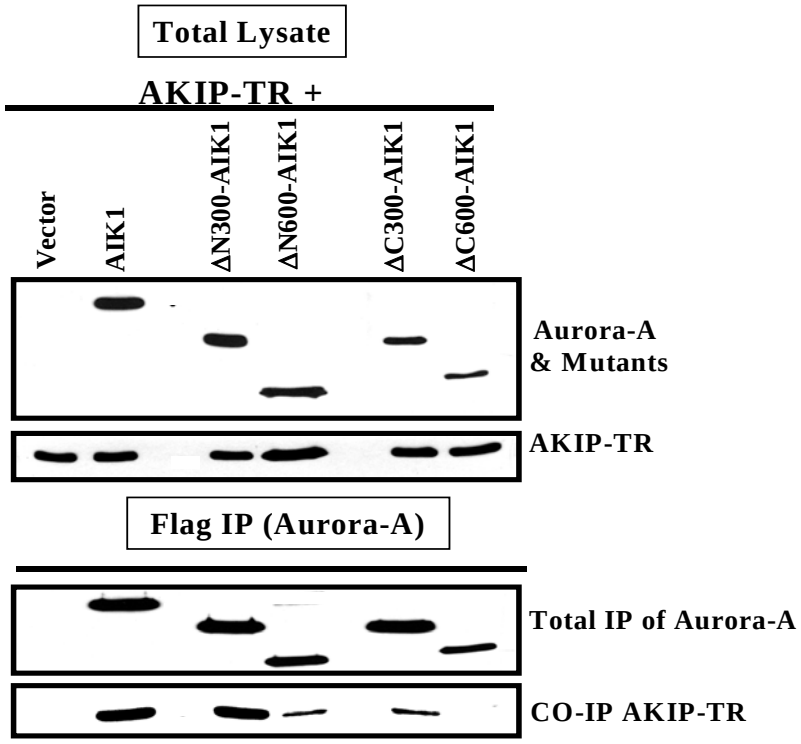


Figure 8-7: Mapping of AKIP-Interacting Domain in Aurora-A

HeLa cells were transfected with HA-tagged AKIP-TR and FLAG-tagged Aurora-A or its various deletion mutants at 1:1 ratio. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The interacting AKIP-TR was detected using the anti-AKIP rabbit polyclonal antibody.

8.2.3. Existence of the Ubiquitin-Independent Degradation Pathway for Aurora-A Kinase

Since AKIP-TR can target the ubiquitination-defective A-Box mutant of Aurora-A for

degradation, alternative degradation pathway, which is independent of ubiquitination, should exist for Aurora-A kinase. To study the existence of this alternative pathway, it is necessary to completely inhibit the Ub-dependent mode of degradation and in the meantime monitor the turnover of the Aurora-A kinase. A cell line, which harbours the temperature-sensitive mutation and hence the inactivation of E1 ubiquitin-activating enzyme at the restrictive temperature, would be very helpful for such a study. Under the permissive temperature condition, both Ub-dependent and Ub–independent pathways are functional, however, when the growth temperature is shifted to the non-permissive one, the Ub-dependent pathway will be switched off, leaving only the Ub-independent pathway still operational.

8.2.3.1. Exogenous Aurora-A

To verify the existence of an Ub-independent pathway for Aurora-A degradation, the turnover of Aurora-A, p21 and cyclin B1 was assessed in the presence of cycloheximide in ts20-CHO temperature-sensitive E1 Ub-activating mutant cells. p21 was previously demonstrated to be able to serve as a target for Ub-independent degradation whereas cyclin B1 has been a prototype target for Ub-dependent degradation.

From the results presented in Figure 8-8, temperature shift to the non-permissive 40^{o}C did not completely stabilize the p21, supporting the previous finding that p21 can be degraded in the absence of ubiquitination, and hence can act as the substrate for Ub-independent degradation.

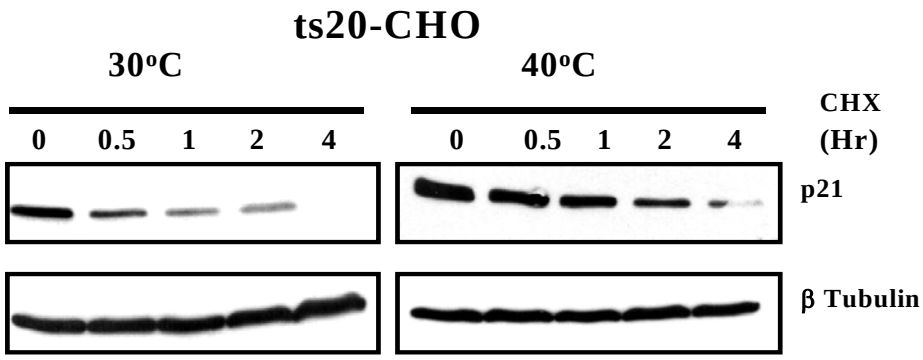


Figure 8-8: p21, A Target for Ubiquitin-Independent Degradation Pathway.

To determine the half-life of p21 in ts20-CHO cells in the presence and absence of polyubiquitination, cells were transfected with HA-tagged p21 expression plasmid at 30°C. 24 hrs post-transfection, the cells were divided into two sets, one set was maintained at 30°C permissive temperature whereas the other set was shifted to 40°C for 16 hrs. After 16 hrs, the cells were treated with 50 μg/ml cycloheximide and both sets were harvested at 0, 0.5, 1, 2 and 4 hrs post-treatment. The level of p21 was analyzed by anti-HA mouse monoclonal antibody. β tubulin was used as the loading control.

In contrast, cyclin B1 level was completely stabilized upon temperature shift to 40°C, as shown in Figure 8-9, indicating that cyclin B1 can only be targeted for the Ub-dependent degradation.

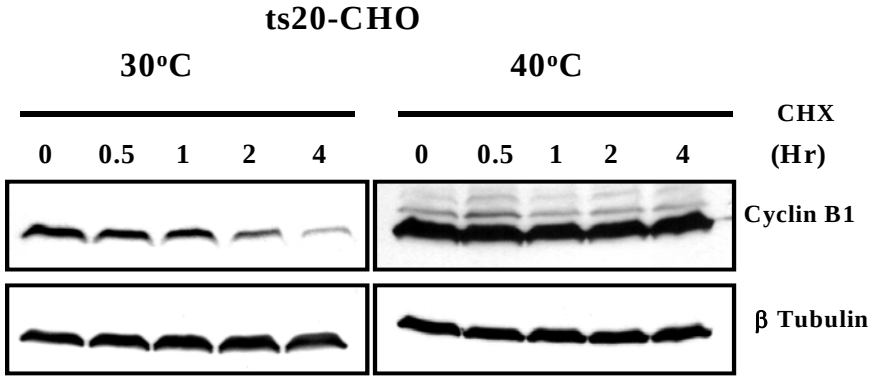


Figure 8-9: Cyclin B1, A Target for Ubiquitin-Dependent Degradation Pathway.

To determine the half-life of cyclin B1 in ts20-CHO cells in the presence and absence of polyubiquitination, cells were transfected with cyclin B1 expression plasmid at 30°C. 24 hrs post-transfection, the cells were divided into two sets, one set was maintained at 30°C permissive temperature whereas the other set was shifted to 40°C for 16 hrs. After 16 hrs, the cells were treated with 50 μg/ml cycloheximide and both sets were harvested at 0, 0.5, 1, 2 and 4 hrs post-treatment. The level of cyclin B1 was analyzed by anti-cyclin B1 rabbit polyclonal antibody. β tubulin was used as the loading control.

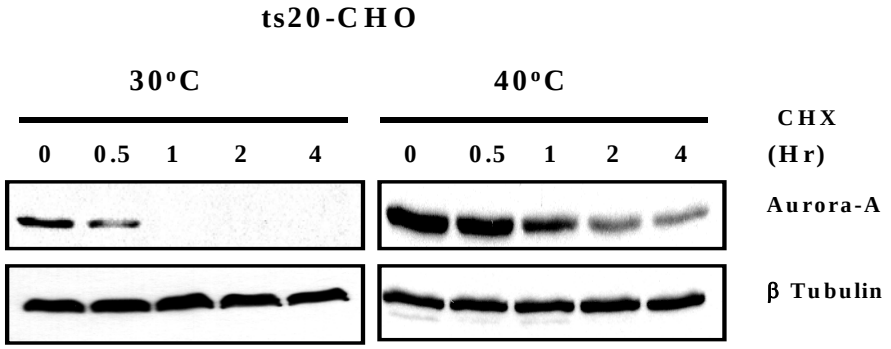


Figure 8-10: Aurora-A, A Target for Ubiquitin-Independent Degradation Pathway

To determine the half-life of Aurora-A in ts20-CHO cells in the presence and absence of polyubiquitination, cells were transfected with FLAG-tagged Aurora-A expression plasmid at 30°C. 24 hrs post-transfection, the cells were divided into two sets, one set was maintained at 30°C permissive temperature whereas the other set was shifted to 40°C for 16 hrs. After 16 hrs, the cells were treated with 50 μg/ml cycloheximide and both sets were harvested at 0, 0.5, 1, 2 and 4 hrs post-treatment. The level of Aurora-A was analyzed by anti-FLAG mouse monoclonal antibody. β tubulin was used as the loading control.

In the case for Aurora-A, it was evident that even within 30 minutes of cycloheximide treatment, there was a sharp decline in Aurora-A steady state level, indicating that Aurora-A is normally an unstable protein. However, upon suppression of the Ub-dependent pathway at 40°C, its steady state level was increased but not stabilized completely, as displayed in Figure 8-10. The lack of complete stabilization implied that Aurora-A could also be targeted for degradation in the absence of ubiquitin similar to p21. Both Aurora-A and p21 can therefore be targeted for degradation, not only through the Ub-dependent pathway, but also through the alternative Ub-independent route.

8.2.3.2. Endogenous Aurora-A

To address the question whether the endogenous Aurora-A also follows the same fate as the exogenous protein with respect to the stabilization in the absence of ubiquitination, we chose to carry out the experiment in the temperature-sensitive mouse cell line ts20-TG, also harbouring a mutation in E1 ubiquitin-activating enzyme, to facilitate the detection of endogenous proteins with the available antibodies. As described previously with ts20-CHO cells, identical experiments were carried out in this mouse cell line to investigate the turnover of endogenous Aurora-A, p21 and cyclin B1 in the presence of cycloheximide. The results presented in Figure 8-11 demonstrated that the endogenous Aurora-A, p21 and cyclin B1 behave similar to their exogenous counterparts with respect to their turnover in the absence of polyubiquitination.

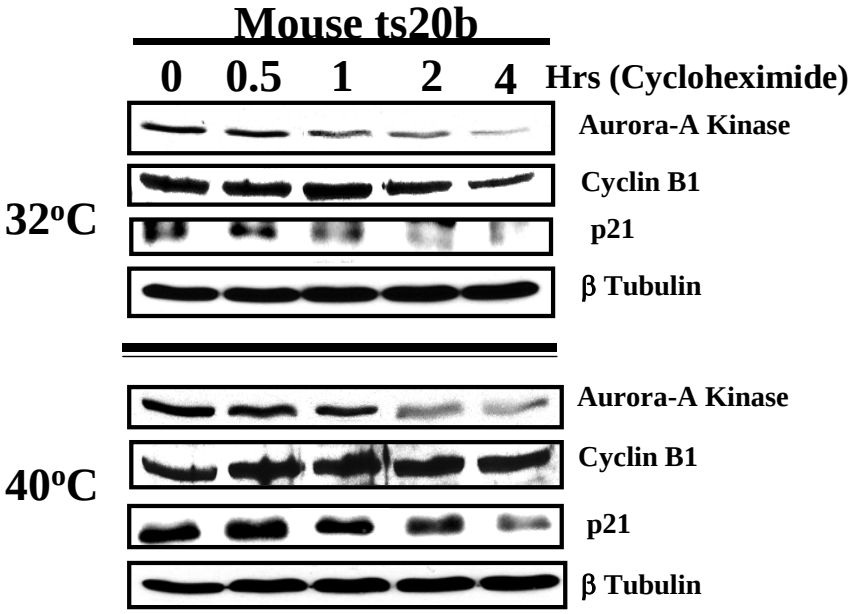


Figure 8-11: Ubiquitin-Independent Degradation of Endogenous Aurora-A Kinase

Mouse ts20b cells were incubated at 32°C or 40°C for 18 hrs followed by the cycloheximide treatment for the indicated times. The protein levels of the endogenous Aurora-A, p21 and cyclin B1 were analyzed by Western Blot analysis using the anti-IAK1 mouse monoclonal, p21 and cyclin B1 rabbit polyclonal antibodies, respectively. β tubulin was used as the loading control.

8.2.4. Role of AKIP-TR in the Ubiquitin-Independent Degradation Pathway for Aurora-A Kinase

At present, Aurora-A can serve as the target for Ub-independent degradation pathway. The previous use of ubiquitination-defective A Box mutant of Aurora-A had identified AKIP-TR as the regulator for the Ub-independent mode of Aurora-A degradation. To further confirm the Ub-independent nature of AKIP-mediated Aurora-A degradation, we employed other approaches, where polyubiquitination was inhibited, such as (i) overexpression of the dominant negative K48R ubiquitin mutant; (ii) previously described temperature-sensitive inactivation in E1 ubiquitin-activating enzyme.

8.2.4.1. Inhibition of Cellular Polyubiquitination by Dominant Negative K48R Ubiquitin Mutant

Ub-dependent protein degradation involves the covalent attachment of multiple ubiquitins to the lysine residues of the target protein(s), facilitating the substrate recognition by the 26S proteasome. To verify whether the AKIP-mediated Aurora-A degradation is affected under the condition where the cellular polyubiquitylation was inhibited, we performed the AKIP-TR-mediated Aurora-A degradation assay in the presence of K48R dominant negative ubiquitin mutant. Incorporation of this dominant negative mutant ubiquitin led to the chain terminating effect, thereby blocking further ubiquitin chain extension, as shown in Figure 8-12. This impaired ubiquitination of Aurora-A was expected to result in less efficient recognition of Aurora-A by the proteasomal degradation machinery.

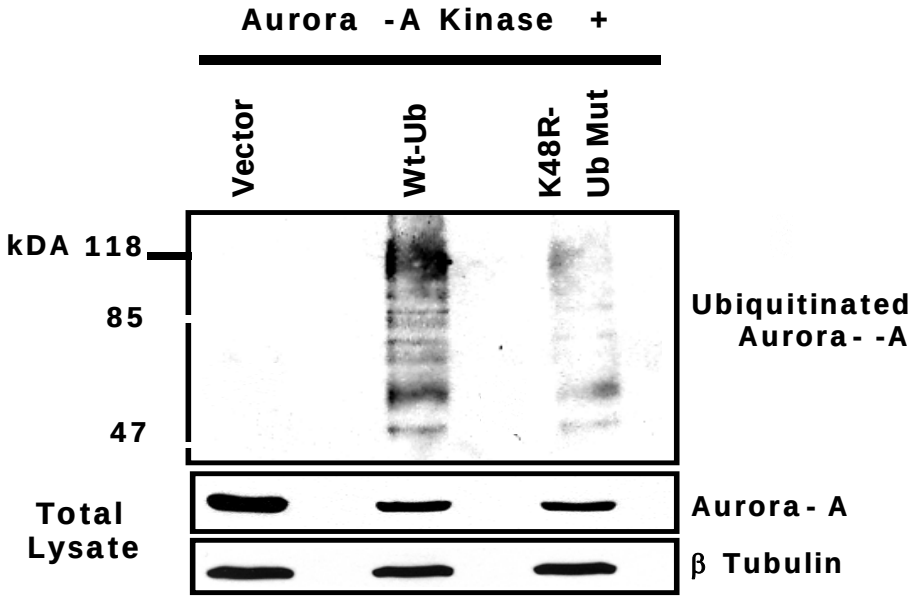


Figure 8-12:

Suppression of Cellular Polyubiquitination of Aurora-A via Overexpression of K48R Ubiquitin Mutant

HeLa cells were co-transfected with FLAG-tagged Aurora-A and either empty vector or His-tagged wild-type or K48R mutant ubiquitin expression construct. Total ubiquitinated proteins were pulled down with NTA-agarose and the ubiquitinated FLAG-tagged Aurora-A was detected with anti-FLAG mouse monoclonal antibody. The protein levels of Aurora-A in the total lysates under different conditions were analyzed using FLAG mouse monoclonal antibody. β tubulin was used as the loading

However, as shown clearly in Figure 8-13, the AKIP-TR-mediated Aurora-A degradation was unaffected in the presence of K48R mutant ubiquitin overexpression and was still as efficient as under the condition with the presence of wild-type ubiquitin. Again, this implied that the targeting of Aurora-A for degradation, mediated by AKIP–TR, did not require the prior ubiquitination of Aurora-A.

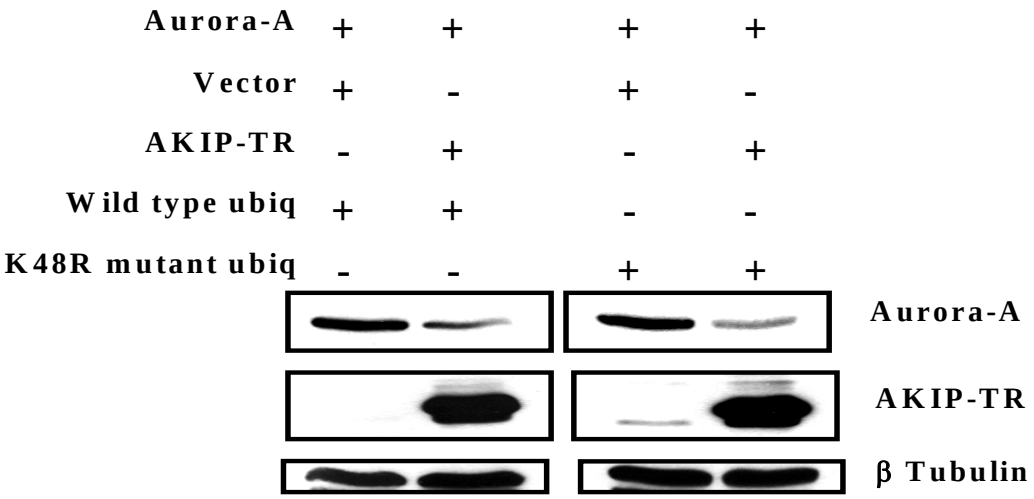


Figure 8-13:

Effect of Polyubiquitination Suppression on AKIP-TR-mediated Aurora-A Degradation: Overexpression of K48R Ubiquitin Mutant

HeLa cells were co-transfected with FLAG-tagged Aurora-A and either empty vector pCDNA3 or FLAG-tagged AKIP-TR at 1:9 ratio, in the presence of either His-tagged wild-type or K48R mutant ubiquitin expression construct. 36 hrs post-transfection, the cells were harvested and analyzed for Aurora-A and AKIP-TR, both detected with anti-FLAG mouse monoclonal antibody. β tubulin was used as the loading control.

8.2.4.2. Inhibition of Cellular Polyubiquitination by Inactivation of E1 Ub-Activating Enzyme

To further confirm the Ub-independent nature of AKIP-TR-mediated Aurora-A degradation, *in vivo* degradation assays had been performed in ts20-CHO cells. In support of the results from the previous approach using the dominant negative K48R ubiquitin mutant, suppression of polyubiquitination by temperature-sensitive mutation of the E1 Ub-activating enzyme at the non-permissive temperature, increased the basal levels of Aurora-A but still did not abolish the AKIP-TR-mediated Aurora-A degradation, as shown in Figure 8-14. This data confirmed again that AKIP-TR mediates Aurora-A degradation without the help of ubiquitin as the targeting factor.

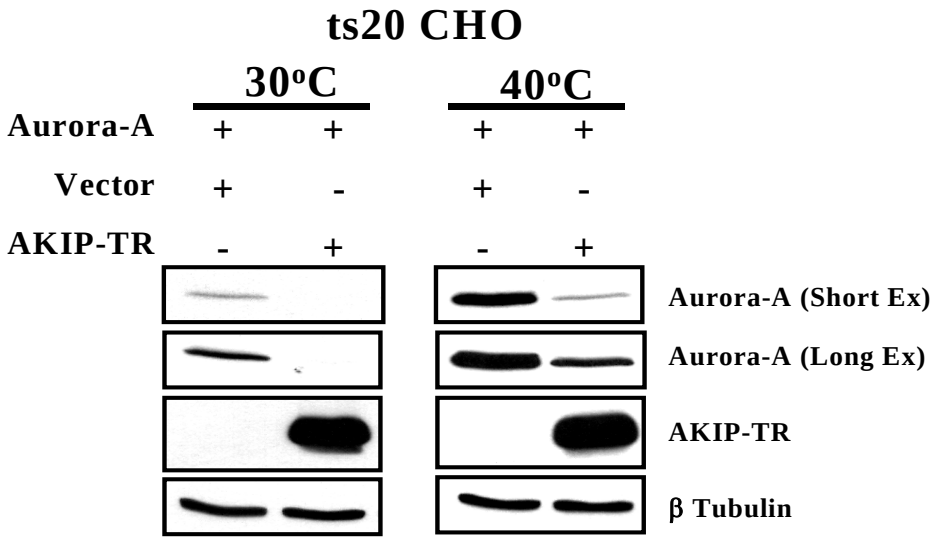


Figure 8-14:

Effect of Polyubiquitination Suppression on AKIP-TR-mediated Aurora-A Degradation: Inactivation of E1 Ub-Activating Enzyme

ts20-CHO were co-transfected with FLAG-tagged Aurora-A and either empty vector pCDNA3 or FLAG-tagged AKIP-TR at 1:9 ratio. The transfected cells were divided into two sets, both sets were initially incubated at 30°C permissive temperature for 24 hrs. After 24 hrs, one set was maintained at 30°C while the other set was incubated at 40°C non-permissive temperature for 16 hrs. The cells were harvested and analyzed for Aurora-A and AKIP-TR using anti-FLAG mouse monoclonal antibody. β tubulin was used as the loading control.

As observed in Figure 8-14, in the absence of AKIP-TR, inhibition of the polyubiquitination leads to a higher basal level of Aurora-A. This implies that under the normal condition, the Ub-dependent degradation is a major pathway operational for Aurora-A turnover and AKIP-TR potentiates Aurora-A degradation through an Ub-independent pathway.

8.2.4.3. Specificity

To verify the specificity of AKIP-mediated Ub-independent Aurora-A degradation, the effect of AKIP overexpression on human Aurora-B, p21 and cyclin B1 stability was investigated in ts20-CHO cells. Aurora-B belongs to one of the closely related members of the Aurora kinase family and shares high sequence homology in the kinase domain with Aurora-A. Therefore, it will be interesting to know if AKIP-TR targets Aurora-A member specifically even among other closely related members of the same family.

On the other hand, p21 has been well documented for its ability to serve as the substrate for Ub-independent degradation whereas cyclin B1 is a prototype substrate solely targeted for Ub-dependent degradation. As shown in Figure 8-15 to 8-17, in contrast to the AKIP-TR effect on Aurora-A, overexpression of AKIP did not influence the protein stability of Aurora-B (Figure 8-15) or p21 (Figure 8-16) or cyclin B1 (Figure 8-17), implying the high specificity of the AKIP-TR-mediated Aurora-A degradation.

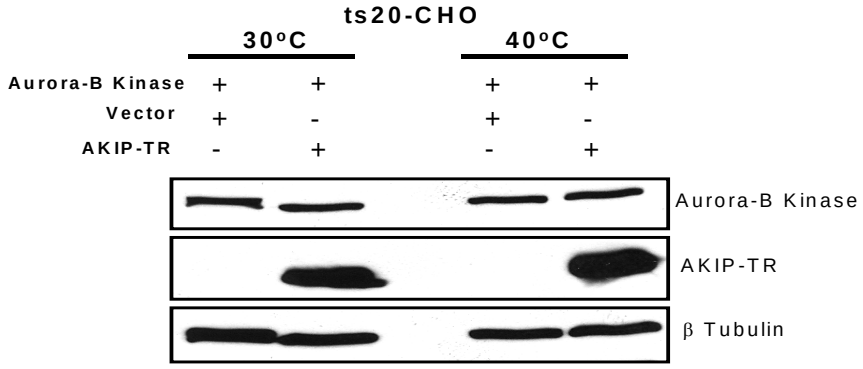


Figure 8-15: Effect of AKIP-TR Overexpression on Aurora-B Protein Stability

ts20-CHO were co-transfected with FLAG-tagged human Aurora-B and either empty vector pCDNA3 or HA-tagged AKIP-TR at 1:5 ratio. The effect of AKIP-TR overexpression on Aurora-B kinase stability was assessed at 36 hrs post-transfection at both permissive and non-permissive temperature. The cells were harvested and analyzed for Aurora-B and AKIP-TR using anti-FLAG and anti-HA mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

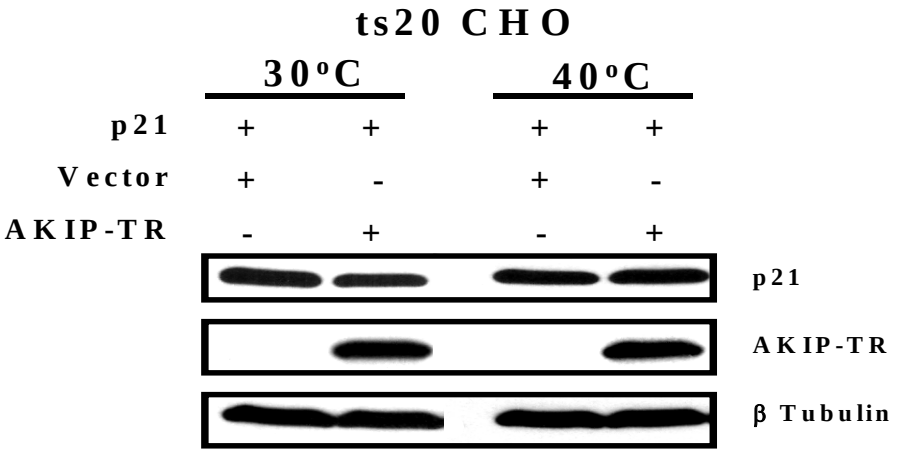


Figure 8-16: Effect of AKIP-TR Overexpression on p21 Protein Stability

ts20-CHO were co-transfected with HA-tagged p21 and either empty vector pCDNA3 or FLAG-tagged AKIP-TR at 1:9 ratio. The effect of AKIP-TR overexpression on p21 stability was assessed at 36 hrs post-transfection at both permissive and non-permissive temperature. The cells were harvested and analyzed for p21 and AKIP-TR using anti-HA and anti-FLAG mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

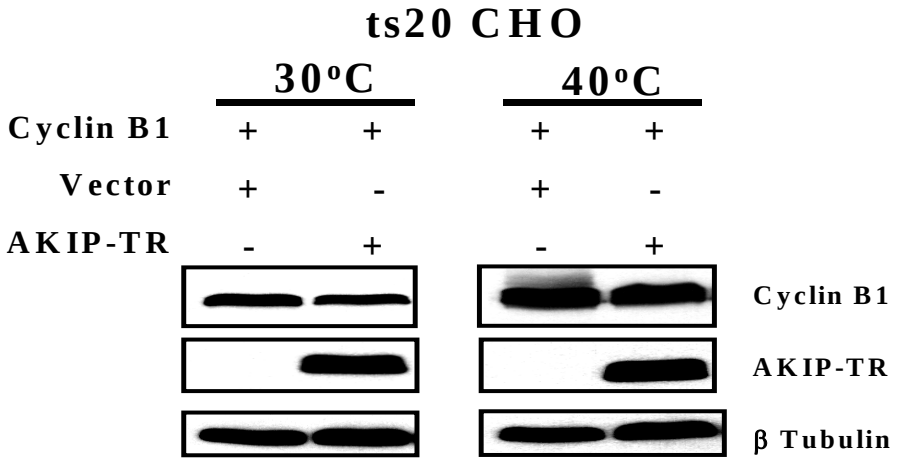


Figure 8-17: Effect of AKIP-TR Overexpression on Cyclin B1 Protein Stability

ts20-CHO were co-transfected with cyclin B1 and either empty vector pCDNA3 or FLAG-tagged AKIP-TR at 1:9 ratio. The effect of AKIP-TR overexpression on cyclin B1 stability was assessed at 36 hrs post-transfection at both permissive and non-permissive temperature. The cells were harvested and analyzed for cyclin B1 and AKIP-TR using anti-cyclin B1 rabbit polyclonal antibody and anti-FLAG mouse monoclonal antibody, respectively. β tubulin was used as the loading

8.2.5. Proteasome-Dependence of the AKIP-mediated Ubiquitin-Independent Degradation of Aurora-A

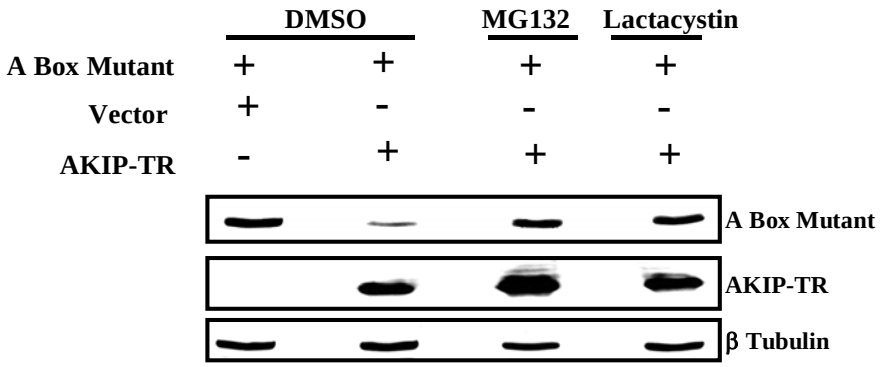
Previous studies had shown that AKIP-mediated Aurora-A degradation was susceptible to inhibition by the specific proteasome inhibitors, implying the proteasome-dependence of AKIP-TR-mediated Aurora-A degradation. To address even more specifically the proteasome-dependency of the AKIP-mediated Ub-independent Aurora-A degradation, we had performed the *in vivo* AKIP-TR-mediated Aurora-A degradation in the absence or presence of proteasome inhibitor, MG132 under the condition that ubiquitination was blocked either by (i) the ubiquitination-defective A Box mutant of Aurora-A; and/or (ii) the overexpression of dominant negative K48R ubiquitin mutant.

8.2.5.1. A Box Mutant

As demonstrated earlier, the A Box mutant, which lacked the poyubiquitination, was specifically targeted by AKIP-TR for protein degradation. Addition of proteasome inhibitor MG132 and Lactacystin reversed the AKIP-TR-mediated degradation of A Box mutant, suggesting that the Ub-independent degradation of Aurora-A is proteasome-dependent, as shown in Figure 8-18.

Figure 8-18:
Proteasome-Dependence of Ub-Independent Degradation of Aurora-A via AKIP-TR: A-Box Mutant

HeLa cells were co-transfected with HA-tagged A-Box mutant of Aurora-A and either empty pCDNA3 vector or FLAG-tagged AKIP-TR at 1:9 ratio. 24 hours post-transfection, the cells were treated with either DMSO or 20 μM MG132 or Lactacystin for 16 hours before harvested for Western Blot analysis. A Box mutant and AKIP-TR were detected using the anti-HA and anti-FLAG mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.



8.2.5.2. Dominant Negative K48R Ubiquitin Mutant

To further verify the proteasome-dependent nature of the Ub-independent pathway of AKIP-mediated Aurora-A degradation, we also looked at the effect of proteasome inhibitor MG132 on the AKIP-TR-mediated degradation of Aurora-A in the presence of dominant negative K48R ubiquitin mutant. As displayed in Figure 8-19, the proteasome inhibitor MG132 could reverse the AKIP-TR-mediated degradation of wild type Aurora-A even in the presence of dominant negative K48R ubiquitin mutant.

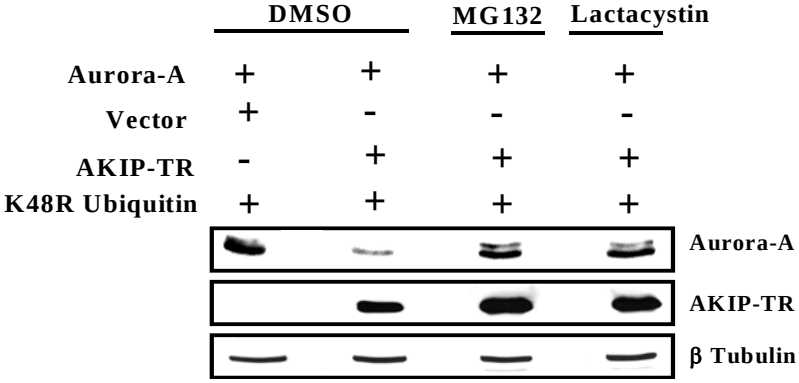


Figure 8-19:

Proteasome-Dependence of Ub-Independent Degradation of Aurora-A via AKIP-TR: K48R Ubiquitin Mutant

HeLa cells were co-transfected with HA-tagged Aurora-A and either empty pCDNA3 vector or FLAG-tagged AKIP-TR at 1:9 ratio in the presence of K48R ubiquitin mutant overexpression. 24 hours post-transfection, the cells were treated either with DMSO or 20 μM MG132 or Lactacystin for 16 hours before harvest for Western Blot analysis. Aurora-A and AKIP-TR were detected using the anti-HA and anti-FLAG mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

8.3. Discussion

The abundance of Aurora-A protein is tightly controlled by the balance of its synthesis and degradation, as its overexpression could lead to checkpoint disruption [1-2], induction of aneuploidy and oncogenic transformation [3-4]. The cell cycle-dependent degradation of Aurora-A is mediated by Cdh1 through the Ub-dependent proteasomal degradation pathway [5-7]. Chfr [8] and hCDC4 [9] represent two other new candidate regulators, which are involved in the destabilization of Aurora-A. However, there is a shared feature among these three regulators, in which they all target Aurora-A for proteasomal degradation with prior ubiquitination of their substrate, Aurora-A. Herein, we provide the first demonstration that there exists an alternative Ub-independent pathway for Aurora-A protein degradation and AKIP facilitates the Aurora-A degradation through this alternative pathway.

Protein degradation via the Ub-independent pathway is no longer a new concept. Though ubiquitination is a pre-requisite for the majority of extralysosomal proteolysis by the 26S proteasome, some proteins can be degraded in the Ub-independent manner, either by the 20S proteasome directly or the 26S proteasome in the presence of ATP [10]. Ornithine decarboxylase (ODC) was the first protein demonstrated to be degraded by the 26S proteasome through this alternative pathway [11]. Other proteins, which can be degraded by 26S proteasome in the Ub-independent manner, include c-Jun [12], Cdk inhibitor p21 [13-14], tumor suppressor p53 [15-16] and calmodulin [17]. In the absence of help from ubiquitin tag, targeting of protein substrates to 26S proteasome relies on other accessory proteins or a

degradation signal within the substrate itself to replace the targeting role of ubiquitin.

For example, ODC is directed to the proteasome by its specific binding partner—antizyme [18-19]. Tax, a protein encoded by human T cell leukemia virus, promotes the binding of the IκBα substrate to the HsN3 subunit of 20S proteasome, thereby facilitating the constitutive degradation of IκBα in the Ub- and phosphorylation-independent manner [20]. Similarly, hyperphosphorylated form of retinoblastoma (Rb) protein is targeted by the viral protein pp71 for the Ub-independent proteasomal degradation [21]. Besides, the proteasome-dependent Ub-independent cellular degradation of Rb protein is shown to be mediated by MDM2, which itself can interact with the C8 subunit of 20S proteasome and thereby facilitating or stabilizing the Rb-C8 interaction [22]. It is interesting to note that MDM2, being an ubiquitin ligase, can play alternative role in the Ub-independent protein degradation. Moreover, NQO1, which can bind both p53 and p73 as well as the 20S proteasome, functions as a gatekeeper of the 20S proteasome and negatively regulates the degradation of p53 and p73 [15]. Inhibition of NQO1 has been shown to induce the p53 and p73 degradation through the 20S proteasome-dependent, Ub-independent pathway [15]. On the other hand, p21, a transcriptional target of p53, represents an example of a protein, which targets itself for Ub-independent degradation by direct binding to the 20S proteasome. It has been shown that p21 directly interacts with the C8 subunit of 20S proteasome *in vitro* and the turnover of mutant p21 *in vivo* directly correlates with its affinity for the C8 subunit *in vitro* [23]. Taken

together, it is apparent that interaction of the target substrate with the proteasomal machinery is a pre-requisite for the Ub-independent degradation, which is similar to the Ub-dependent degradation.

On the other hand, our study also uncovered another role of AKIP in the Ub-independent degradation of Aurora-A, in which binding of AKIP interfered with the normal ubiquitination of Aurora-A. Using the Aurora-A deletion mutants, we had identified a region of Aurora-A, which might contain signal(s) for ubiquitination and we demonstrated that AKIP targeted this region. The region deleted in that ubiquitination-defective Aurora-A mutant (ΔC600-AIK1) contained the D1 and D2 destruction boxes. This result was consistent with the previous findings from another group. Through their mutagenesis analysis of the D boxes of Aurora-A, they showed that D2 box might serve as a signal for polyubiquitination, but not a signal for proteolysis [26]. Therefore, this implied that AKIP might also target the D2 box of the Aurora-A and mask the ubiquitination signal for recognition by the ubiquitination machinery. The speculation of AKIP targeting of the ubiquitination domain of Aurora-A was further supported by the observation that the non-interactive AKIP mutant, which was defective in binding Aurora-A, restored the ubiquitination of Aurora-A.

The AKIP-mediated inhibition of Aurora-A ubiquitination is unlikely due to the potentiation of Aurora-A deubiquitination. At present, no evidence has indicated any role of deubiquitination in the Ub-independent protein degradation pathway, instead, recent study [27]

had demonstrated that deubiquitination event plays a crucial role in the proteolysis of ubiquitinated substrates and it is tightly coordinated with the substrate unfolding and translocation. Proteasomes represent the important player in the deubiquitination process as they exhibit a broad spectrum of intrinsic and associated deubiquitinating activities [28]. The same study also showed that the constitutively ubiquitinated protein substrates were trapped in the proteasome and underwent incomplete proteolysis.

As our study had clearly demonstrated that AKIP promoted the Ub-independent degradation of Aurora-A. An interesting question posed would be how AKIP targets Aurora-A to the proteasome in the absence of ubiquitination. Generally, marking of the substrates serves two functions, unfolding and targeting [20-21, 24]. Since AKIP can bind Aurora-A, it is unclear yet whether the binding of AKIP assists in the unfolding of Aurora-A, hence making Aurora-A a better substrate to be recognized by the 20S proteasome. Alternatively, AKIP may replace the protein targeting role of ubiquitin and AKIP binding to Aurora-A targets Aurora-A directly to the proteasome. In this context, it will be interesting to investigate whether AKIP interacts directly with the 20S proteasome and whether Aurora-A can be degraded by 20S proteasome *in vitro* in the presence of AKIP. However, it is also possible that AKIP may not play a direct role in targeting of Aurora-A to the proteasome, instead AKIP may induce modification of Aurora-A by other ubiquitin-like small molecule [25] or there could be involvement of other secondary proteins in the targeting of Aurora-A to the proteasome. We

cannot rule out another possibility that Aurora-A itself can also bind to C8 subunit of 20S proteasome and AKIP promotes or stabilizes the Aurora-A: C8 interaction, similar to the MDM2-mediated Rb degradation.

Using the ubiquitination-defective Aurora-A mutant, dominant negative ubiquitin mutant and the temperature-sensitive mutant cell lines defective in E1 ubiquitin activating enzyme at the restrictive temperature, we had clearly demonstrated that Aurora-A could be degraded even in the absence of polyubiquitination. Furthermore, AKIP, which constitutively targeted Aurora-A for degradation in proteasome-dependent manner, did not require polyubiquitination. Our results presented herein conform to the trend of a protein being degraded by both the Ub-dependent and Ub-independent pathways, such as p53 [15], Rb [21-22] and p21 [14]. As Ub-independent pathway can co-exist with the Ub-dependent pathway for Aurora-A degradation, could AKIP be one of the determinants for the switch between these alternative pathways? It is possible as AKIP can inhibit the polyubiquitination of Aurora-A and we had shown that binding of AKIP to Aurora-A might interfere with the interaction of the ubiquitination machinery with Aurora-A. Future investigation on the cross-talk between AKIP and Aurora-A ubiquitination is absolutely required to understand the role of AKIP as the molecular switch between Ub-dependent and Ub-independent pathways.

8.4. Conclusion

In summary, we have added Aurora-A to the increasing list of substrates that can be targeted for Ub-independent proteasomal degradation and AKIP facilitates the degradation of Aurora-A through this Ub-independent pathway. At this juncture, it is not clear why there should be two pathways for the degradation of the same protein and what is the physiological significance of AKIP-mediated Ub-independent Aurora-A degradation in the cellular context. These questions remain unanswered for most of the substrates targeted for the Ub-independent proteasomal degradation. However, irrespective of the mechanism and cellular context, AKIP-mediated degradation of Aurora-A still provides an alternative strategy to down-regulate this oncogene and makes AKIP a prospective anti-cancer target.

8.5. References

1. Marumoto T, Hirota T, Morisaki T, Kunitoku N, Zhang D, Ichikawa Y, Sasayama T, Kuninaka S, Mimori T, Tamaki N, Kimura M, Okano Y, Saya H. **Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells.** Genes Cells. (2002) 7(11):1173-82.

2. Anand S, Penrhyn-Lowe S, Venkitaraman AR. **AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol.** Cancer Cell. (2003) 3(1):51-62.

3. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR, Sen S. **Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation.** Nat Genet. (1998) 20(2):189-93.

4. Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B, Schryver B, Flanagan P, Clairvoyant F, Ginther C, Chan CS, Novotny M, Slamon DJ, Plowman GD. **A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers.** EMBO J. (1998) 17(11):3052-65.

5. Littlepage LE, Ruderman JV. **Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit.** Genes Dev. (2002) 16(17):2274-85.

6. Taguchi S, Honda K, Sugiura K, Yamaguchi A, Furukawa K, Urano T. **Degradation of human Aurora-A protein kinase is mediated by hCdh1**. FEBS Lett. (2002) 519(1-3):59-65.

7. Castro A, Arlot-Bonnemains Y, Vigneron S, Labbe JC, Prigent C, Lorca T. **APC/Fizzy-Related targets Aurora-A kinase for proteolysis.** EMBO Rep. (2002) 3(5):457-62. Epub 2002 Apr 18.

8. Yu X, Minter-Dykhouse K, Malureanu L, Zhao WM, Zhang D, Merkle CJ, Ward IM, Saya H, Fang G, van Deursen J, Chen J. **Chfr is required for tumor suppression and Aurora A regulation.** Nat Genet. (2005) 37(4):401-6.

9. Mao JH, Perez-Losada J, Wu D, Delrosario R, Tsunematsu R, Nakayama KI, Brown K, Bryson S, Balmain A. **Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene.** Nature. (2004) 432(7018):775-9.

10. Orlowski M, Wilk S. **Ubiquitin-independent proteolytic functions of the proteasome.** Arch Biochem Biophys. (2003) 415(1):1-5.

11. Murakami Y, Matsufuji S, Kameji T, Hayashi S, Igarashi K, Tamura T, Tanaka K, Ichihara A. **Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination.** Nature. (1992) 360(6404):597-9.

12. Jariel-Encontre I, Pariat M, Martin F, Carillo S, Salvat C, Piechaczyk M. **Ubiquitinylation is not an absolute requirement for degradation of c-Jun protein by the 26 S proteasome**. J Biol Chem. (1995) 270(19):11623-7.

13. Sheaff RJ, Singer JD, Swanger J, Smitherman M, Roberts JM, Clurman BE. **Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination**. Mol Cell. (2000) 5(2):403-10.

14. Jin Y, Lee H, Zeng SX, Dai MS, Lu H. **MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation.** EMBO J. (2003) 22(23):6365-77.

15. Asher G, Tsvetkov P, Kahana C, Shaul Y. **A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73.** Genes Dev. (2005) 19(3):316-21.

16. Asher G, Lotem J, Sachs L, Kahana C, Shaul Y. **Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1.** Proc Natl Acad Sci U S A. (2002) 99(20):13125-30.

17. Tarcsa E, Szymanska G, Lecker S, O'Connor CM, Goldberg AL. **Ca2+-free calmodulin and calmodulin damaged by in vitro aging are selectively degraded by 26 S proteasomes without ubiquitination.** J Biol Chem. (2000) 275(27):20295-301.

18. Coffino P. **Antizyme, a mediator of ubiquitin-independent proteasomal degradation.** Biochimie. (2001) 83(3-4):319-23.

19. Mangold U. **The antizyme family: polyamines and beyond.** IUBMB Life. (2005) 57(10):671-6.

20. Krappmann D, Wulczyn FG, Scheidereit C. **Different mechanisms control**

signal-induced degradation and basal turnover of the NF-kappaB inhibitor IkappaB alpha in vivo. EMBO J. (1996) 15(23):6716-26.

21. Kalejta RF, Shenk T. **Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein.** Proc Natl Acad Sci U S A. (2003) 100(6):3263-8.

22. Sdek P, Ying H, Chang DL, Qiu W, Zheng H, Touitou R, Allday MJ, Xiao ZX. **MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein.** Mol Cell. (2005) 20(5):699-708.

23. Touitou R, Richardson J, Bose S, Nakanishi M, Rivett J, Allday MJ. **A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 alpha-subunit of the 20S proteasome.** EMBO J. (2001) 20(10):2367-75.

24. Ghislain M, Dohmen RJ, Levy F, Varshavsky A. **Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in Saccharomyces cerevisiae.** EMBO J. (1996) 15(18):4884-99.

25. Jentsch S, Pyrowolakis G. **Ubiquitin and its kin: how close are the family ties?** Trends Cell Biol. (2000) 10(8):335-42.

26. Honda K, Mihara H, Kato Y, Yamaguchi A, Tanaka H, Yasuda H, Furukawa K, Urano T. **Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway**. Oncogene. (2000) 19(24):2812-9.

27. Zhu Q, Wani G, Wang Q, M El-mahdy, Snapka R.M., Wani A.A. **Deubiquitination by proteasome is coordinated with substrate translocation for proteolysis *in vivo.*** Exp Cell Res. (2005) 307(2):436-451.

28. Glickman M.H., Ciechanover A. **The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction.** Physiol. Rev. (2002) 82(2):373-428.

(Result and Discussion)

CHAPTER 9

Mechanism of Ubiquitin-Independent AKIP-TR -mediated Aurora-A Degradation: Role of Antizyme (AZ)

9.1. Summary

We had previously demonstrated the existence of Ub-independent pathway for Aurora-A protein degradation, which was specifically potentiated by its negative regulator, AKIP. The present work aims to further characterize the detailed mechanism for the Ub-independent AKIP-mediated Aurora-A degradation. In this chapter, we have investigated antizyme, another modulator of Aurora-A protein stability. Antizyme is a well-studied mediator for the Ub-independent protein degradation pathway. Our study had shown that antizyme could also directly regulate the protein stability of Aurora-A in the Ub-Independent manner, either by ectopic or polyamine-induced expression of antizyme. These interesting findings had prompted us to search for any functional link between the antizyme and AKIP in regulating the Ub-independent degradation of Aurora-A. Inhibition of antizyme function blocked the AKIP-mediated degradation of Aurora-A. Moreover, our studies had identified *in vivo* interaction between Aurora-A and antizyme, which might be important for the *in vivo* [Aurora-A]-[AKIP]-[antizyme] ternary complex formation. The interaction between Aurora-A and antizyme was not only essential for the antizyme-mediated Aurora-A degradation, but also important for the AKIP-mediated Aurora-A degradation. Hence, antizyme played an important regulatory role in the AKIP-mediated Aurora-A degradation. Further study had demonstrated that AKIP acted upstream of the antizyme by enhancing the binding affinity of antizyme to Aurora-A, thereby enhancing the recognition and targeting to proteasome and the subsequent degradation.

9.2. Results

9.2.1 Antizyme can Directly Target Aurora-A for Degradation

We had previously demonstrated that Aurora-A could be a substrate for the Ub-independent protein degradation pathway. On the other hand, antizyme had been well studied for its role as a mediator of Ub-independent protein degradation. Hence, we were interested to investigate whether antizyme might somehow be involved in the Ub-independent degradation of Aurora-A.

9.2.1.1. Effect of Exogenous Antizyme Overexpression on Protein Stability of Exogenous or Endogenous Aurora-A

To study the possible role of antizyme in regulating the protein stability of Aurora-A, we followed the protein stability of Aurora-A, in the presence of ectopically expressed antizyme in HeLa and/or CHO cells. Cyclin D1 was used as the positive control for the antizyme effect as it was reported to be degraded by antizyme, whereas the cyclin B1 was used as the negative control. As shown in Figure 9-1 and 9-2, the exogenously overexpressed antizyme decreased the protein stability of both the endogenous and transfected Aurora-A while cyclin B1 levels were unaltered. Thus, antizyme could play a role in the negative regulation of the Aurora-A protein stability.

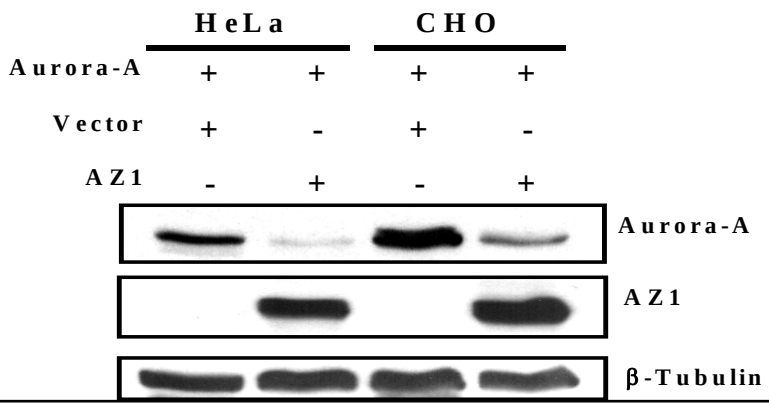


Figure 9-1: Effect of Antizyme Overexpression on Exogenous Aurora-A Stability

HeLa or CHO cells were co-transfected with HA-tagged Aurora-A and either empty vector or His-tagged AZ1 at 1:9 ratio. 36 hours post-transfection, the transfected cells were harvested for Western Blot analysis of exogenous Aurora-A protein stability. Aurora-A and AZ1 were detected using anti-HA mouse monoclonal antibody and anti-AZ rabbit polyclonal antibody, respectively. β tubulin was used as the loading control.

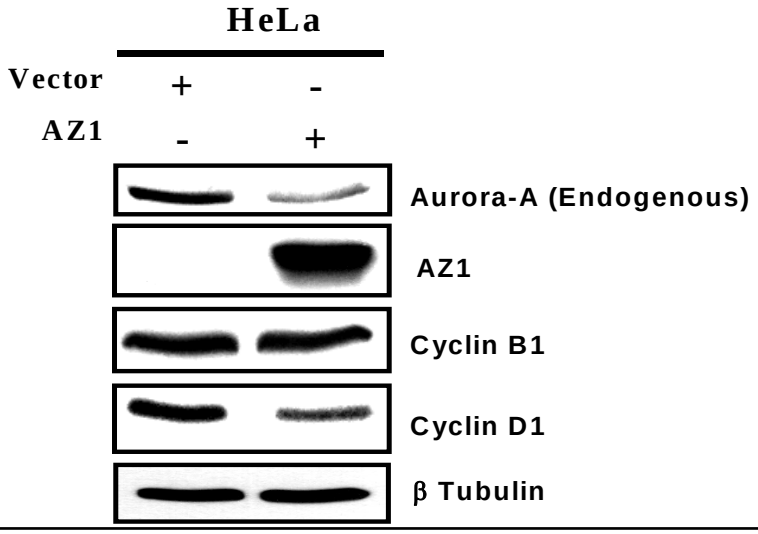


Figure 9-2: Effect of Antizyme Overexpression on Endogenous Aurora-A Stability

HeLa cells were transfected with either empty vector or His-tagged AZ1. 36 hours post-transfection, the transfected cells were harvested for Western Blot analysis of the endogenous Aurora-A protein stability. Aurora-A and AZ1 were detected using anti-IAK1 mouse monoclonal antibody and anti-AZ rabbit polyclonal antibody, respectively. Cyclin D1 was used as the positive control and detected using the anti-cyclin D1 mouse monoclonal antibody, whereas cyclin B1 was used as the negative control and detected with anti-cyclin B1 rabbit polyclonal antibody. β tubulin was used as the loading control.

9.2.1.2. Effect of Polyamine-Induced Antizyme Expression on Protein Stability of Endogenous Aurora-A

To verify whether upregulation of the endogenous level of antizyme promotes Aurora-A protein degradation, we exploited the polyamine putrescine, which is the physiological inducer of antizyme frameshifting and expression. As expected, treatment of the rat prostate carcinoma AT2.1 cell line with putrescine led to the induction of the endogenous antizyme expression with a concomitant decrease in the protein stability of the endogenous Aurora-A and the positive control cyclin D1, while the protein stability of the negative control cyclin A was unaffected (Figure 9-3). These data confirmed a role for antizyme in the negative regulation of Aurora-A protein stability.

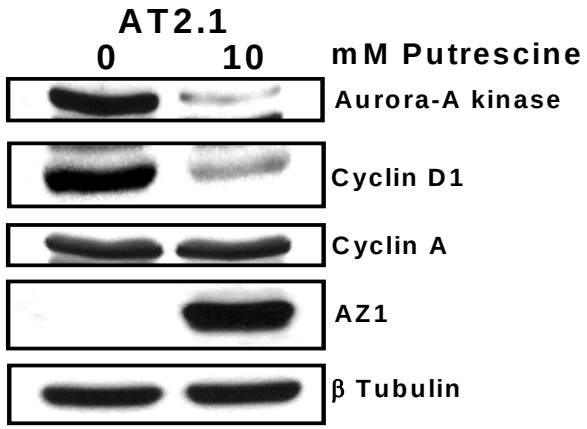


Figure 9-3: Effect of Endogenous Antizyme Induction on Endogenous Aurora-A Stability
AT 2.1 cells were treated with 10 mM of Putrescine for 24 hours post-transfection prior to harvest for Western Blot analysis of endogenous protein stability of Aurora-A, positive control cyclin D1, negative control cyclin A and endogenous induction of AZ1. Aurora-A and AZ1 were detected using anti-IAK1 and anti-AZ rabbit polyclonal antibody, respectively. Cyclin D1 was used as the positive control and detected using the anti-cyclin D1 mouse monoclonal antibody, whereas cyclin A was used as the negative control and detected with anti-cyclin A rabbit polyclonal antibody. β -tubulin was used as the loading control.

9.2.1.3. AZ-mediated Aurora-A Degradation Occurs via The Proteasome-Dependent but Ubiquitin-Independent Pathway

Since antizyme plays a crucial role in the Ub-independent protein degradation pathway, we would like to verify if antizyme also targets Aurora-A for degradation through the Ub-independent pathway. An *in vivo* antizyme-mediated Aurora-A degradation assay in the presence of Aurora-A polyubiquitination suppression was carried out. As shown in Figure 9-4, antizyme could still target the ubiquitination-defective A box mutant of Aurora-A for protein degradation. Similarly, inactivation of E1 Ub-activating enzyme at the non-permissive temperature did not affect the antizyme-mediated Aurora-A degradation, as shown in Figure 9-5. Therefore, similar to other targets of antizyme, antizyme-mediated Aurora-A degradation is also Ub-independent. Furthermore, the use of specific proteasome inhibitors like MG132 and Lactacystin in the *in vivo* antizyme-mediated Aurora-A degradation assay (Figure 9-6) had demonstrated that antizyme targets Aurora-A to proteasome for degradation.

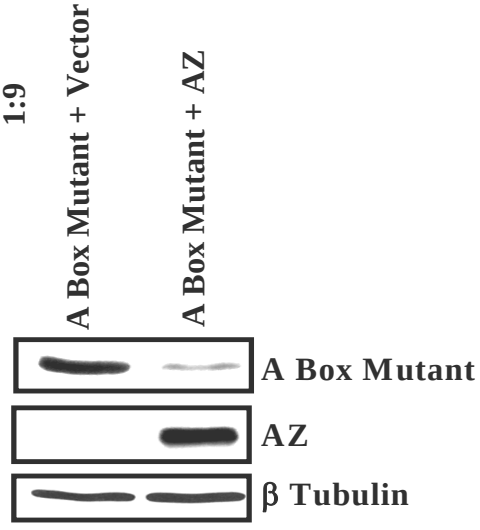


Figure 9-4:
Effect of AZ Overexpression on Aurora-A A Box mutant Protein Stability an Ubiquitination-Defective Mutant.
HeLa cells were co-transfected with HA-tagged A Box Mutant of Aurora-A and either empty vector pCDNA3 or His-tagged AZ at 1:9 ratio. 36 hours post-transfection, the transfected cells harvested and analyzed for A Box mutant and AZ using the anti-HA and anti-AZ rabbit polyclonal antibody. β tubulin was used as the loading control.

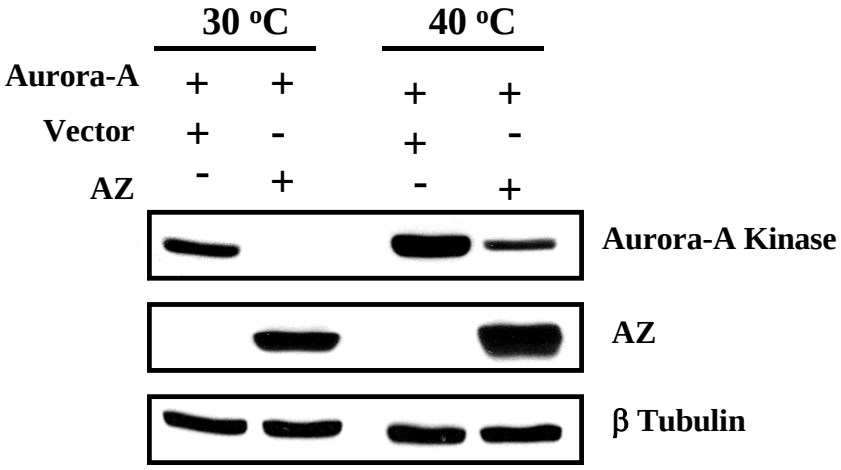


Figure 9-5:

Effect of Polyubiquitination Suppression on AZ-mediated Aurora-A Degradation: Inactivation of E1 Ubiquitin-Activating Enzyme

ts20-CHO cells were co-transfected with HA-tagged Aurora-A and either empty vector pCDNA3 or His-tagged AZ at 1:9 ratio. 24 hours post-transfection, the transfected cells were divided into two sets, one set was maintained at 30ºC permissive temperature, while the other set was incubated at 40ºC non-permissive temperature for 16 hours. The cells were harvested and analyzed for Aurora-A and AZ using the anti-HA and anti-AZ rabbit polyclonal antibody. β tubulin was used as the loading control.

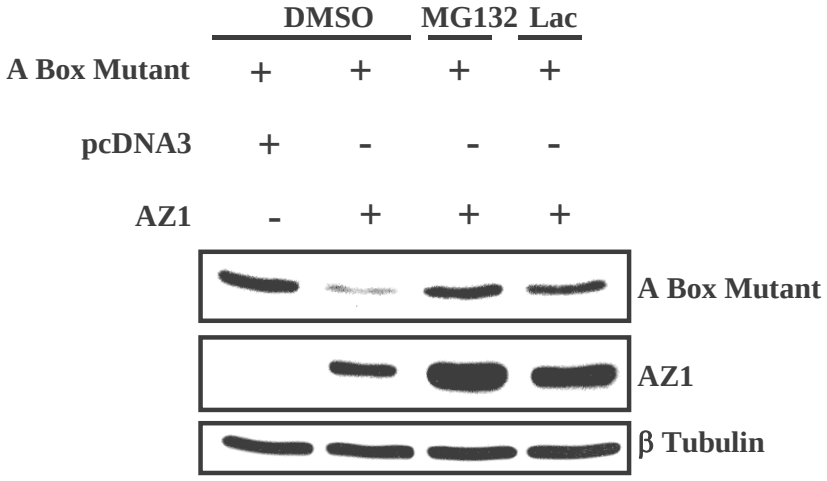


Figure 9-6: Proteasome-Dependence of AZ-mediated Aurora-A Degradation

HeLa cells were co-transfected with HA-tagged A Box mutant of Aurora-A and either empty vector pCDNA3 or His-tagged AZ at 1:9 ratio. 24 hours post-transfection, the transfected cells were treated with either DMSO or 20 mM MG132 or Lactacystin for 16 hours. The cells were harvested and analyzed for Aurora-A and AZ using the anti-HA and anti-AZ rabbit polyclonal antibody. β tubulin was used as the loading control.

9.2.1.4. *In vivo* Interaction between Antizyme and Aurora-A

It had been shown that the antizyme bound to ODC and enhanced the targeting of ODC to proteasome and subsequent Ub-independent degradation. It will be interesting to know if there exists a similar interaction between the antizyme and Aurora-A, which targets Aurora-A to the proteasome. To demonstrate an interaction between Aurora-A and antizyme, an *in vivo* interaction assay as described previously was carried out. Results from Figure 9-7 showed that antizyme could be coimmunoprecipitated with Aurora-A, suggesting that antizyme was capable of interacting with Aurora-A.

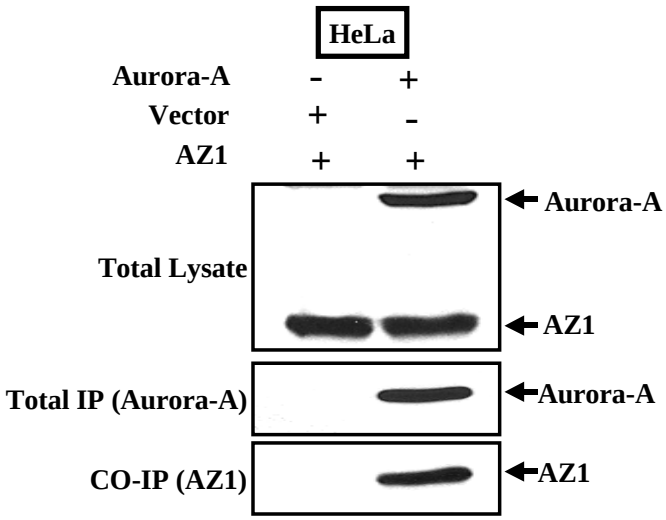


Figure 9-7: *In vivo* Interaction between Aurora-A and Antizyme

HeLa cells were co-transfected with His-tagged AZ1 and either empty vector or FLAG-tagged Aurora-A at 1:1 ratio. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The interacting AZ1 was detected using the anti-AZ rabbit polyclonal antibody. Aurora-A was detected using the anti-FLAG rabbit polyclonal antibody.

9.2.1.5. Isolation of Antizyme Non-Interacting Mutant of Aurora-A

As shown in the previous section, antizyme could directly interact with Aurora-A *in vivo*. The next pondering question would be whether the antizyme-Aurora-A interaction is essential for antizyme-mediated Aurora-A degradation. To address this question, we carried out *in vitro* interaction assays with antizyme and previously described Aurora-A deletion mutants (ΔN300-AIK1, ΔN600-AIK1, ΔC300-AIK1, ΔC600-AIK1) to identify a mutant Aurora-A that is defective in interaction with antizyme. We immunoprecipitated the wild-type Aurora-A and its various deletion mutants and checked if their interactions with the antizyme had been impaired by the deletion in Aurora-A.

Results from Figure 9-8 showed that all Aurora-A deletion mutants, except the ΔC600-AIK1 mutant, retained their ability to interact with antizyme, suggesting the region of Aurora-A from a.a. 610-909 was essential for its interaction with antizyme, which coincided with the interaction domain for AKIP-TR.

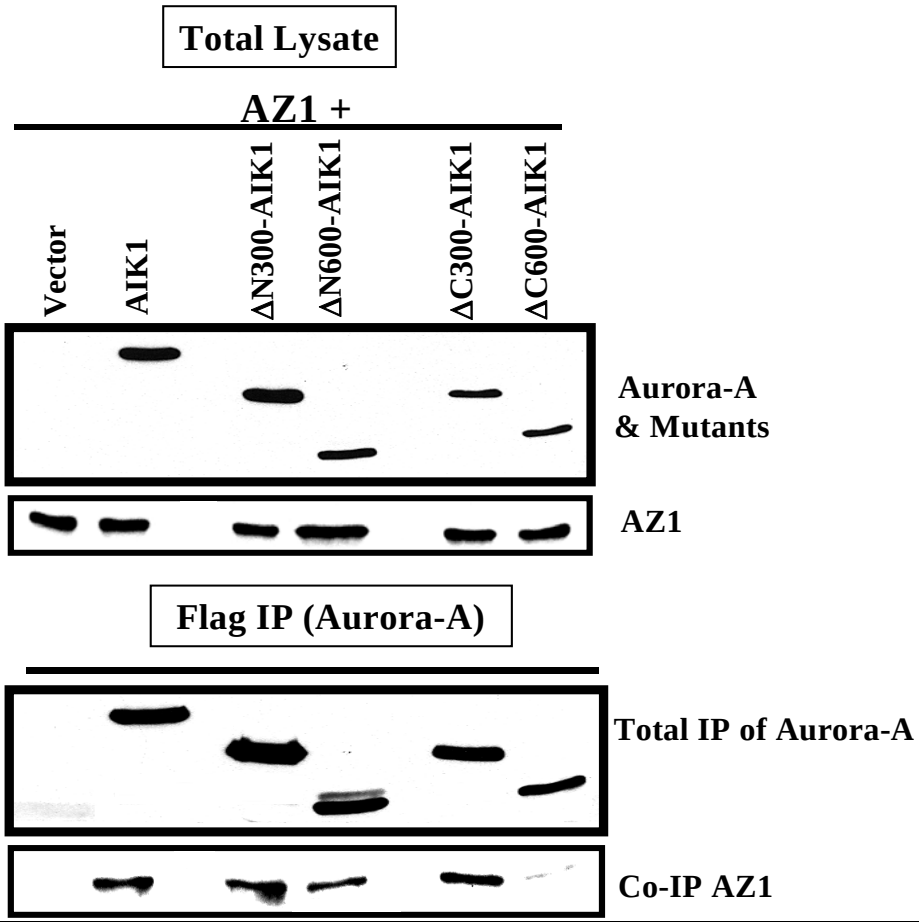


Figure 9-8: Domain Mapping of AZ1-Interacting Domain in Aurora-A

HeLa cells were co-transfected with His-tagged AZ1 and either empty vector or FLAG-tagged wild-type or various deletion mutants of Aurora-A at 1:1 ratio. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The interacting AZ1 was detected using the anti-AZ rabbit polyclonal antibody. Aurora-A was detected using the anti-FLAG rabbit polyclonal antibody.

9.2.1.6. Dependency of Aurora-A Degradation on antizyme-Aurora-A interaction

With the identification of the Aurora-A mutant (ΔC600-AIK1) which was defective in binding antizyme, we could now study the effect of impaired antizyme:Aurora-A interaction on the antizyme-mediated Aurora-A degradation. We used the ΔC600-AIK1 non-interacting mutant for the *in vivo* antizyme-mediated degradation assay and compared to the wild-type (AIK1) or other antizyme-interacting Aurora-A mutants (ΔN300-AIK1, ΔN600-AIK1,

ΔC300-AIK1) with respect to their susceptibilities to antizyme-mediated degradation. As shown in Figure 9-9, both the wild-type and the antizyme-interacting Aurora-A mutants could be targeted by antizyme for degradation, whereas non-interacting ΔC600-AIK1 mutant was not susceptible for antizyme-mediated degradation, implying that the antizyme:Aurora-A interaction played an essential role in the antizyme-mediated Aurora-A degradation.

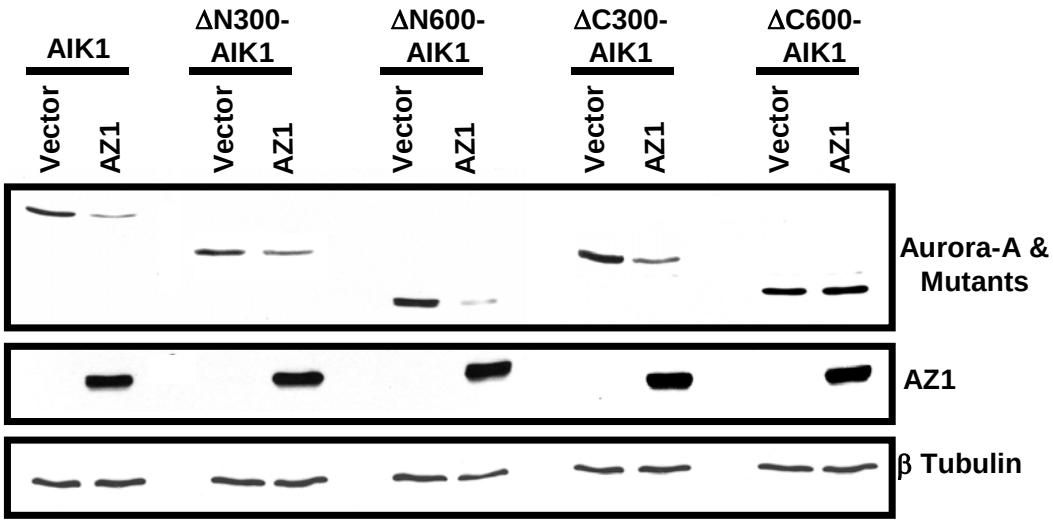


Figure 9-9: Effect of Impaired Aurora-A:AZ1 Interaction on AZ1-mediated Aurora-A Degradation

ts20-CHO cells were co-transfected with His-tagged AZ1 and either FLAG-tagged wild-type or various deletion mutants of Aurora-A at 5:1 ratio. 36 hours post-transfection, the transfected cells were harvested for Western Blot analysis of the protein stability of Aurora-A, both wild-type and mutants in the presence of AZ1 overexpression. The Aurora-A and AZ1were detected using the anti-FLAG mouse monoclonal antibody and anti-AZ rabbit polyclonal antibody, respectively. β tubulin was used as the loading control.

9.2.2. Functional Link between Antizyme and AKIP-TR-mediated Aurora-A Degradation

The results presented thus far pointed to the fact that both AKIP-TR and antizyme could interact with and target Aurora-A for degradation. Since AKIP-TR targeted Aurora-A for degradation through an Ub-independent pathway, and antizyme had also been implicated in

the regulation of Ub-independent degradation of other proteins like ODC and cyclin D1, it will be interesting to explore whether there exists a functional link between AKIP-TR and antizyme in the Ub-independent degradation of Aurora-A.

9.2.2.1. Antizyme Inhibition by Antizyme Inhibitor (AZI)

It had been established that the functional activity of antizyme could be inactivated by its negative regulator, antizyme inhibitor (AZI) [28]. To search for any interdependency between AKIP-TR and antizyme in Aurora-A degradation, we carried out the AKIP-TR-mediated Aurora-A degradation assay in the presence of antizyme inhibitor (AZI). We presumed that if the AKIP-TR-mediated Aurora-A degradation is antizyme-dependent, it should be sensitive to antizyme inhibition by the AZI overexpression.

For this experiment, we generated recombinant expression plasmid co-expressing AZI and AKIP-TR from a single bicistronic expression vector. Transfection of cells with constructs expressing AKIP-TR alone or with AZ1 showed (Figure 9-10) that overexpression of AKIP-TR alone led to the efficient down-regulation of Aurora-A. However, to our excitement, the coexpression of AZI and thus inhibition of antizyme function did impair the AKIP-TR-mediated Aurora-A degradation. This suggested that antizyme did play a role in the AKIP-TR-mediated Aurora-A degradation. On the other hand, to rule out the possibility that AZI overexpression might affect the Aurora-A protein stability directly, we included the negative control where we overexpressed AZI alone, however, it did not have any effect on

the Aurora-A protein stability.

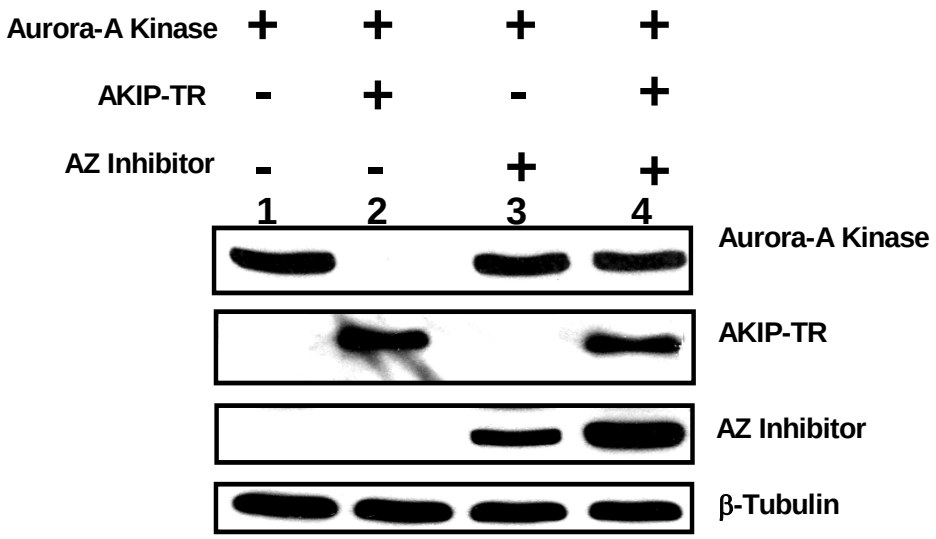


Figure 9-10: Effect of Antizyme Inhibition via Antizyme Inhibitor (AZI) on AKIP-TR-mediated Aurora-A Degradation

ts20-CHO cells were co-transfected with HA-tagged Aurora-A and (i) pIRES (Lane 1); (ii) pIRES-[Flag-AKIP-TR] (Lane2); (iii) [HA-AZI]-pIRES (Lane3); (iv) [HA-AZI]-pIRES-[Flag AKIP-TR] (Lane 4) at 1:9 ratio. 36 hours post-transfection, transfected cells were harvested for Western Blot analysis of the protein stability of Aurora-A kinase in the presence of AKIP-TR or AZI or both AKIP-TR and AZI overexpression. Both Aurora-A and AZI were detected using the anti-HA mouse monoclonal antibody and AKIP-TR was detected using the anti-FLAG mouse monoclonal

9.2.2.2. Defective Degradation of Antizyme Non-Interacting Mutant of Aurora-A by AKIP-TR

As shown in the previous section, inhibition of antizyme function by the antizyme inhibitor blocked the AKIP-TR-mediated Aurora-A degradation. Moreover, both AKIP-TR and antizyme bound Aurora-A and targeted Aurora-A for degradation. To further demonstrate the existence of a functional link between AKIP-TR and antizyme in mediating Aurora-A degradation, we went on to investigate the consequence of impaired antizyme:Aurora-A interaction on the AKIP-TR-mediated Aurora-A degradation.

To this end, we carried out *in vivo* degradation assay in the presence of AKIP-TR using the antizyme non-interacting ΔC600-AIK1 mutant. Its susceptibility to AKIP-TR-mediated degradation was compared to that of wild type and other antizyme-interacting Aurora-A mutants (ΔN300-AIK1, ΔN600-AIK1, ΔC300-AIK1). As shown in Figure 9-11, only the ΔC600-AIK1 mutant was defective in being targeted by AKIP-TR for degradation, suggesting that antizyme: Aurora-A interaction was essential for the AKIP-TR-mediated Aurora-A degradation.

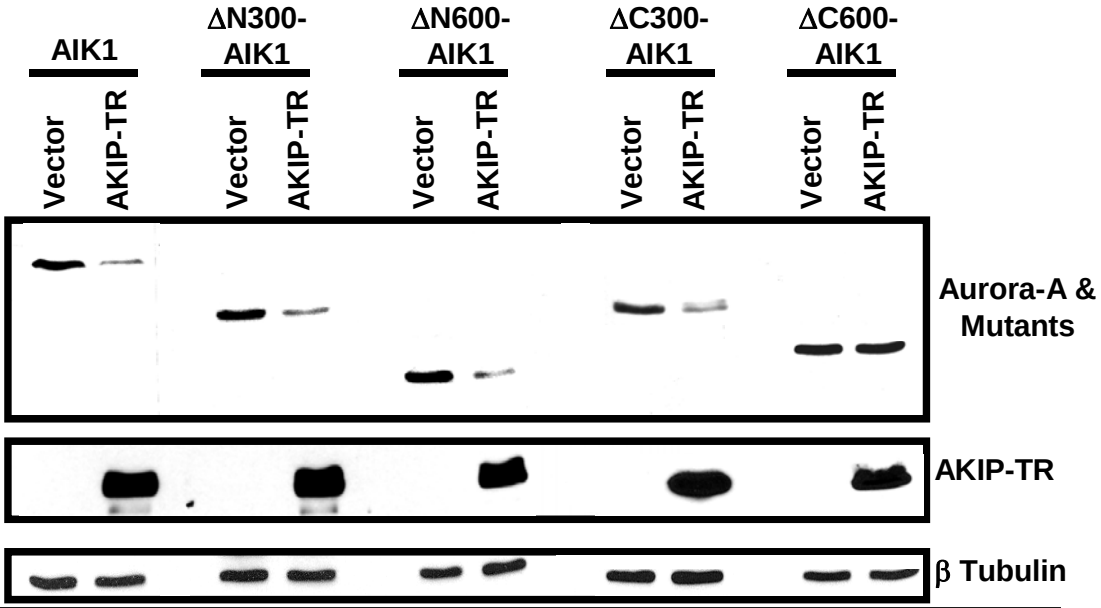


Figure 9-11: Effect of Impaired Aurora-A:AZ1 Interaction on AKIP-TR-mediated Aurora-A Degradation

ts20-CHO cells were co-transfected with HA-tagged AKIP-TR and either FLAG-tagged wild-type or various deletion mutants of Aurora-A at 5:1 ratio. 36 hours post-transfection, the transfected cells were harvested for Western Blot analysis of the protein stability of Aurora-A, both wild-type and mutants, in the presence of AKIP-TR overexpression. The Aurora-A and AKIP-TR were detected using the anti-FLAG and anti-HA mouse monoclonal antibodies, respectively. β tubulin was used as the loading control.

9.2.2.3. *In vivo* Interaction between Antizyme and AKIP-TR

To further understand how the antizyme participates the AKIP-TR-mediated Aurora-A degradation, we decided to probe into the possibility of any *in vivo* interaction between the antizyme and AKIP-TR. We had previously demonstrated the *in vivo* interaction between the AKIP-TR and Aurora-A and the AKIP-TR-mediated Aurora-A degradation involved antizyme. To investigate the relationship between AKIP-TR and antizyme further, we carried out *in vivo* interaction assays between AKIP-TR and antizyme. However, as shown in Figure 9-12, no *in vivo* interaction could be detected between AKIP-TR and antizyme, indicating that the functional role of antizyme in AKIP-TR-mediated Aurora-A was independent of antizyme-AKIP-TR interaction.

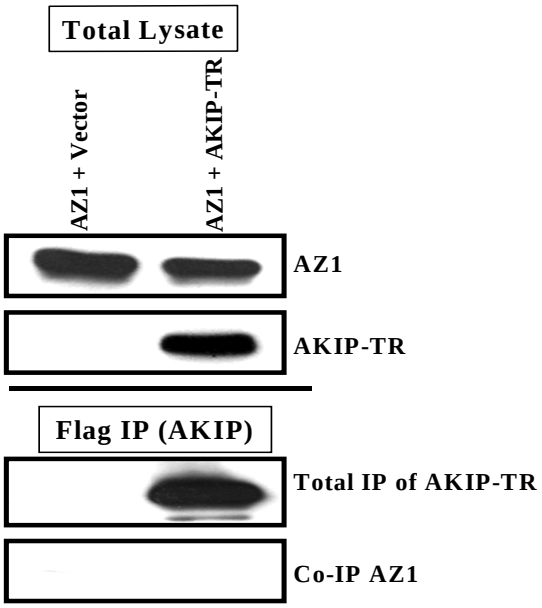


Figure 9-12: *In vivo* Interaction between AKIP-TR and Antizyme

HeLa cells were co-transfected with His-tagged AZ1 and either empty vector or FLAG-tagged AKIP-TR at 1:1 ratio. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The interacting AZ1 was detected using the anti-AZ rabbit polyclonal antibody. AKIP-TR was detected using the anti-FLAG rabbit polyclonal antibody.

9.2.2.4. [Aurora-A] : [Antizyme] : AKIP-TR] Ternary Complex

At present, *in vivo* interaction had been detected between Aurora-A and AKIP-TR and between Aurora-A and antizyme, though *in vivo* interaction between AKIP-TR and antizyme was undetectable. Therefore, it is possible that Aurora-A, AKIP-TR and antizyme might form a ternary complex *in vivo*.

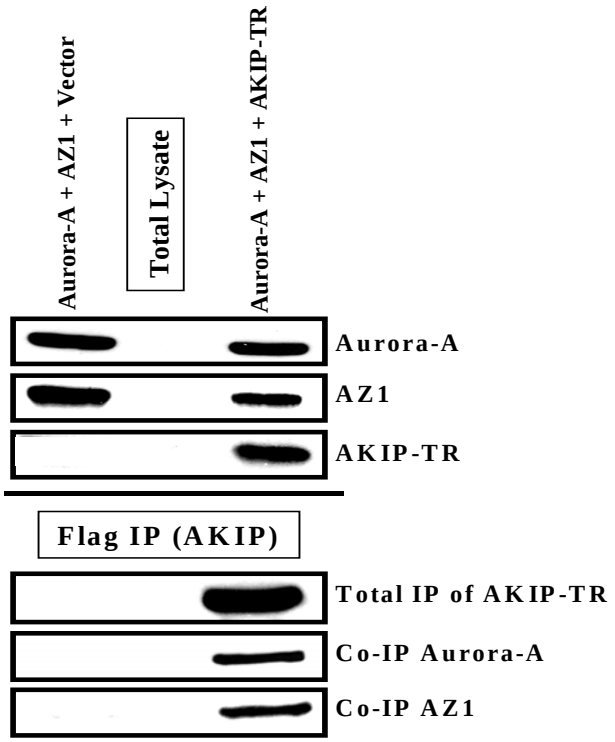


Figure 9-13: *In vivo* Ternary Complex of [Aurora-A] : [AZ1] : [AKIP-TR]
HeLa cells were co-transfected with His-tagged AZ1, HA-tagged Aurora-A and either empty vector or FLAG-tagged AKIP-TR at 1:1:1 ratio. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The interacting AZ1 and Aurora-A were detected using the anti-AZ and anti-HA rabbit polyclonal antibodies, respectively. AKIP-TR was detected using the anti-FLAG rabbit polyclonal antibody.

To study the formation of the [Aurora-A]: [antizyme]: [AKIP-TR] ternary complex *in vivo*, we immunoprecipitated AKIP-TR from cell lysate overexpressing Aurora-A, antizyme and AKIP-TR and attempted to detect the co-immunoprecipitated Aurora-A and antizyme.

Immunoprecipitation study had shown that the immunoprecipitated AKIP-TR pulled down both Aurora-A and antizyme, as shown in Figure 9-13. This suggested that AKIP-TR, Aurora-A and antizyme could exist as a ternary complex *in vivo*.

9.2.3. Role of AKIP-TR and Antizyme in Aurora-A Degradation

9.2.3.1. Affinity of Antizyme to Aurora-A in the Presence of AKIP-TR

Now we had clearly demonstrated the existence of a functional link between the AKIP-TR and antizyme in mediating the degradation of Aurora-A, the next question that looms is the order of these modulators in the pathway. Based on the current knowledge on the mechanism of antizyme-mediated degradation where binding of antizyme to its protein substrate enhances the recognition and targeting of substrate to the proteasome, we speculate that AKIP-TR acts upstream of antizyme in targeting the Aurora-A for protein degradation. There are two possible mechanisms for the Aurora-A Ub-independent degradation. In one case, AKIP-TR might increase the affinity of Aurora-A to antizyme and therefore Aurora-A can be targeted more efficiently to the proteasome for degradation in the Ub-independent manner. On the other hand, it is also possible that AKIP-TR could stimulate the frameshifting and expression of antizyme and hence increase binding of antizyme to Aurora-A and subsequent enhancement of the Aurora-A targeting to proteasome for Ub-independent degradation.

To assess whether AKIP-TR acts upstream of antizyme by enhancing the antizyme: Aurora-A interaction, we employed the co-immunoprecipitation assay to study the efficiency

of interaction between Aurora-A and antizyme in the presence and absence of AKIP-TR overexpression. As shown in Figure 9-14, in the presence of AKIP-TR overexpression, the binding of antizyme to Aurora-A was significantly enhanced.

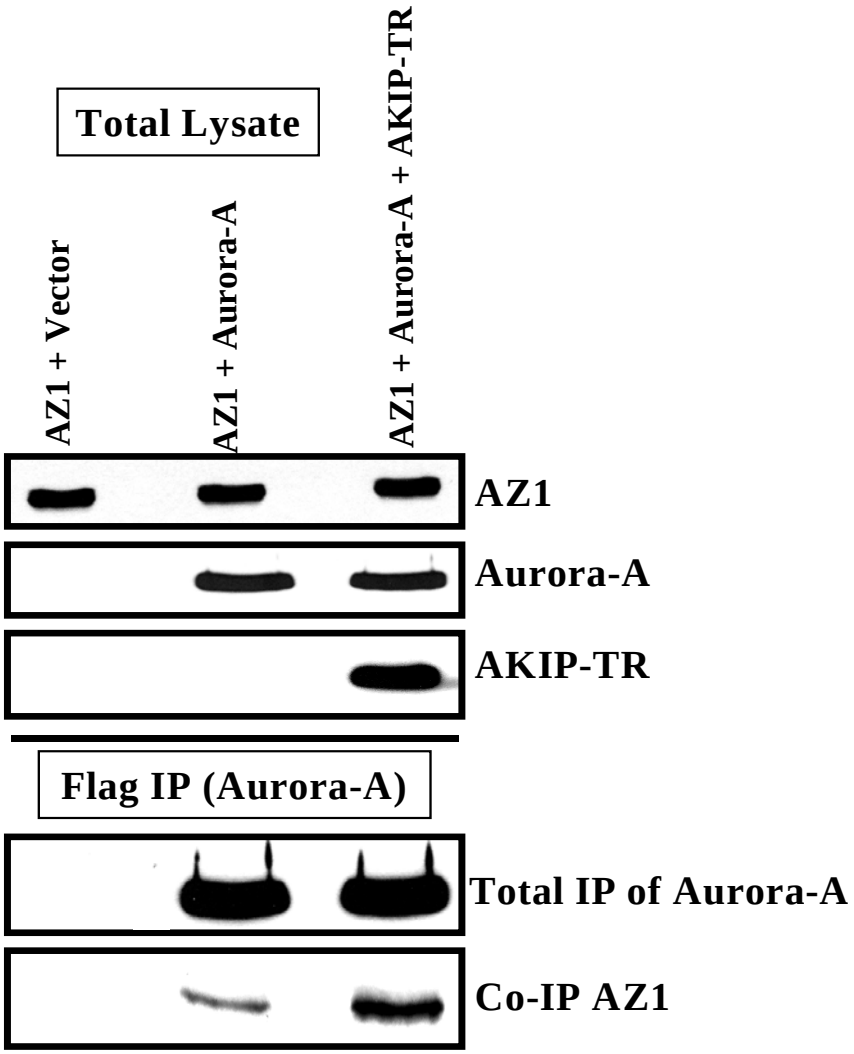


Figure 9-14: Binding Affinity of Antizyme to Aurora-A in the Presence of AKIP-TR
HeLa cells were co-transfected with His-tagged AZ1 and FLAG-tagged Aurora-A at 1:1 ratio in the absence or presence of HA-tagged AKIP-TR overexpression. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The interacting AZ1 was detected using the anti-AZ rabbit polyclonal antibody. The Aurora-A and AKIP-TR were detected using the anti-FLAG and anti-HA rabbit polyclonal antibody.

9.2.3.2. Affinity of AKIP-TR to Aurora-A in the Presence of Antizyme

On the other hand, we also studied the efficiency of interaction between Aurora-A and AKIP-TR in the presence and absence of antizyme overexpression. As expected, the Aurora-A:AKIP-TR interaction was not influenced even in the presence of antizyme overexpression, as demonstrated in Figure 9-15, supporting our initial hypothesis that AKIP-TR acted upstream of antizyme in mediating the degradation of Aurora-A.

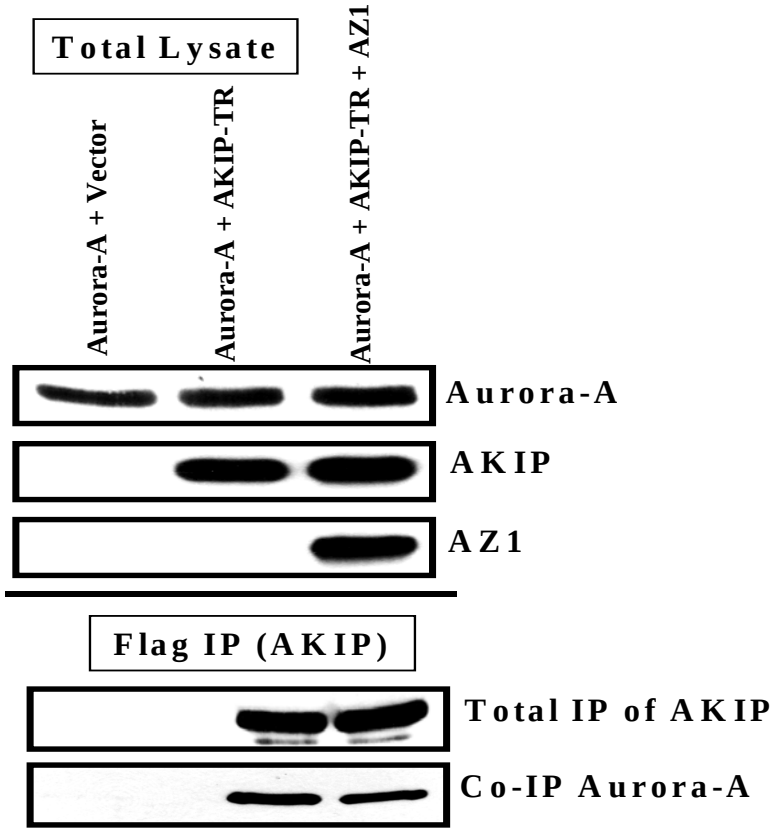


Figure 9-15: Binding Affinity of AKIP to Aurora-A in the Presence of Antizyme

HeLa cells were co-transfected with FLAG-tagged AKIP-TR and HA-tagged Aurora-A at 1:1 ratio in the absence or presence of His-tagged AZ1 overexpression. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The interacting Aurora-A was detected using the anti-HA rabbit polyclonal antibody. The AZ1 and AKIP-TR were detected using the anti-AZ and anti-FLAG rabbit polyclonal antibody.

9.2.3.3. No Induction of Antizyme Expression in the Presence of AKIP-TR

Finally, we also probed into the other possibility that AKIP-TR might stimulate the translational frameshifting and expression of antizyme, thereby promoting the interaction between Aurora-A and antizyme. A wild-type form of antizyme without the pre-incorporated frameshift mutation, was used in the present study to assay if the AKIP-TR overexpression induces the translational frameshift and expression of antizyme. Putrescine-induced frameshifting of the wild-type antizyme was used as the positive control. Results from Figure 9-16 had shown that putrescine did induce the antizyme expression by translational frameshifting whereas the AKIP-TR overexpression did not, suggesting AKIP-TR did not promote the antizyme binding to Aurora-A through the induction of antizyme expression by translational frameshift.

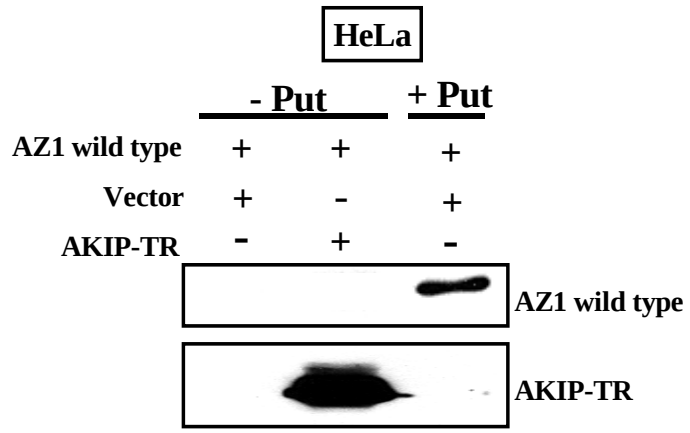


Figure 9-16: Effect of AKIP-TR Overexpression on Translational Frameshifting and Expression of Antizyme

HeLa cells were co-transfected with His-tagged wild type AZ1 and either empty vector or FLAG-tagged AKIP-TR at 1:3 ratio in the absence or presence of putrescine treatment for 24 hours. 24 hours post-transfection, the transfected cells were harvested for FLAG immunoprecipitation. The expression of frameshifted AZ1 in the presence of AKIP-TR overexpression or putrescine treatment was monitored. The AZ1 and AKIP-TR were detected using the anti-AZ and anti-FLAG rabbit polyclonal antibody.

9.3. Discussions

The protein degradation of increasing number of cell cycle regulators is found to deviate from the prevailing ubiquitin-proteasome paradigm of destruction, instead their degradation can be completely Ub-independent or coexist with the Ub-dependent pathways [1]. These cell cycle regulators include Cdk inhibitor-p21 [2-3] and p27 [4], cyclin D1 [5] as well as the tumor suppressor-p53, p73 [6-7] and Rb [8-9]. We had previously demonstrated that Aurora-A kinase, one of important regulators for mitosis, could also be targeted for its protein degradation through the Ub-independent pathway and this Ub-independent degradation of Aurora-A was potentiated by AKIP. In view of the existence of an Ub-independent degradation for these cell cycle proteins, this alternative pathway, which uncouples degradation from ubiquitin modification, may reflect a specialized regulatory mechanism for cell cycle regulation, which remains an interesting question yet to be answered.

With respect to the detailed mechanism on how the protein substrates could be targeted to proteasome in the absence of the polyubiquitin tag, the knowledge is still very limited though a few studies had shed some lights on the possible ways to protein targeting. Our present work focusing on the mechanism AKIP-mediated targeting of Aurora-A for Ub-independent proteasomal degradation, had successfully discovered a functional link between AKIP-mediated Aurora-A degradation to antizyme, currently the most well-studied mediator for Ub-independent protein degradation. To date, the protein degradation of ornithine decarboxylase (ODC) [10] and cyclin D1 [5] had been shown to be regulated by antizyme

(AZ) [11-12] in the Ub-independent manner, however, the mechanism of AZ-mediated degradation of ODC is more well characterized.

AZ can interact with ODC and this binding is essential to target ODC for degradation by the 26S proteasome. With respect to role of AZ in mediating ODC protein degradation, studies had found that the attachment of AZ changed the ODC protein conformation, thereby exposing its C-terminal degradation signal for recognition by 26S proteasome and hence enhancing the association of ODC with proteasome [13]. Moreover, AZ was also thought to be a potential functional equivalent of polyubiquitin tag as studies had shown that substrate-linked or free polyubiquitin chains could compete for the AZ-mediated ODC degradation [14]. In other word, AZ and polyubiquitin chain might share the same recognition element on the proteasome [14]. Similarly, AZ also interacts with cyclin D1 and this interaction facilitates the cyclin D1 Ub-independent protein degradation [5]. However, whether or not AZ enhances the cyclin D1 association with proteasome in a similar way to ODC has not been addressed and demonstrated.

The role of AZ in Aurora-A degradation was clearly shown in our study, as either ectopic expression of AZ or endogenous AZ induction by polyamine could lead to the down-regulation of Aurora-A protein stability, which was consistent with cases with ODC and cyclin D1. Furthermore, AZ could also interact with Aurora-A and the AZ:Aurora-A interaction was essential for the AZ-mediated Aurora-A degradation, as had been observed for

the AZ-mediated ODC and cyclin D1 degradation. Based on the role of AZ in proteasome-targeting of ODC, it would be interesting to investigate whether it was also the same case for the role of AZ in the recognition and targeting of Aurora-A to proteasome. However, our study had added another level of complexity in the AZ-mediated degradation of Aurora-A, which involved the regulation from AKIP. Our study had shown that AKIP acted upstream AZ in the Ub-independent degradation pathway for Aurora-A, where AKIP enhanced the affinity of Aurora-A to AZ. It is yet to be demonstrated whether this enhanced binding of AZ to Aurora-A in the presence of AKIP helps targeting Aurora-A more efficiently to proteasome for ubiquitin-independent degradation. However, the results from our study showed that AKIP overexpression exerted no effect on induction of AZ translational frameshifting and expression, suggesting that AKIP may regulate the AZ-mediated Aurora-A degradation at the post-translational level of AZ expression. It should be noted that the study was carried out with the N-terminally truncated form of AKIP, which we had previously shown to be sufficient for mediating Aurora-A degradation as well as more efficient in Aurora-A degradation. However, it is possible that the N-terminus truncated portion of AKIP, though may be unnecessary for mediating Aurora-A degradation, may instead important for inducing the translational frameshifting and expression of AZ. This possibility awaits future investigations.

Our studies on *in vivo* ternary complex formation of AKIP:Aurora-A:AZ and the

characterization of their interactions in any combination of two, had suggested the potential of *in vivo* ternary complex formation of endogenous AKIP:Aurora-A:AZ and this ternary complex formation depended on both the Aurora-A:AKIP interaction and Aurora-A:AZ interaction with no direct involvement of the AKIP:AZ interaction. There is a possibility that the binding of AKIP to Aurora-A may change its protein conformation, thereby exposing the important domain for the recognition and binding by AZ. A successful testing of this possibility probably accounts for the mechanism on how AKIP promotes the AZ binding to Aurora-A.

Our two main studies showing the abrogation of AKIP-mediated Aurora-A degradation in the presence of either antizyme inhibition or impaired Aurora-A:AZ interaction, had clearly identified the important role of antizyme in AKIP-mediated Aurora-A degradation. Though antizyme had been previously shown to target ODC for degradation via 26S proteasome in the ATP-dependent manner [15], it was unclear yet whether AKIP-mediated Aurora-A degradation, which we had shown to be also AZ- and proteasome-dependent, is mediated through the ATP-dependent 26S proteasome or ATP-independent 20S proteasome.

Surprisingly, another study uncovered a novel but contradictory role of AZ against its normal regulation of the Ub-independent protein degradation. AZ together with the proteasomal subunit HsN3 were shown to be involved in targeting the Smad 1 to the proteasome for degradation through the Ub-dependent mechanism [16-17], the first and only

demonstration of AZ playing a role in the regulation of Ub-dependent protein degradation.

Several studies had shown that perturbation of the antizyme level could affect the cell cycle progression [18-21], but the mechanism by which antizyme influences the cell cycle is still a doubt. The previous study with AZ-mediated cyclin D1 degradation [5] and our current study on AZ-mediated Aurora-A degradation suggested this effect of antizyme could be mediated by a direct interaction between antizyme and cell cycle regulators. Antizyme overexpression or induction could lead to cell cycle arrest in G1 phase and growth inhibition and this growth phenotype was observed in many organisms [22-24], implying that direct regulation of the cell cycle by antizyme is an evolutionary conserved biological mechanism. Therefore, AZ-mediated Aurora-A degradation may play a potential tumour suppressor role in those cancer cells overexpressing Aurora-A, probably also through the suppression of cell proliferation and induction of apoptosis. Since Aurora-A also plays a positive role in cell migration, down-regulation of Aurora-A in cancer cells via AZ induction may inhibit the metastasis, thereby reducing the invasiveness of the cancer. So, it would be interesting and crucial to know how significance is the role AZ-mediated Aurora-A down-regulation in the suppression of cell proliferation and metastasis.

During the cell cycle, cyclin D1 is phosphorylated at Thr-286 and this phosphorylation is essential for promoting the ubiquitination and cell cycle-dependent degradation of cyclin D1 [5]. In addition, this phosphorylation also controls the nuclear localization of cyclin D1 in

which phosphorylation induces the nuclear export of cyclin D1 to cytoplasm [25]. This redistribution of cyclin D1 depends on the nuclear exportin CRM1 [25]. Similarly, nuclear localization signals had been identified in antizyme and antizyme had also been shown to shuttle between nucleus and cytoplasm in a CRM1-dependent manner [26]. Interestingly, localization of antizyme and ODC to the nucleus had been demonstrated during mouse development [27]. All these findings suggested antizyme could bind to ODC and other proteins in the nucleus and escort them to cytoplasm for degradation [26]. This implication may be relevant to the possible physiological role of AKIP-mediated Aurora-A degradation. As AKIP is a nuclear protein and Aurora-A is regulated by phosphorylation in the cell cycle-dependent manner, it will be interesting to investigate the subcellular localization of AKIP-mediated Aurora-A degradation as well as how AKIP co-operates with antizyme in Aurora-A degradation.

9.4. Conclusion

In summary, our identification of antizyme role in Aurora-A degradation has further strengthened the implicated role of antizyme in cell cycle regulation, though its physiological significance is yet to be discovered. Moreover, the identification of AKIP as an upstream regulator of antizyme in Aurora-A degradation provides us a stepping stone towards a better understandings of the ubiquitin-independent protein degradation mediated by antizyme. I believe a clearer understanding of the physiological roles of AKIP and antizyme in Aurora-A

degradation in the future will shed some lights on the tumour suppressor potential of AKIP and make AKIP a good target for the anti-cancer drug.

9.5. References

1. Hoyt MA, Coffino P. **Ubiquitin-free routes into the proteasome**. Cell Mol Life Sci. (2004) 61(13):1596-600.

2. Sheaff RJ, Singer JD, Swanger J, Smitherman M, Roberts JM, Clurman BE. **Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination.** Mol Cell. (2000) 5(2):403-10.

3. Jin Y, Lee H, Zeng SX, Dai MS, Lu H. **MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation.** EMBO J. (2003) 22(23):6365-77.

4. Shirane M, Harumiya Y, Ishida N, Hirai A, Miyamoto C, Hatakeyama S, Nakayama K, Kitagawa M. **Down-regulation of p27(Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing.** J Biol Chem. (1999) 274(20):13886-93.

5. Newman RM, Mobascher A, Mangold U, Koike C, Diah S, Schmidt M, Finley D, Zetter BR. **Antizyme targets cyclin D1 for degradation. A novel mechanism for cell growth repression.** J Biol Chem. (2004) 279(40):41504-11.

6. Asher G, Tsvetkov P, Kahana C, Shaul Y. **A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73.** Genes Dev. (2005) 19(3):316-21.

7. Asher G, Lotem J, Sachs L, Kahana C, Shaul Y. **Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1.** Proc Natl Acad Sci U S A. (2002) 99(20):13125-30.

8. Kalejta RF, Shenk T. **Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein**. Proc Natl Acad Sci U S A. (2003) 100(6):3263-8.

9. Sdek P, Ying H, Chang DL, Qiu W, Zheng H, Touitou R, Allday MJ, Xiao ZX. **MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein.** Mol Cell. (2005) 20(5):699-708.

10. Murakami Y, Matsufuji S, Kameji T, Hayashi S, Igarashi K, Tamura T, Tanaka K, Ichihara

A. **Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination.** Nature. (1992) 360(6404):597-9.

11. Coffino P. **Antizyme, a mediator of ubiquitin-independent proteasomal degradation.** Biochimie. (2001) 83(3-4):319-23.

12. Mangold U. **The antizyme family: polyamines and beyond.** IUBMB Life. (2005) 57(10):671-6.

13. Li X, Coffino P. **Degradation of ornithine decarboxylase: exposure of the C-terminal target by a polyamine-inducible inhibitory protein.** Mol Cell Biol. (1993) 13(4):2377-83.

14. Zhang M, Pickart CM, Coffino P. **Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate.** EMBO J. (2003) 22(7):1488-96.

15. Murakami Y, Matsufuji S, Hayashi SI, Tanahashi N, Tanaka K. **ATP-Dependent inactivation and sequestration of ornithine decarboxylase by the 26S proteasome are prerequisites for degradation.** Mol Cell Biol. (1999) 19(10):7216-27.

16. Lin Y, Martin J, Gruendler C, Farley J, Meng X, Li BY, Lechleider R, Huff C, Kim RH, Grasser WA, Paralkar V, Wang T. **A novel link between the proteasome pathway and the signal transduction pathway of the bone morphogenetic proteins (BMPs).** BMC Cell Biol. (2002) 3:15.

17. Gruendler C, Lin Y, Farley J, Wang T. **Proteasomal degradation of Smad1 induced by bone morphogenetic proteins.** J Biol Chem. 2001 Dec 7;276(49):46533-43.

18. Oredsson SM. **Polyamine dependence of normal cell-cycle progression.** Biochem Soc Trans. (2003) 31(2):366-70.

19. Koike C, Chao DT, Zetter BR. **Sensitivity to polyamine-induced growth arrest correlates with antizyme induction in prostate carcinoma cells.** Cancer Res. (1999) 59(24):6109-12.

20. Bettuzzi S, Davalli P, Astancolle S, Pinna C, Roncaglia R, Boraldi F, Tiozzo R, Sharrard M, Corti A. **Coordinate changes of polyamine metabolism regulatory proteins**

during the cell cycle of normal human dermal fibroblasts. FEBS Lett. (1999) 446(1):18-22.

21. Fredlund JO, Johansson MC, Dahlberg E, Oredsson SM. **Ornithine decarboxylase and S-adenosylmethionine decarboxylase expression during the cell cycle of Chinese hamster ovary cells.** Exp Cell Res. (1995) 216(1):86-92.

22. Tsuji T, Usui S, Aida T, Tachikawa T, Hu GF, Sasaki A, Matsumura T, Todd R, Wong DT. **Induction of epithelial differentiation and DNA demethylation in hamster malignant oral keratinocyte by ornithine decarboxylase antizyme.** Oncogene. (2001) 20(1):24-33.

23. Murakami Y, Matsufuji S, Miyazaki Y, Hayashi S. **Destabilization of ornithine decarboxylase by transfected antizyme gene expression in hepatoma tissue culture cells.** J Biol Chem. (1992) 267(19):13138-41

24. Ivanov IP, Matsufuji S, Murakami Y, Gesteland RF, Atkins JF. **Conservation of polyamine regulation by translational frameshifting from yeast to mammals.** EMBO J. (2000) 19(8):1907-17.

25. Alt JR, Cleveland JL, Hannink M, Diehl JA. **Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation.** Genes Dev. (2000) 14(24):3102-14.

26. Murai N, Murakami Y, Matsufuji S. **Identification of nuclear export signals in antizyme-1.** J Biol Chem. (2003) 278(45):44791-8.

27. Gritli-Linde A, Nilsson J, Bohlooly-Y M, Heby O, Linde A. **Nuclear translocation of antizyme and expression of ornithine decarboxylase and antizyme are developmentally regulated.** Dev Dyn. (2001) 220(3):259-75.

28. Mangold U., Leberer E. **Regulation of all members of the antizyme family by antizyme inhibitor.** Biochem. J. (2005) 385: 21-28

CHAPTER 10

Current View and Future Outlook

10.1. Current View and Hypothesis

Possible Anti-Tumour Role of AKIP-mediated Ub-Independent Degradation of Aurora-A

Based on the information and data presented in this thesis, a hypothesis on the possible anti-tumour role of AKIP through its interplay with AZ in the Ub-independent degradation of Aurora-A is summarized in Figure 10-1.

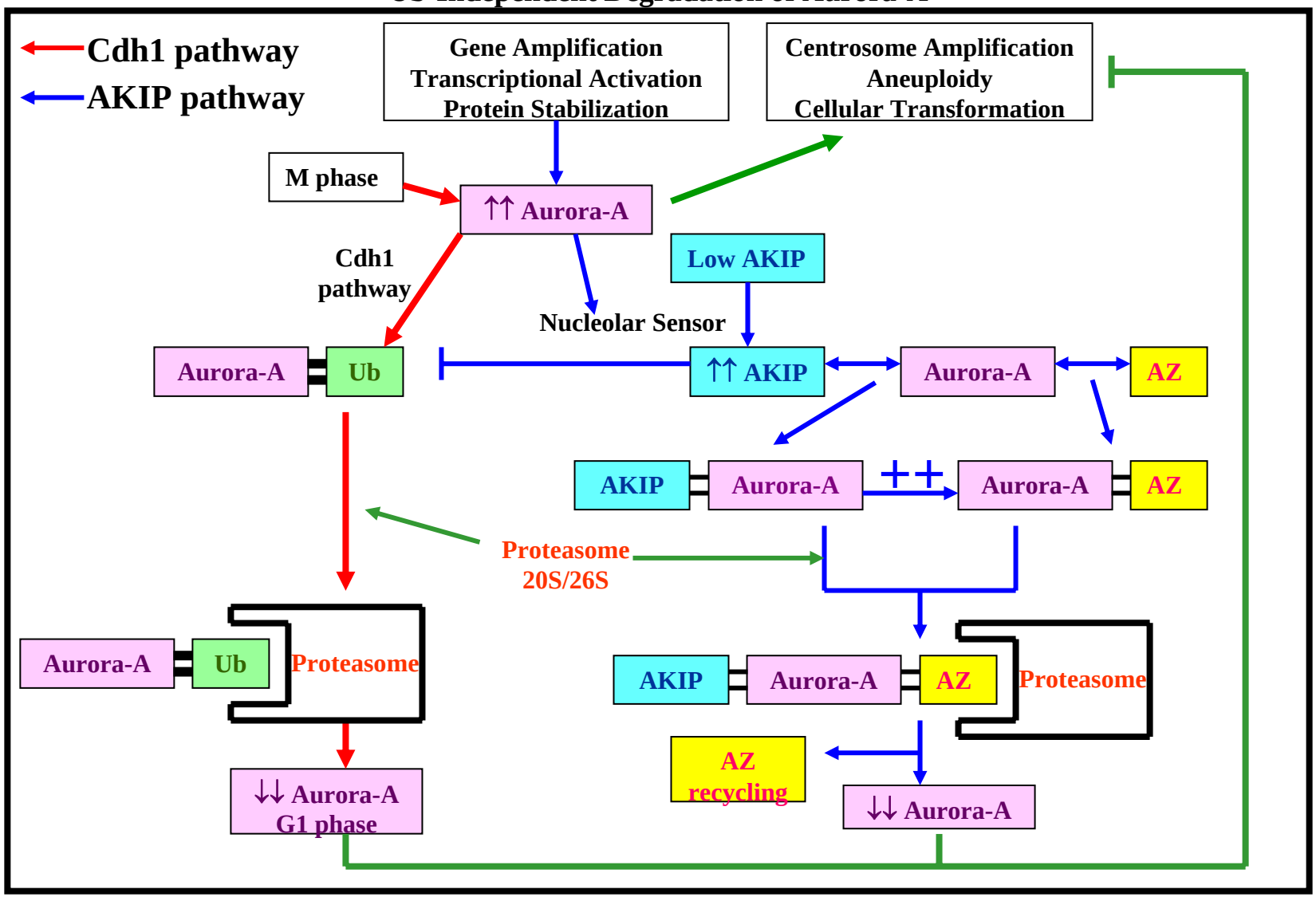
The protein levels of Aurora-A can be upregulated under two situations: (1) physiological induction during M phase of the cell cycle; (2) abnormal induction through acquired gene amplification, transcriptional activation and protein stabilization. Under normal condition, Aurora-A undergoes cell cycle-dependent protein degradation, with its protein expression peaking at G2/M and rapidly degraded upon mitotic exit into next G1 phase. This cell cycle-dependent degradation of Aurora-A is mediated by the Cdh1-Ub-dependent pathway. Defective Aurora-A degradation during M to G1 transition could also lead to aberrant mitosis and subsequently induction of chromosomal instability and oncogenic transformation.

Alternatively Aurora-A can be degraded in an Ub-independent mode, which is mediated through the AKIP. Normally, AKIP protein may be unstable, present at low level or sequestered in nucleolus, thereby protecting Aurora-A from the AKIP-induced constitutive Ub-independent degradation. However, under certain physiological setting, which is yet to be discovered, AKIP protein stability may increase or the AKIP sequestered in the nucleolus may be released. The induced or released AKIP can then bind Aurora-A. Binding of Aurora-A by

AKIP blocks the ubiquitination of Aurora-A, thereby turning off the Ub-dependent pathway for Aurora-A degradation. The Ub-dependent mode of degradation may not be not favored under certain abnormal situation as it is more energy consuming and more complex involving protein modification by ubiquitin tag. Instead, Aurora-A will be channeled through the Ub-independent degradation pathway.

Normally Aurora-A associates weakly with antizyme (AZ) and plays important role in targeting protein to proteasome for subsequent Ub-independent degradation. Binding of AKIP to Aurora-A increases the affinity of AZ to Aurora-A, hence enhancing the recognition and targeting of Aurora-A to proteasome for degradation. AZ is spared from destruction and thus recycled for another round of AKIP-mediated Aurora-A degradation until normal level of Aurora-A protein is reached. Therefore, this protective mechanism against abnormal Aurora-A accumulation via AKIP may therefore prevent the transformed phenotypes induced by Aurora-A overexpression.

Figure 10-1: Hypothesis of Possible Anti-Tumour Role of AKIP-Mediated Ub-Independent Degradation of Aurora-A



10.2. Future Outlook

The data obtained from current studies could lead us to speculate various interesting possibilities for future studies. The localization of AKIP to nucleolus suggests the possibility of a role for AKIP in ribosomal biogenesis, which is essential for the protein synthesis in the cell [1-2]. Interestingly, recent studies had uncovered a wider and more dynamic role of nucleolus. Nucleolus is not just a site for the ribosomal synthesis machinery, but also for some newfound nucleolar oncogenes and tumor suppressors [3]. It can function as a cellular stress sensor and adapt to stress through not only ribosome production and subsequent protein translation but also through activation of downstream growth suppressors, such as ARF and p53 [4]. Nucleolar dysfunction has now been closely linked to tumorigenesis [3]. Hence, it might be interesting to investigate the link between AKIP-mediated Aurora-A degradation and cellular stress response, which may have significant implications in tumorigenesis. Our preliminary data showed that cellular stress by UV could influence the subcellular localization of AKIP (data not shown). We had observed that UV treatment of AKIP-expressing cells induced the translocation of AKIP out from nucleolus into nucleoplasm. The significance of this finding in relation to AKIP-mediated Aurora-A degradation could be addressed in the future.

On the other hand, the change in AKIP localization from nucleolus during interphase to mitotic structure during mitosis showed similarity to that of a group of proteins, called mitotic

exit network (MEN) proteins. MEN functions as a checkpoint protein that is different from that of the spindle assembly checkpoint, where it regulates the processes at the end of mitosis. MEN is found on the spindle pole body where it controls the activation of APC/C by the Cdh1 activator. It is normally sequestered in nucleolus throughout most of the cell cycle and released from nucleolus only at the end of mitosis. Much works on MEN were performed in yeast, knowledge on the role of MEN in higher eukaryotes is still very limited. One example of MEN protein is the Cdc14 from *S.cerevisiae*. Cdc14 is localized in the nucleolus during most of the cell cycle as part of the RENT complex (REgulation of Nucleolar silencing and Telophase exit) and is then liberated from nucleolus at the end of mitosis. Cdc14 activates APC^{Cdh1}-dependent degradation of Clb2 and increase the expression and stability of Cdk inhibitor Sic1, thereby promoting the mitotic exit and assembly of pre-replication complexes for DNA synthesis in next cycle. Cdc14 is bound to Net1 in the nucleolus, which anchors and inhibits Cdc14. It would be interesting to investigate whether AKIP also takes up the similar role as MEN, regulating the mitotic exit by ensuring the proteolysis of Aurora-A upon exit into G1 [9-14] and how this is linked with the Cdh1-Ub-dependent degradation of Aurora-A, which also occurs during M to G1 transition.

The identification of AKIP as a negative regulator of Aurora-A oncogene suggests its potential as a target for anti-cancer drugs. Several small molecule inhibitors of Aurora kinases, which target specifically their kinase activity by occupying the catalytic ATP-binding sites,

had been developed recently [6-7]. One of them, called VX-680 had shown very promising results in its anti-tumor potential and treatment of tumors that are resistant to the standard therapies [8]. The development of these inhibitors has strengthened our confidence that targeting Aurora kinases could be useful for cancer therapy. However, these inhibitors did not selectively inhibit a particular member of Aurora kinase family and this might increase the potential for toxic effects in the clinical setting. Therefore, if inhibition of kinase activity of a single Aurora family member can mediate the anti-tumor activity, then it could be beneficial to develop selective inhibitors of that particular Aurora kinase.

In this context, AKIP may be a good candidate as shown by its specificity towards Aurora-A. Still, it is yet to be demonstrated that Aurora-A is the most suitable and useful target among the Aurora kinase family for therapeutic intervention. In the context of cancer therapy by targeting Aurora-A kinase, drugs that target the protein stability of Aurora-A may be a better choice than those that target the kinase activity of Aurora-A. The centrosome amplification induced by Aurora-A overexpression, which is implicated in subsequent failure of bipolar spindle assembly and aneuploidy, does not require the kinase activity of the Aurora-A, although kinase activity is still necessary for the oncogenic transformation [15]. AKIP, being a negative regulator of Aurora-A targeting its protein stability, can therefore serve as a better anti-cancer target in preventing transformation phenotypes caused by both levels of high protein expression and kinase activity. Moreover, if Aurora-A exists as part of a

multiprotein complex that has structural or catalytic roles, destabilization of Aurora-A protein may affect the stoichiometry of the complex and disrupt the normal localization and function of its binding partners. Therefore, future works focusing on the tumour suppressor role of AKIP is of utmost importance. The role of AKIP in the reversal of Aurora-A-induced transformed phenotype, like increased cell proliferation, centrosome amplification, aneuploidy, *in vitro* transformation and formation of tumour in nude mice, should be addressed and investigated next.

In a recent attempt to address the possibility of Aurora-A as the therapeutic target for pancreatic cancer, it was found that specific knockdown of Aurora-A in pancreatic cancer cells strongly suppressed the *in vitro* cell growth and *in vivo* tumorigenicity, by inducing G2/M arrest and subsequent apoptosis. Furthermore, the knockdown of Aurora-A also synergistically enhanced the sensitivity of the pancreatic cancer cells to the chemodrug Taxol [5]. As AKIP also knocks down Aurora-A protein level through potentiation of its degradation, it would be interesting to investigate the possibility that down-regulation of Aurora-A by AKIP might also have similar anti-tumor or taxol chemosensitizing activity.

Apart from its role in the destabilization of Aurora-A kinase when overexpressed, the normal function of AKIP is yet to be established. It will be interesting to investigate whether AKIP is also the normal physiological trigger for Aurora-A degradation. Other pertinent questions to be answered include the subcellular location where interaction between AKIP

and Aurora-A occurs. Being a negative regulator of Aurora-A oncogene, there is a possibility that down-regulation of AKIP could play a role in tumorigenesis. Systematic analysis of AKIP mRNA and protein expression in the tumor tissues may be necessary to address this question.

10.3. References

1. Lam YW, Trinkle-Mulcahy L, Lamond AI. **The nucleolus**. J Cell Sci. (2005) 118(Pt 7):1335-7.

2. Nazar RN. **Ribosomal RNA processing and ribosome biogenesis in eukaryotes.** IUBMB Life. (2004) 56(8):457-65.

3. Maggi LB Jr, Weber JD. **Nucleolar adaptation in human cancer.** Cancer Invest. (2005) 23(7):599-608.

4. Mayer C, Grummt I. **Cellular stress and nucleolar function.** Cell Cycle. (2005) 4(8):1036-8.

5. Hata T, Furukawa T, Sunamura M, Egawa S, Motoi F, Ohmura N, Marumoto T, Saya H, Horii A. **RNA interference targeting aurora kinase a suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells.** Cancer Res. (2005) 65(7):2899-905.

6. Keen N, Taylor S. **Aurora-kinase inhibitors as anticancer agents.** Nat Rev Cancer. (2004) 4(12):927-36.

7. Andrews PD. **Aurora kinases: shining lights on the therapeutic horizon?** Oncogene. (2005) 24(32):5005-15.

8. Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A, Su M, Golec JM, Miller KM. **VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo.** Nat Med. (2004) 10(3):262-7.

9. Cerutti L, Simanis V. **Controlling the end of the cell cycle**. Curr Opin Genet Dev. (2000) 10(1):65-9.

10. Jaspersen SL, Charles JF, Tinker-Kulberg RL, Morgan DO. **A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae**. Mol Biol Cell. (1998) 9(10):2803-17.

11. Shou W, Seol JH, Shevchenko A, Baskerville C, Moazed D, Chen ZW, Jang J, Shevchenko A, Charbonneau H, Deshaies RJ. **Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex.** Cell. (1999) 97(2):233-44.

12. Straight AF, Shou W, Dowd GJ, Turck CW, Deshaies RJ, Johnson AD, Moazed D. **Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity.** Cell. (1999) 97(2):245-56.

13. Visintin R, Hwang ES, Amon A. **Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus.** Nature. (1999) 398(6730):818-23.

14. Bachant JB, Elledge SJ. **Mitotic treasures in the nucleolus.** Nature. (1999) 398(6730):757-8.

15. Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B, Schryver B, Flanagan P, Clairvoyant F, Ginther C, Chan CS, Novotny M, Slamon DJ, Plowman GD. **A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers.** EMBO J. (1998) 17(11):3052-65.

SECTION 4

Appendices

Appendix I

Published papers arising from thesis work

Lim Shen Kiat, Kam Man Hui, and Ganesan Gopalan. **Aurora-A Kinase Interacting Protein (AIP), a Novel Negative Regulator of Human Aurora-A Kinase.** J Biol. Chem. (2002); 277(47):45558-45565.

Lim Shen Kiat, and Ganesan Gopalan. **Aurora-A Kinase Interacting Protein (AURKAIP1) Promotes Aurora-A Degradation through Alternative Ubiquitin-Independent Pathway.** Biochem. J (2006); Nov 27 [Epub ahead of print]

Lim Shen Kiat, and Ganesan Gopalan. **Antizyme1 mediates AURKAIP1-dependent degradation of Aurora-A.** (2006) Submission to Oncogene for 2nd review

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 47, Issue of November 22, pp. 45558–45565, 2002

Printed in U.S.A.

Aurora-A Kinase Interacting Protein (AIP), a Novel Negative Regulator of Human Aurora-A Kinase*

Received for publication, July 9, 2002, and in revised form, September 16, 2002
Published, JBC Papers in Press, September 18, 2002, DOI 10.1074/jbc.M206820200

Lim Shen Kiat, Kam Man Hui, and Ganesan Gopalan‡

From the Division of Cellular and Molecular Research, National Cancer Centre, Singapore 169610

Aurora kinases have evolved as a new family of mitotic centrosome- and microtubule-associated kinases that regulate the structure and function of centrosomes and spindle. One of its members, Aurora-A, is a potential oncogene. Overexpression of Aurora-A is also implicated in defective centrosome duplication and segregation, leading to aneuploidy and tumorigenesis in various cancer cell types. However, the regulatory pathways for mammalian Aurora-A are not well understood. Exploiting the lethal phenotype associated with the overexpression of Aurora-A in yeast, we performed a dosage suppressor screen in yeast and report here the identification of a novel negative regulator of Aurora-A, named AIP (Aurora-A kinase Interacting Protein). AIP is a ubiquitously expressed nuclear protein that interacts specifically with human Aurora-A *in vivo*. Ectopic expression of AIP with Aurora-A in NIH 3T3 and COS cells results in the down-regulation of ectopically expressed Aurora-A protein levels, and this down-regulation is demonstrated to be the result of destabilization of Aurora-A through a proteasome-dependent protein degradation pathway. A noninteracting deletion mutant of AIP does not down-regulate Aurora-A protein, suggesting that the interaction is important for the protein degradation. AIP could therefore be a potential useful target gene for anti-tumor drugs.

Faithful chromosome segregation and cytokinesis are two essential steps in mitosis, which is responsible for the viability and genetic stability of daughter cells. This involves the concerted spatial and temporal interactions among various components such as the chromosomes, centrosomes, actin, and the microtubule cytoskeleton. Defined at the molecular level, the stability and the activation/inactivation of the proteins associated with these structures result in the regulation of the chromosome, centrosome, spindle microtubules, and actin dynamics. Reversible protein phosphorylation plays an important regulatory role in orchestrating the interactions among various proteins during mitosis (1–6). Many of the protein kinases and their opposing phosphatases, which are involved in these signaling cascades, have been identified (7). For example, the cyclin-dependent kinases are activated by cdc25 phosphatase (8) to regulate different stages of M phase, starting from triggering the mitotic entry, spindle formation, anaphase to cytokinesis (9–12). Similarly, polo-like kinases regulate several processes, which include centrosome maturation, cyclin-dependent kinase 1 activation and inactivation, and cytokinesis (9, 13–15). Other participating kinases include Bub1 kinase, which localizes to the kinetochore and regulates the anaphase checkpoint (16), and the centrosome-associated NimA-related kinase, which primarily regulates the centrosome cycles (17). The stability of the mitotic regulators also plays a pivotal role in the progression and completion of mitosis. In mitotic cells, progression into anaphase depends on the activation of the anaphase-promoting complex/cyclosome (APC/C)[1] by phosphorylation to degrade the mitotic regulators (18, 19).

Recently, the Aurora kinase family emerged as a new family of mitotic serine threonine kinases regulating the centrosomal and microtubule function, ensuring the accurate chromosome segregation and efficient completion of cytokinesis (20, 21). The Aurora kinase family was first identified in the budding yeast as Ipl1 (22), and subsequently various homologs of Ipl1 have been isolated from diverse organisms, ranging from *Drosophila*, *Xenopus*, *Caenorhabditis elegans*, mouse, rat, to human (20, 21). Ipl1 is the only representative of this family in yeast, two Aurora-related kinases are found in *Drosophila* and *C. elegans*, and three in mammals (20, 21). Their roles in chromosome segregation are implicated in the phenotypes of various mutants. *S. cerevisiae ipl1* mutants showed abnormal chromosome segregation and ploidy (22–24). *Drosophila Aurora* mutants showed defective centrosome separation resulting in the formation of monopolar spindles (25). They share similarity in their kinase catalytic domain but no or little similarity in their N-terminal domain, which seems to be species- and member-specific. Their expression and kinase activity are tightly cell cycle-regulated, peaking at M phase and disappearing rapidly upon mitotic exit (26–30). In mammals, there are three members of this family, designated Aurora-A, Aurora-B, and Aurora-C. Orthologs of Aurora-A kinase localize to the centrosome and mitotic spindle and function during the early part of the mitosis from prophase (27, 29, 31, 49). Aurora-B kinase localizes to the mid-body and postmitotic bridge, functioning during the late mitosis, and plays a role in cytokinesis (30, 32). Human Aurora-C kinase localizes to the anaphase centrosomes (33), and its function remains to be elucidated.

Increased attention has now been focused on Aurora-A kinase because of its suggestive role in tumorigenesis. Overexpression of Aurora-A kinase is observed in more than 50% of primary colorectal tumors and 6–18% of primary breast tumors (29, 31). Human Aurora-A kinase maps to chromosome

* This work was supported by the National Medical Research Council of Singapore, Singapore National Science and Technology Board, and the Singapore Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Laboratory of Gene Structure and Expression, Division of Cellular and Molecular Research, National Cancer Centre, Level 6, 11 Hospital Dr., Singapore 169610. Tel.: 65-6321-4075; Fax: 65-6321-3606; E-mail: cmrgop@nccs.com.sg.

[1] The abbreviations used are: APC/C, anaphase-promoting complex/cyclosome; ALLM, *N*-acetyl-Leu-Leu-methional; ALLN, *N*-acetyl-Leu-Leu-norleucinal; EST, expressed sequence tag; HsAurora-A, human Aurora-A; MmAurora-B, mouse Aurora-B; SD, synthetic dropout; TBS, Tris-buffered saline; TR, N-terminal truncated.

20q-13.2 (30), which is frequently amplified in several human tumors (29, 34–38). Ectopic overexpression of Aurora-A in near diploid normal breast epithelial cells causes centrosome hyperamplification and aneuploidy (31). Also, overexpression of Aurora-A in rodent cells displayed cellular transforming activity, suggesting that when overexpressed, Aurora-A could function as a potential oncogene (29, 31). The extensive research done on the yeast homolog Ipl1, *Xenopus* homolog Eg2, and *Drosophila aurora* had shed some light into the functional role of Aurora-A kinase in mitosis. Kinesin-related proteins CIN8 (39), Pav (40) and Eg5 (41) were found to interact directly with the Aurora kinase homologs in yeast (Ipl1), *Drosophila* (*aurora*), and *Xenopus* (Eg2), respectively. Also, yeast Ipl1 is also found to interact with the kinetochore protein Ndc10, implying the possible role of Aurora kinase in the establishment of the mitotic checkpoint via monitoring the capture of the chromosome kinetochores by the spindle microtubule (24, 42, 43). Human Aurora-A kinase is shown to interact with Cdc20 (44), which is involved in the mitotic activation of anaphase-promoting complex APC/C (45).

Presently, only very limited knowledge is available on the function(s) of Aurora-A kinase in mammals. Understanding the functions of Aurora-A kinase and delineation of the Aurora-A kinase signaling pathway would definitely help us to have a clearer understanding of the role of the kinase in chromosome segregation and neoplastic transformation. Hence, in an effort to identify any interacting proteins as well as the negative regulators of Aurora-A kinase, a dosage suppressor screen in which HeLa cell cDNAs that can alleviate Aurora-A-mediated cytotoxicity in yeast has been carried out. In this paper, we report the identification of AIP, one such potential negative regulator of Aurora-A kinase.

EXPERIMENTAL PROCEDURES

Yeast Dosage Suppressor Screening—Yeast strain EGY188 (**MATa** *trp1his3ura3leu2*::2 LexAop-LEU2) was maintained in the rich YPD medium. Yeast transformation, plasmid isolation, and protein extracts were prepared as described (46). For cDNA library screening, EGY188 cells were grown to log phase in YPD and cotransformed with plasmids containing 150 μg of Aurora-A cDNA in pEG202 and 150 μg HeLa cell cDNAs in pJG4-5 using the LiOAc method (47). The resulting transformants were selected on galactose containing synthetic dropout media lacking histidine and tryptophan (SD−His−Trp). Yeast clones, which survived the Aurora-A-mediated cytotoxicity, were reconfirmed by streaking onto glucose- and galactose-containing synthetic dropout media, and the clones, which grew only on the galactose-containing plates, were characterized further by sequencing.

Cloning of AIP and Plasmid Constructs—To clone a full-length AIP cDNA, a PCR-based approach was employed. Two primers, GG8 (5′-CGC TGC CGA TCG GGG CCG ACT-3′) and GG10 (5′-ACT ACG GAT CAC AGC AGC AAC-3′), were designed for PCR cloning of AIP from the HeLa cell cDNA library. All Aurora-A and AIP constructs were made in the mammalian expression vector pCDNA3 (Invitrogen). The cyclin B1 expression plasmid pAPuro-CyclinB1 was a kind gift from Dr. Prochownik, Pittsburgh, PA. To trace the transfected AIP, a FLAG epitope was introduced at the N terminus of both truncated AIP-TR (87–600 bp) and full-length AIP (1–600 bp) constructs by PCR as described previously (28).

Northern Blot Analysis—Pre-made blots containing poly(A) RNA isolated from adult human tissues and a human cancer cell line panel were purchased from Clontech and used for hybridization with AIP-specific probe. Blots were hybridized according to Church and Gilbert (48) with a 477-bp AIP 3′-end fragment labeled using a random prime labeling kit. Blots were then stripped and reprobed with β-actin to quantitate RNA loading.

Cell Culture, Transfection, and Drug Treatment—NIH 3T3 and COS cells were maintained in Dulbecco's modified Eagle's medium, and HeLa cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Transfections of cultured cell lines have been carried out using LipofectAMINE (Invitrogen) according to the manufacturer's recommended protocol. Typically, 8×10^5 HeLa cells were seeded in a 60-mm dish 24 h prior to the transfection and transfected with different plasmids at a total concentration of 3 μg. For the *in vivo* interaction assay, equal amounts of either HsAurora-A or MmAurora-B plasmids were cotransfected with different combinations of control or AIP-expressing constructs using the LipofectAMINE PLUS reagent (15 μl of LipofectAMINE and 8 μl of PLUS reagent) for 5 h. Similarly, 7×10^5 NIH 3T3 cells or 1.8×10^6 COS cells were plated in a 60-mm dish 24 h prior to the transfection. For Aurora-A degradation study, COS7 cells were cotransfected with HsAurora-A and FLAG-tagged AIP at different ratios while maintaining the total amount of DNA transfected to 3 μg. The same optimized transfection conditions were used. For immunofluorescence staining, 3×10^5 cells were seeded on the coverslip placed in the 35-mm dish 1 day prior to the transfection. A total of 1 μg of plasmid DNA and 6 μl of LipofectAMINE/6 μl of PLUS reagent were used for transfection. To inhibit 26 S proteasome-mediated protein degradation, COS cells were treated with 20 μM *N*-Cbz-Leu-Leu-Leu-AL (MG132; Sigma), 25 μM ALLM (Calbiochem), 25 μM lactocystin β-lactone (Calbiochem), and 150 μM ALLN (Calbiochem) for 12 h.

Cell Lysis, Immunoprecipitation, and Immunoblotting—The cells were lysed for 15 min on ice in lysis buffer (1× TBS, 10% glycerol, 1% Nonidet P-40) containing protease inhibitors mixture (Roche Molecular Biochemicals). The lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. The protein concentration of the lysates was measured by the Bio-Rad Protein Assay (Pierce). Prior to the immunoprecipitation, 1 mg of lysates was precleared by incubation with 80 μl of 50% slurry of protein G-agarose (Sigma) for 1 h at 4 °C. For antibody coupling to the protein G-agarose, 20 μl of rabbit anti-HsAurora-A serum (44) or 6 μg of FLAG M2 mouse monoclonal antibody (Stratagene) was incubated with 80 μl of 50% slurry of protein G-agarose (Sigma) for 1 h at room temperature. For immunoprecipitation, the precleared lysate and antibody-coupled protein G-agarose were mixed and rotated for 2 h at 4 °C. Immune complexes were washed twice with wash buffer I (1× TBS, 10% glycerol, 0.5% Nonidet P-40, 1% bovine serum albumin) and twice with wash buffer II (1× TBS, 10% glycerol, 0.5% Nonidet P-40). The immune complexes were solubilized by boiling with SDS sample buffer and resolved by SDS-PAGE. The proteins were subsequently transferred to Hybond C+ nylon membrane (Amersham Biosciences). After blocking with 5% nonfat milk in TBS, the blots were incubated with rabbit anti-HsAurora-A (1:1,500) or mouse monoclonal anti IAK1 (Transduction Laboratories) at a dilution of 1:1,000 or FLAG M2 mouse monoclonal antibody (Stratagene) at 1:2,000 overnight at 4 °C. The horseradish peroxidase-conjugated secondary antibodies were also diluted accordingly in blocking buffer (goat anti-rabbit horseradish peroxidase (Bio-Rad), 1:5,000; goat anti-mouse horseradish peroxidase (Pierce), 1:8,000) and incubated with the blot for 1 h at room temperature. The secondary antibodies were detected by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to Kodak Biomax MR film.

Construction of AIP Deletion Mutants—Four AIP deletion mutants were created by PCR-based deletion mutagenesis. A 99-bp and a 198-bp deletion, each separately from the N and C terminus of AIP, were synthesized using four pairs of primers flanking the desired domain. The forward primers were designed to add the 8-amino acid FLAG tag to the N terminus of each mutant protein. The amplified fragments spanning different regions of AIP were cloned into pCDNA3 for expression purposes. The expected sequences of the deletion mutants were confirmed by sequencing.

In vivo interaction and degradation assays were carried out with these AIP mutants as described previously.

Immunofluorescence Staining—Cells grown on coverslips were fixed in −20 °C methanol for 5 min at room temperature. After blocking for 30 min in blocking buffer (1× TBS, 1% bovine serum albumin, 0.1% Triton X-100, 10% goat serum, 0.02% sodium azide), cells were incubated with the primary antibody, mouse anti-FLAG (Stratagene; 1:800), for 1 h at room temperature. The cells were washed thoroughly in 1× TBS and incubated further with the respective secondary antibodies. Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) was used as the secondary antibody. For propidium iodide staining, cells were incubated with 0.05 μg/ml propidium iodide. Cells were analyzed by using a Leica epifluorescence microscope (Bio-Rad) equipped with a multiband filter set and/or confocal microscopy.

RESULTS

Molecular Cloning of AIP—Overexpression of Aurora-A kinase is lethal in yeast (29). By exploiting the lethal phenotype of Aurora-A kinase, we attempted to isolate mammalian proteins that can suppress the lethal phenotype when cotransformed and rescue the yeast from Aurora-A-mediated death.

a.

```
DTGTAGTVAP PQSYQCPPSQ IGEGAEQGDE GVADAPQIQC KNVLKIRRRK  50
MNHHKYRKLV KKTRFLRRKV QEGRLRRKQI KFEKDLRRIW LKAGLKEAPE 100
GWQTPKIYLR GK                                          112
```

b.

```
hAIP    MLLGRLTSQL LRAV.PWAGG RPPWPVSGVL GSRVCGPLYS TSPAGPGRAA   49
rAIP    .......... .......... .......... .SHAFRPLYS LQPASPSRAA   19
mAIP    MFLARLTSRL ARTVVPWAGF SRSCPGSGVI GSYAFRPLYS LQPASPSRAA   50

hAIP    SLPRKGAQLE LEEMLVPRKM SVSPLESWLT ARCFLPRLDT GTAGTVAPPQ   99
rAIP    SLPGKRAQLE LEEFLVPRKM AISPLESWLT VQYLLPRLNV EVPVTLAPSQ   69
mAIP    SLPGKRTQSE LEEFLVPRKM AISPLESWLT AQYLLPRRNV EVPVTLAPSQ  100

hAIP    SYQCPPSQIG EGAEQGDEGV ADAPQIQCKN VLKIRRRKMN HHKYRKLVKK  149
rAIP    FYKCPPSQGE EEAKQGDREV WDATPMQCKN VLKIRRRKMN HHKYRKLIKR  119
mAIP    FYECPPRQGE EEAQQGVREA WDATPVQCKN VLKIRRRKMN HHKYRKLVKR  150

hAIP    TRFLRRKVQE GRLRRKQIKF EKDLRRIWLK AGLKEAPEGW QTPKIYLRGK  199
rAIP    TRFLRRKVRE GRLKRKQIKF EKDLKRIWLK AGLKEAPENW QTPKIYMKNK  169
mAIP    TRFLRRKVRE GRLKKKQIKF EKDLKRIWLK AGLKEAPENW QTPKIYLKNK  200
```

FIG. 1. **AIP amino acid sequence alignment.** *a*, the deduced amino acid sequence of AIP isolated by dosage suppressor screen in yeast is presented. This sequence lacks the 87 amino acids from the N terminus of the full-length AIP protein. The location of the tandem bipartite nuclear localization signal is highlighted with *italics* and *underlining*. *b*, amino acid sequence alignment of human AIP (hAIP) with those of mouse (mAIP) and rat (rAIP). The mouse and rat sequences were derived from the EST data base. The mouse AIP sequence was derived from EST clones AI425574 and AA545527, and the corresponding rat AIP sequence was derived from EST clone AI104388. Identical amino acids in the sequences are presented in *bold face*.

For this purpose, a plasmid construct was made in yeast expression vector pEG202 where constitutive expression of the full-length Aurora-A kinase in yeast is achieved using the alcohol dehydrogenase promoter. Yeast strain EGY188 was cotransformed with this Aurora-A plasmid and a HeLa cell cDNA library in pJG4-5 where cDNAs were expressed under a galactose-inducible *GAL1* promoter. 0.5×10^6 cotransformants were screened, and the resulting positive clones were selected for galactose-dependent reversal of Aurora-A-mediated cell death and characterized further by sequencing. Interestingly, sequence analysis of a total of 141 positive clones revealed that a 477-bp cDNA fragment, which we designate as AIP, containing the 3′-end of the mRNA, was represented 17 times. The authenticity of these 17 clones was verified by the galactose-dependent rescue from Aurora-A-mediated cell death. The high frequency (12% of the total) and the reproducible rescue from the Aurora-A- mediated lethality by AIP led us to characterize this cDNA fragment further. The predicted translation product of the cDNA fragment isolated by the suppressor screen in yeast is presented in Fig. 1*a*. Comparison of the protein and nucleotide sequence of AIP with the sequences in the GenBank data base revealed that it is identical to the sequence corresponding to an uncharacterized protein with the accession number AK000615, a sequence submitted to the data base as a part of the human genome sequence project. Sequences similar to human AIP were found in mouse and rat EST data bases also. Fig. 1*b* compares the deduced amino acid sequence of AIP with the homologous sequences available in the data bases. Human AIP shares 72 and 73% identity at the amino acid level over its entire length with mouse and rat AIP, respectively. However, AIP-related sequences were not found in the lower eukaryotic genomes such as yeast, *Drosophila*, and *C. elegans*. Based on the above information, we concluded that AIP is a novel gene and cloned the putative full-length AIP cDNA by 5′-rapid amplification of cDNA ends. The full-length AIP cDNA contains a 597-bp open reading frame that encodes a 199-amino acid polypeptide with a predicted molecular mass of 22 kDa. RNA blot analysis of human tissues and cancer cell lines indicated that AIP is ubiquitously expressed in a wide variety of tissues, especially high in heart, skeletal muscles, and testis (Fig. 2*a*). Computer-assisted search for the motifs presented in AIP protein found a tandem bipartite nuclear localization signal, suggesting AIP could be a nuclear protein. Indeed, ectopically expressed FLAG epitope-tagged AIP was localized to the nuclear compartment of the cell (Fig. 2*b*).

AIP Interacts with HsAurora-A in Vivo—The dosage suppressor screen employed here to isolate AIP is capable of identifying both the direct and indirect regulator(s) of Aurora-A kinase. Preliminary information that AIP might interact directly with Aurora-A kinase came from the yeast two-hybrid *in vivo* interaction assay where the partial AIP cDNA interacted with Aurora-A to activate the *LEU* reporter in yeast (data not shown). To verify whether a similar interaction between AIP and Aurora-A occurs in mammalian cell context, we overexpressed the FLAG-tagged AIP cDNA into HeLa cells and attempted to coimmunoprecipitate the Aurora-A with the transfected AIP protein. The results presented in Fig. 3 indicate that AIP associates with Aurora-A *in vivo* and that AIP can be coimmunoprecipitated with Aurora-A, and conversely, Aurora-A can be coimmunoprecipitated with FLAG-tagged AIP using FLAG antibody. However, it is noted that the interaction of transfected AIP with the endogenous Aurora-A *in vivo* was difficult to demonstrate. We presumed that the difficulty in demonstrating the coimmunoprecipitation could be caused by the lower amounts of Aurora-A available in AIP-transfected cells. The result presented in Fig. 3*a* is the best that is achieved under the given experimental conditions. To explore the interaction further, HeLa cells that are otherwise contained in comparatively lower levels of Aurora-A protein (data not shown) were transfected with HsAurora-A together with FLAG-tagged AIP, and coimmunoprecipitation followed by Western blot analysis were carried out. The results presented in Fig. 3*b* demonstrate that the transfected AIP protein and Aurora-A protein can be coimmunoprecipitated independently of whether

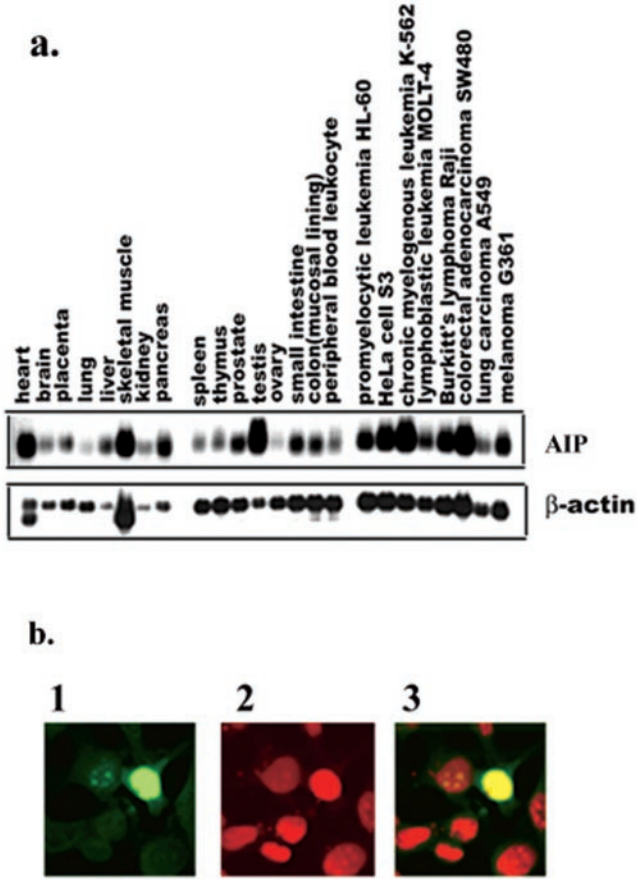


FIG. 2. **AIP mRNA expression and nuclear localization.** *a*, Northern blot analysis of AIP mRNA in adult human tissues and cancer cell lines was carried out with pre-made Northern blots purchased from Clontech. The blots were hybridized with the 477-bp AIP cDNA derived from the yeast dosage suppressor screen. The blot was stripped and reprobed with β-actin. *b*, HeLa cells were transiently transfected with a FLAG epitope-tagged AIP cDNA, and the subcellular localization of the transfected AIP protein (*panel 1*) was detected by staining with FLAG M2 monoclonal antibody (Stratagene) followed by confocal microscopy. Counterstaining of DNA was carried out with propidium iodide (*panel 2*). *Panel 3* represents the merged image to show the nuclear localization of the transfected AIP protein.

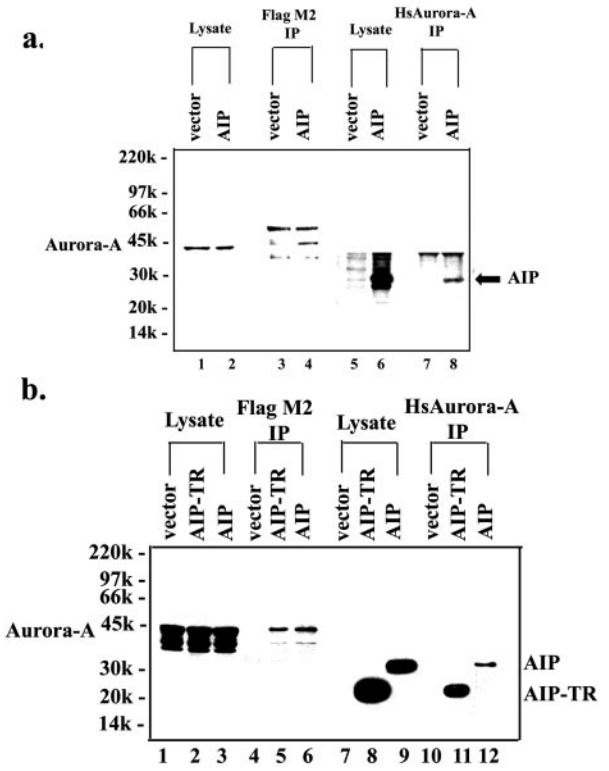


FIG. 3. **AIP interacts with HsAurora-A kinase *in vivo*.** *a*, HeLa cells were transfected with empty vector or FLAG-tagged AIP as described under "Experimental Procedures." Cell lysates equivalent to 1 mg of protein were used for immunoprecipitation (*IP*) with rabbit antiserum against HsAurora-A protein as well as mouse FLAG M2 monoclonal antibody. The cell lysates and the corresponding immunoprecipitates were separated by PAGE and blotted onto nitrocellulose filters. The blots were probed reciprocally with HsAurora-A (*lanes 1–4*) and FLAG M2 (*lanes 5–8*) antibodies. *b*, HeLa cells were transfected with HsAurora-A and FLAG-tagged AIP constructs at a 1:1 ratio as described before. Cell lysates equivalent to 1 mg of protein were used for immunoprecipitation with rabbit antiserum against HsAurora-A as well as FLAG M2 monoclonal antibody. The immunoprecipitates and the corresponding lysates were separated by SDS-PAGE and blotted onto nitrocellulose filters. The blots were probed reciprocally with HsAurora-A (*lanes 1–6*) antibodies and FLAG M2 (*lanes 7–12*).

FLAG M2- or Aurora-A-specific antibodies were used.

Overexpression of AIP Down-regulates Aurora-A Protein—Because AIP has been isolated as the negative regulator of Aurora-A kinase, we presumed that direct interaction of AIP with Aurora-A kinase should result in the down-regulation of either the stability and/or activity of Aurora-A kinase. In an attempt to study the impact of AIP-Aurora-A interaction on the Aurora-A protein, the levels of Aurora-A protein in AIP-transfected cells were investigated. Initial attempts to study the effect of AIP overexpression on the levels of Aurora-A protein were unsuccessful because of lower transfection efficiency, which was not sufficient to demonstrate the effect of AIP overexpression on endogenous Aurora-A protein. Hence, dividing NIH 3T3 or COS cells were cotransfected with FLAG-tagged AIP and HsAurora-A expression constructs at different ratios, and the levels of HsAurora-A protein were followed by Western blot analysis. A human Aurora-A-specific peptide antiserum was used to detect the transfected human Aurora-A in the background of the endogenous mouse and monkey Aurora-A protein. Ectopic expression of Aurora-A protein in human or monkey cell lines resulted in multiple Aurora-A-specific bands. These protein bands were verified to be Aurora-A-specific by Western blot analysis with two different Aurora-A-specific antibodies (data not shown). In COS cells, ectopic expression of Aurora-A results in two Aurora-A-specific bands of which the top band comigrated with the 46,000 endogenous Aurora-A protein from HeLa cells (data not shown). The nature of these other fragment(s), at present, is not clear. However, the results presented demonstrate that AIP, when overexpressed, could down-regulate the Aurora-A protein-specific bands in both a dose-dependent (Fig. 4*a*) and time-dependent manner (Fig. 4*b*). Both full-length AIP as well as the N-terminal truncated form of AIP (AIP-TR) were able to down-regulate Aurora-A protein (data not shown) although the truncated AIP was more efficient in that it could completely deplete the ectopic expressed Aurora-A protein in COS cells (Fig. 4, *a* and *b*).

AIP Interaction with Aurora-A Is Important for the Down-regulation of Aurora-A—To address the question of whether the interaction between AIP and Aurora-A is a necessary step for the down-regulation of Aurora-A, we attempted to isolate a deletion mutant of AIP protein, which does not interact with Aurora-A protein. A total of four deletion mutants lacking regions from either the N or C terminus of AIP protein were constructed and used for Aurora-A interaction studies as described previously. The size and location of these deletions in the different deletion mutants in relation to the wild type AIP protein are given in Fig. 5*a*. Expression of these deletion mutants in HeLa as well as COS cells showed that these mutant proteins have comparable stability (data not shown) except the

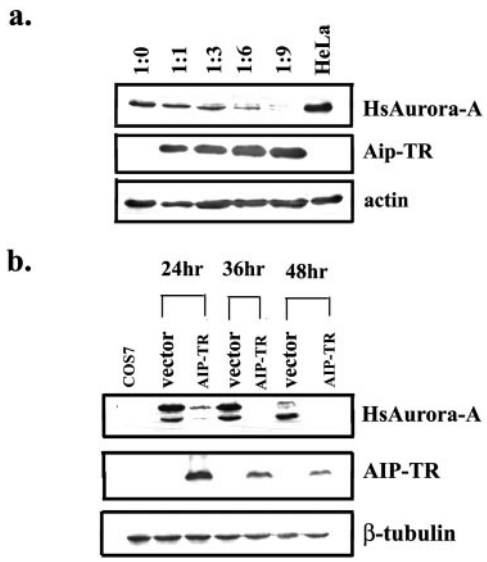


FIG. 4. **AIP down-regulates HsAurora-A level *in vivo*.** *a*, NIH 3T3 cells were transfected with HsAurora-A and FLAG-tagged, truncated AIP construct at different ratios starting from 1:0 to 1:9, respectively, for 36 h. Cell extracts were prepared, and HsAurora-A and AIP proteins were detected with the corresponding antibodies described before. The blot was reprobed with goat anti-actin to verify loading. HeLa cell extract was used as the positive control on the Western blot. *b*, COS cells were transfected with HsAurora-A and FLAG-tagged, truncated AIP constructs at a 1:9 ratio, respectively, for different time points until 48 h, and the cell extracts were analyzed for HsAurora-A and AIP proteins using monoclonal IAK1 and FLAG M2 antibodies. Extract from COS7 cells was used as the negative control. The blot was reprobed with mouse anti β-tubulin to verify loading.

mutant ΔC198-AIP, which showed lower stability. This mutant lacks the bipartite nuclear localization signal, and it remains to be shown whether the lack of nuclear localization signal is responsible for the lower stability and function of this mutant. However, all of the deletion mutants can be expressed successfully in both HeLa and COS cells. To compensate for the lower stability of the ΔC198-AIP mutant, a higher ratio of ΔC198-AIP to Aurora-A (9:1) was used instead of the usual 1:1 in the interaction assay without compromising the levels of Aurora-A protein. Under these conditions, the expression of ΔC198-AIP protein was comparable with that of other mutant AIP proteins. To rule out further the possibility that the lower levels of ΔC198-AIP protein in the lysate are responsible for the absence of detectable protein in the interaction assay, the coimmunoprecipitation was carried out from 1 mg as well as 2 mg of ΔC198-AIP protein lysates. The data presented in Fig. 5*b* suggest that the mutants ΔN99-AIP, ΔN198-AIP, and ΔC99-AIP can interact with Aurora-A protein efficiently (*lanes 7–9*), like the wild type protein (Fig. 3*b*). However, the C-terminal deletion mutant ΔC198-AIP did not show any interaction with Aurora-A protein irrespective of the levels of the mutant protein (*lanes 10* and *11*). This suggests that amino acids 127–166 of AIP contain elements that are necessary for the interaction with Aurora-A protein. To investigate further the efficacy of the noninteracting ΔC198-AIP mutant in degrading Aurora-A protein, an *in vivo* degradation assay as described previously (Fig. 4) was performed with the wild type AIP as well as the AIP mutants. The results presented in Fig. 5*c* demonstrate that the noninteracting ΔC198-AIP mutant was less efficient in degrading Aurora-A protein compared with the wild type and other deletion mutants. This suggests that the AIP/Aurora-A interaction is important for the degradation of Aurora-A protein.

AIP Specifically Down-regulates Aurora-A—To verify the specificity of the effect of AIP overexpression on the down-regulation of Aurora-A protein, the effect of AIP overexpression on MmAurora-B, another member of the Aurora kinase family, as well as cyclin B1 was investigated. The rationale for selecting cyclin B1 is that, like Aurora-A protein, the proteasome-dependent pathway (50, 51) also degrades it. COS cells were transfected with FLAG-tagged AIP-TR together with either MmAurora-B or human cyclin B1 at ratio of 9:1, respectively, and the effects of AIP-TR overexpression on the levels of these proteins were analyzed. The data presented in Fig. 6, *a* and *b*, indicate that the overexpression of AIP-TR does not affect the down-regulation of either MmAurora-B or human cyclin B1 and support the notion that AIP down-regulates Aurora-A protein specifically. Also, the failure to down-regulate cyclin B1 suggests that the effect of AIP is not mediated by the generalized activation of the proteolytic machinery.

Proteasome Inhibitors Reverse the AIP-mediated Down-regulation of Aurora-A—It has been shown the proteasome plays a major role in the regulation of Aurora-A stability (50). Hence, it is possible that the effect of AIP overexpression on the down-regulation of Aurora-A could be mediated through the potentiation of proteasome-dependent degradation of Aurora-A. To address this question, COS cells were transfected with FLAG-tagged AIP-TR together with empty vector or HsAurora-A expression constructs, and the effect of AIP overexpression on the down-regulation of Aurora-A was followed in the presence and absence of proteasome inhibitors such as MG132, ALLN, and clasto-lactacystin β-lactone. As shown in Fig. 7, proteasome inhibitors could reverse the AIP-mediated down-regulation of Aurora-A protein to different levels depending on their potencies to inhibit the proteasome machinery. Calpain inhibitor ALLM could not reverse the AIP-mediated degradation of Aurora-A protein, suggesting that the cysteine protease calpain is unlikely to play a role in the AIP-mediated down-regulation of Aurora-A. Taken together, these results indicate that the proteasome plays a major role in AIP-mediated down-regulation of Aurora-A protein.

DISCUSSION

Aurora-A kinase is a member of a serine/threonine kinase family implicated in equal segregation of chromosomes between daughter cells. Aurora-A kinase is suggested to play a role also in tumorigenesis (29). Overexpression of Aurora-A kinase transforms cultured rodent cells and causes aneuploidy in near diploid mammary epithelial cells (31). Regulation of Aurora-A kinase expression and activity occurs at multiple levels such as gene amplification, transcription, phosphorylation, and degradation through the proteasome-dependent pathway (29, 31, 50). Currently, attempts are being made to understand the functions of Aurora-A kinase at the molecular level. In this paper, using a dosage suppressor screen in yeast, we have isolated and investigated AIP, a novel negative regulator of Aurora-A kinase. We have shown that AIP interacts specifically and down-regulates Aurora-A kinase by potentiating its degradation through the proteasome-dependent pathway. We demonstrated that both the full-length and N-terminal truncated AIP could interact with Aurora-A kinase, suggesting that the C-terminal portion of AIP alone is sufficient for the interaction. However, the interaction of endogenous Aurora-A kinase with AIP in cells overexpressing AIP was difficult to demonstrate probably because of the degradation of endogenous protein by AIP. This inference was supported by the observation that when Aurora-A protein levels were increased by the coexpression of Aurora-A and AIP, AIP and Aurora-A kinase can be coimmunoprecipitated readily (Fig. 3, *a* and *b*). Similarly, both full-length and truncated AIP were effective in the down-regulation of Aurora-A kinase (data not shown).

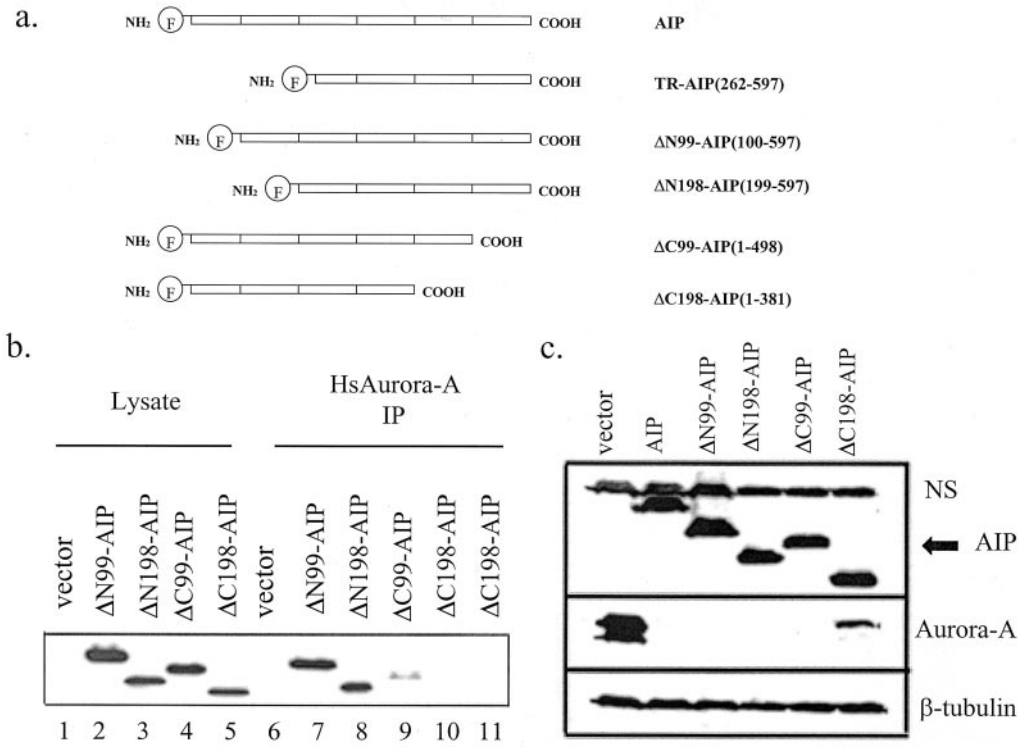


FIG. 5. **AIP/Aurora-A interaction is important for the degradation of Aurora-A.** *a*, comparison of the size and location of the deletions of all the AIP mutant proteins with full-length AIP protein. All of the AIP variants contain a FLAG tag at the N terminus. The *numbers* within *parentheses* denote the nucleotides of AIP cDNA, and number *1* corresponds to the nucleotide A of the translational start ATG. *b*, HeLa cells were transfected with Aurora-A and FLAG-tagged AIP mutant constructs at 1:1 ratio, respectively, as described before, except that the ΔC198-AIP was cotransfected at a higher ratio of 1:9. For all samples, cell lysates equivalent to 1 mg of protein were used for immunoprecipitation (*IP*) with rabbit antiserum against human Aurora-A. In the case of ΔC198-AIP, coimmunoprecipitation was carried out from 1 mg as well as 2 mg of protein lysates. The immunoprecipitates and the corresponding lysates were separated by SDS-PAGE and blotted onto nitrocellulose filters. The blots were probed FLAG M2 antibody to detect the AIP proteins. *c*, COS cells were cotransfected with HsAurora-A and either pCDNA3 or any of the AIP mutant constructs at a 1:9 ratio, respectively, and the effect of overexpression of the AIP proteins on the degradation of Aurora-A protein was assessed at 36 h after transfection as described previously. Cell extracts were analyzed for HsAurora-A and AIP proteins using monoclonal IAK1 and FLAG M2 antibodies, respectively. The blot was reprobed with mouse anti β-tubulin to verify loading.

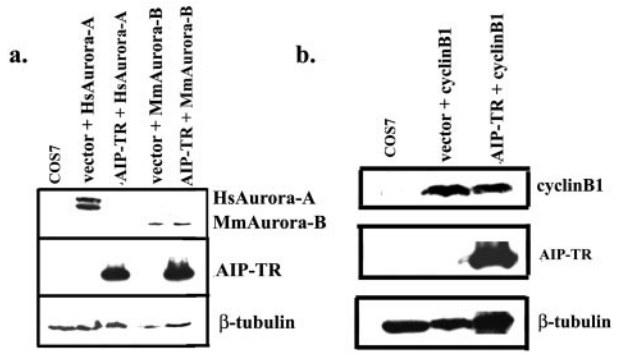


FIG. 6. **Overexpression of AIP does not down-regulate either MmAurora-B or cyclin B1.** HsAurora-A or MmAurora-B or human cyclin B1 was cotransfected with FLAG-tagged AIP-TR expression construct at a ratio of 1:9, respectively, into COS cells, and the effect of overexpression of AIP-TR on the levels of HsAurora-A, MmAurora-B, and cyclin B1 was assessed. AIP-TR, HsAurora-A, and MmAurora-B (*a*) and AIP-TR and cyclin B1 (*b*) levels were analyzed by Western blot analysis using the respective antibodies. Extracts from COS7 cells were used as the negative control. The blot was reprobed with mouse anti-β-tubulin to verify loading.

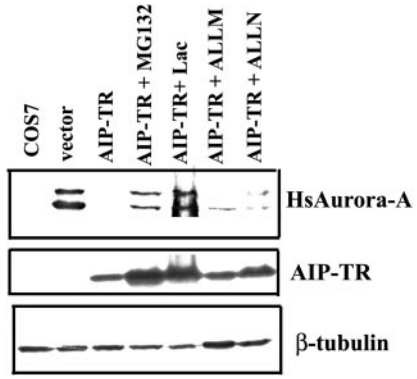


FIG. 7. **AIP degrades HsAurora-A through a proteasome-dependent pathway.** COS cells were transfected with HsAurora-A in combination with empty vector as well as FLAG-tagged, truncated AIP. Cotransfection with AIP construct was carried out in five sets. Four sets were treated with the proteasome inhibitors MG132 (20 μM) ALLN (150 μM), lactocystin β-lactone (25 μM), and the calpain inhibitor ALLM (25 μM), and the last set was treated with the vehicle dimethyl sulfoxide for 12 h. 36 h after transfection, cell lysates were prepared and analyzed for HsAurora-A and AIP protein by Western blotting. The blot was reprobed with mouse anti β-tubulin to verify equal loading.

However, the truncated AIP was more efficient in the down-regulation probably because of either the higher levels of the truncated protein accumulated inside the cell or the better binding to Aurora-A kinase. The increased level of truncated AIP was evident from all of our Western blot analysis that the cells accumulated more of the truncated AIP than the full-length AIP (Fig 3*b*). Analysis of the mutant AIP proteins for the interaction/degradation of Aurora-A protein demonstrated that the mutant ΔC198-AIP lacks the elements

essential for the AIP/Aurora-A interaction, and the interaction is important for the degradation of Aurora-A kinase (Fig. 5, *a* and *b*). Interestingly, this mutant lacks the nuclear localization signal, suggesting that the targeting of AIP to the nucleus may be necessary for the interaction and degradation of Aurora-A kinase.

We have shown that AIP down-regulates Aurora-A kinase possibly through proteasome-dependent degradation. AIP is not unique in that there are other examples of proteins involved in instigating the degradation of cell cycle-related interacting partners through the proteasome pathway. Jab1 has been shown to promote the degradation of the cell cycle regulator p27kip1 in a proteasome-dependent manner (52). However, the exact role of Jab1 in the degradation is still unclear. The WD repeat-containing protein cdc20 interacts with and targets the budding yeast anaphase regulator Pds1 (securin) for degradation through the APC/C (53). Similarly, it has been well documented that MDM2 can facilitate the degradation of p53 (54). In this case, it is evident now that MDM2 itself can act as the ubiquitin ligase facilitating the ubiquitination of p53 (55). The observation that AIP also could destabilize Aurora-A kinase specifically through 26 S proteasome raises an interesting question as to what proteasome-targeting mechanism is employed for AIP-mediated Aurora-A degradation. AIP sequence analyses do not reveal any similarity to either F box proteins (56) or U box proteins (57), which play crucial roles in targeting and ubiquitination, respectively. On the other hand, the failure of AIP-dependent cyclin B1 degradation in cells overexpressing AIP confirms the notion that AIP does not activate the 26 S proteasome machinery in a generic way. The specific interaction of AIP with Aurora-A kinase as well as the essential nature of the AIP/Aurora-A interaction for the degradation of Aurora-A supports interesting possibilities such as AIP directly modifying and/or targeting Aurora-A kinase for destabilization or AIP/Aurora-A kinase interaction being the key rate-limiting step in the Aurora-A kinase degradation pathway. Although it is known that Aurora-A kinase is polyubiquitnated before degradation by APC/cyclosome (50), it still remains to be shown that AIP plays a role in the ubiquitination of Aurora-A kinase. It has been shown that cdc20/p55cdc, which is capable of activating APC, interacts with human Aurora-A (44). However, the question of whether cdc20 targets Aurora-A for degradation also remains to be answered.

Apart from its role in destabilization of Aurora-A kinase when overexpressed, the normal function of AIP is yet to be established. The results obtained so far point to the fact that AIP is a ubiquitously expressed nuclear protein. It will be interesting to investigate whether AIP is the normal physiological trigger for Aurora-A degradation. Other pertinent questions to be answered will include the subcellular location where interaction between AIP and Aurora-A occurs and at which stage of cell cycle AIP-mediated Aurora-A degradation occurs. Being a negative regulator of Aurora-A, a potential oncogene, there is a possibility that down-regulation of AIP could play a major role in tumorigenesis. Currently, experiments are being carried out to address these issues.

In summary, the findings reported in this paper identify a novel negative regulator of Aurora-A kinase. Understanding the normal function of AIP as well as the characterization of the molecular mechanisms involved in the AIP-mediated destabilization of Aurora-A will be the next chapter in this investigation. Moreover, the targeted degradation of Aurora-A by AIP provides us with the handle to manipulate the endogenous level of the oncogenic Aurora-A kinase. Hence, AIP could therefore be a potential target gene for anti-cancer drugs in the future.

Acknowledgment—We thank Dr. Prochownik for the cyclin B1 expression plasmid.

REFERENCES

1. Verde, F., Labbe, J. C., Doree, M., and Karsenti, E. (1990) *Nature* **343,** 233–238
2. Vandre, D. D., Davis, F. M., Rao, P. N., and Borisy, G. G. (1986) *Eur. J. Cell Biol.* **41,** 72–81
3. Vandre, D. D., Davis, F. M., Rao, P. N., and Borisy, G. G. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 4439–4443
4. Nigg, E. A., Blangy, A., and Lane, H. (1996) *Exp. Cell Res.* **229,** 174–180
5. Centonze, V. E., and Borisy, G. G. (1990) *J. Cell Sci.* **95,** 405–411
6. Vandre, D. D., and Borisy, G. G. (1989) *J. Cell Sci.* **94,** 245–258
7. Nigg, E. A. (2001) *Ernst Schering Res. Found. Workshop* **34,** 19–46
8. Hoffmann, I., Clarke, P. R., Marcote, M. J., Karsenti, E., and Draetta, G. (1993) *EMBO J.* **12,** 53–63
9. Nigg, E. A. (2001) *Mol. Cell. Biol.* **2,** 21–31
10. Morgan, D. O. (1995) *Nature* **274,** 131–134
11. Nigg, E. A. (1995) *BioEssays* **17,** 471–480
12. Andersen, S. S. (1999) *BioEssays* **21,** 53–60
13. Donaldson, M. M., Tavares, A. A., Hagan, I. M., Nigg, E. A., and Glover, D. M. (2001) *J. Cell Sci.* **114,** 2357–2358
14. Nigg, E. A. (1998) *Curr. Opin. Cell Biol.* **10,** 776–783
15. Glover, D. M., Hagan, I. M., and Tavares, A. A. (1998) *Genes Dev.* **12,** 3777–3787
16. Straight, A. F. (1997) *Curr. Biol.* **7,** R613–R616
17. Mayor, T., Meraldi, P., Stierhof, Y. D., Nigg, E. A., and Fry, A. M. (1999) *FEBS Lett.* **45,** 292–295
18. Morgan, D. O. (1999) *Nat. Cell Biol.* **1,** E47–E53
19. Kotani, S., Tanaka, H., Yasuda, H., and Todokoro, K. (1999) *J. Cell Biol.* **146,** 791–800
20. Giet, R., and Prigent, C. (1999) *J. Cell Sci.* **112,** 3591–3601
21. Bischoff, J. R., and Plowman, G. D. (1999) *Trends Cell Biol.* **9,** 454–459
22. Chan, C. S., and Botstein, D. (1993) *Genetics* **135,** 677–691
23. Tung, H. Y., Wang, W., and Chan, C. S. (1995) *Mol. Cell. Biol.* **15,** 6064–6074
24. Biggins, S., Severin, F. F., Bhalla, N., Sassoon, I., Hyman, A. A., and Murray, A. W. (1999) *Genes Dev.* **13,** 532–544
25. Glover, D. M., Leibowitz, M. H., McLean, D. A., and Parry, H. (1995) *Cell* **81,** 95–105
26. Niwa, H., Abe, K., Kunisada, T., and Yamamura, K. (1996) *Gene* (*Amst.*) **169,** 197–201
27. Kimura, M., Kotani, S., Hattori, T., Sumi, N., Yoshioka, T., Todokoro, K., and Okano, Y. (1997) *J. Biol. Chem.* **272,** 13766–13771
28. Gopalan, G., Chan, C. S. M., and Donovan, P. J. (1997) *J. Cell Biol.* **138,** 643–656
29. Bischoff, J. R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., Chan, C. S., Novotny, M., Slamon, D. J., and Plowman, G. D. (1998) *EMBO J.* **17,** 3052–3065
30. Shindo, M., Nakano, H., Kuroyanagi, H., Shirasawa, T., Mihara, M., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., Yagita, H., and Okumura, K. (1998) *Biochem. Biophys. Res. Commun.* **244,** 285–292
31. Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. (1998) *Nat. Genet.* **20,** 189–193
32. Terada, Y., Tatsuka, M., Suzuki, F., Yasuda, Y., Fujita, S., and Otsu, M. (1998) *EMBO J.* **17,** 667–676
33. Kimura, M., Matsuda, Y., Yoshioka, T., and Okano, Y. (1999) *J. Biol. Chem.* **274,** 7334–7340
34. Tanner, M. M., Tirkkonen, M., Kallioniemi, A., Collins, C., Stokke, T., Karhu, R., Kowbel, D., Shadravan, F., Hintz, M., and Kuo, W. L (1994) *Cancer Res.* **54,** 4257–4260
35. Kallioniemi, A., Kallioniemi, O. P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H. S., Pinkel, D., Gray, J. W., and Waldman, F. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2156–2160
36. De Angelis, P. M., Clausen, O. P., Schjolberg, A., and Stokke, T. (1999) *Br. J. Cancer.* **80,** 526–535
37. Courjal, F., Cuny, M., Rodriguez, C., Louason, G., Speiser, P., Katsaros, D., Tanner, M. M., Zeillinger, R., and Theillet, C. (1996) *Br. J. Cancer* **74,** 1984–1989
38. Sakakura, C., Hagiwara, A., Yasuoka, R., Fujita, Y., Nakanishi, M., Masuda, K., Shimomura, K., Nakamura, Y., Inazawa, J., Abe, T., and Yamagishi, H. (2001) *Br. J. Cancer* **84,** 824–831
39. Geiser, J. R., Schott, E. J., Kingsbury, T. J., Cole, N. B., Totis, L. J., Bhattacharyya, G., He, L., and Hoyt, M. A. (1997) *Mol. Biol. Cell* **8,** 1035–1050
40. Adams, R. R., Tavares, A. A., Salzberg, A., Bellen, H. J., and Glover, D. M. (1998) *Genes Dev.* **12,** 1483–1494
41. Giet, R., Uzbekov, R., Cubizolles, F., Le Guellec, K., and Prigent, C. (1999) *J. Biol. Chem.* **274,** 15005–15013
42. Francisco, L., Wang, W., and Chan, C. S. (1994) *Mol. Cell. Biol.* **14,** 4731–4740
43. Sassoon, I., Severin, F. F., Andrews, P. D., Taba, M. R., Kaplan, K. B., Ashford, A. J., Stark, M. J., Sorger, P. K., and Hyman, A. A. (1999) *Genes Dev.* **13,** 545–555
44. Farruggio, D. C., Townsley, F. M., and Ruderman, J. V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7306–7311
45. Fang, G., Yu, H., and Kirschner, M. W. (1998) *Mol. Cell* **2,** 163–171
46. Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L., Wang, H. G., and Reed, J. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9238–9242
47. Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet.* **16,** 339–346
48. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1991–1995
49. Tatsuka, M., Katayama, H., Ota, T., Tanaka, T., Odashima, S., Suzuki, F., and Terada, Y. (1998) *Cancer Res.* **58,** 4811–4816
50. Honda, K., Mihara, H., Kato, Y., Yamaguchi, A., Tanaka, H., Yasuda, H.,

Furukawa, K., and Urano, T. (2000) *Oncogene* **19,** 2812–2819
51. Goltzer, M., Murray, A. W., and Kirschner, M. W. (1991) *Nature* **349,** 132–138
52. Tomoda, K., Kubota, Y., and Kato, J. (1999) *Nature* **398,** 160–165
53. Hilioti, Z., Chung, Y. S., Mochizuki, Y., Hardy, C. F., and Cohen-Fix, O. (2001). *Curr. Biol.* 4, 1347–1352
54. Inoue, T., Geyer, R. K., Howard, D., Yu, Z. K., and Maki, C. G. (2001) *J. Biol. Chem.* **276,** 45255–45260
55. Issacs, J. S., Saito, S., and Neckers, L. M. (2001) *J. Biol. Chem.* **276,** 18497–18506
56. Craig, K. L., and Tyers, M. (1999) *Prog. Biophys. Mol. Biol.* **72,** 299–328
57. Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K. (2001) *J. Biol. Chem.* **276,** 33111–33120

Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

AURORA-A KINASE INTERACTING PROTEIN 1 (AURKAIP1) PROMOTES AURORA-A DEGRADATION THROUGH ALTERNATIVE UBIQUITIN-INDEPENDENT PATHWAY

Shen Kiat Lim and Ganesan Gopalan*
Laboratory of Gene Structure and Expression, Division of Cellular and Molecular Research
National Cancer Centre, Singapore 169610.

Running Title: Ubiquitin-independent degradation of Aurora-A

Key words: cancer, oncogene, degradation, proteasome, ubiquitin-independent.

Word count: 7407

* To whom correspondence should be addressed: Ganesan Gopalan, Laboratory of Gene Structure and Expression, Division of Cellular and Molecular Research, #03-03 Singhealth Research Facilities, Block A, 7 Hospital Drive, Singapore 169611. Phone (65) 63214075; Fax: (65) 63213606; Email: cmrgop@nccs.com.sg

Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

ABSTRACT:

Mitotic Aurora-A is an oncogene, which undergoes cell cycle-dependent regulation of both synthesis and degradation. Overexpression of Aurora-A leads to aneuploidy and cellular transformation in cultured cells. It has been shown that the cell cycle dependent turnover of Aurora-A is mediated by cdh1 through the APC/C[1]-ubiquitin-proteasome pathway. We have described earlier the identification of an Aurora-A kinase interacting protein, AURKAIP1 (formerly described as AIP), which is also involved in the destabilization of Aurora-A through proteasome-dependent degradation pathway. In an attempt to investigate the mechanism of AURKAIP1-mediated Aurora-A degradation, we report here that AURKAIP1 targets Aurora-A for degradation through proteasome-dependent but ubiquitin-independent manner. AURKAIP1 inhibits polyubiquitination of Aurora-A. A non-interactive AURKAIP1 mutant that cannot destabilize Aurora-A restores ubiquitination of Aurora-A. An A-box mutant of Aurora-A, which cannot be targeted for proteasome-dependent degradation by cdh1, can still be degraded by AURKAIP1. Inhibition of cellular ubiquitination either by expression of dominant negative ubiquitin mutants or by studies in ts-20 CHO cell line lacking the E1 ubiquitin activating enzyme at the restrictive temperature, cannot abolish AURKAIP1-mediated degradation of Aurora-A. AURKAIP1 specifically decreases the stability of Aurora-A in ts-20 CHO cells at the restrictive temperature, while cyclinB1 and p21 are not affected. This demonstrates that there exists an Ub-independent alternative pathway for Aurora-A degradation and AURKAIP1 promotes Aurora-A degradation through this Ub-independent yet proteasome-dependent pathway.

Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

INTRODUCTION

Protein degradation plays an essential role of in the regulation of many cellular physiological processes, particularly in cell cycle control, where cell cycle proteins are periodically expressed. Aberrant protein degradation could lead to uncontrolled cell cycle and cancers. The ubiquitin-proteasome system has evolved as the key machinery in the selective degradation of most intracellular short-lived regulatory or abnormal proteins [1-4]. Target proteins are covalently tagged with multiple ubiquitins, forming the polyubiquitin chain, which not only serves as the recognition signal for the 26S proteasome, but also assists in the unfolding of target proteins. Ubiquitination requires the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3), where E3 confers the substrate specificity. The Ub-dependent degradation pathway is presumed to be involved in the degradation of most of the proteins. However, some of the proteins can also be degraded in the absence of detectable prior ubquitination either directly by 20S proteasome or by 26S proteasome in the presence of ATP (5).

Aurora-A represents one of the many mitotic proteins, whose protein levels are temporally regulated by the Ub-dependent proteolysis at the end of mitosis before the cells progress into the G1 phase of next cell cycle. Aurora-A is ubiquitinated by the Cdh1-activated APC/C, an E3 ubiquitin ligase through the recognition of C-terminal destruction box (D-box) and N-terminal A-Box. Dephosphorylation of the highly conserved serine 51 in A-box during mitotic exit could control the timing of Aurora-A degradation [6-8]. Regulation of Aurora-A degradation is very important as ectopic expression of Aurora-A in human and rodent cells induces centrosome amplification, aneuploidy, transformed phenotype and tumor formation in nude mice [9,10]. Aurora-A is overexpressed in many cancer types and mapped to chromosome 20q13 region, frequently amplified in many human cancers [11-13]. Overexpression of Aurora-A significantly correlates with induction of aneuploidy, centrosome anomaly, poor prognosis and invasiveness of the primary human tumours and of experimental tumours in animal model systems [14,15].

Previously, in our attempt to understand the negative regulation of Aurora-A, we have isolated a novel direct negative regulator of Aurora-A, named as Aurora-A Kinase Interacting Protein 1 (AURKAIP1) [16]. AURKAIP1 targets Aurora-A for degradation in a proteasome-dependent manner. AURKAIP1-Aurora-A interaction is necessary for AURKAIP1-mediated Aurora-A degradation. The exact mechanism of AURKAIP1-mediated Aurora-A degradation is unclear. Presently, we try to explore the mechanism of Aurora-A degradation in the AURKAIP1-regulated

pathway. The results presented here demonstrate that AURKAIP1 facilitates proteasome-dependent degradation of Aurora-A by an alternative mechanism that is independent of ubiquitination. This implies that Aurora-A can be delivered to the proteasome via two distinct Ub-dependent and Ub-independent pathways.

Materials and Methods

Plasmids and Cloning

HA-tagged p21 expression construct is a gift from Dr. Michele Pagano; His-tagged wild type ubiquitin and HA-tagged K48R mutant ubiquitin expression construct is a gift from Dr. Ivan Dikic. Addition of a histidine tag to the K48R mutant ubiquitin was carried out by PCR and cloned into pCDNA3 (Invitrogen); His-tagged wild type ubiquitin plasmid was used as the template to generate the K48R/K63R double mutant using the Gene Editor in vitro Site-directed mutagenesis system (Promega); FLAG-, HA-tagged human Aurora-A were PCR amplified and cloned into pCDNA3; the A-box mutant (S51D) of Aurora-A mutant in pCDNA3 was also generated using the Gene Editor in vitro Site-Directed Mutagenesis System (Promega); HA-, FLAG-tagged human AURKAIP1 and TR-AURKAIP1 were PCR amplified and cloned into pCDNA3. All cloned sequences were verified by sequencing.

Antibodies

Mouse monoclonal anti-FLAG M2 antibody (Stratagene), 1:2000; rabbit polyclonal anti-FLAG (Sigma), 1: 2000; mouse monoclonal anti-β tubulin antibody (Sigma), 1:1000; mouse monoclonal anti-HA-tag (Sigma), 1:2000; mouse monoclonal anti-IAK1 (BD Transduction), 1:1000; rabbit polyclonal anti-Cyclin B1 antibody (Santa Cruz), 1:3000 and mouse monoclonal anti-His-tag antibody (Sigma), 1:1000 were used. All HRP-conjugated secondary antibodies (Pierce) were used at 1:6000 to 1:8000 dilutions.

Cell Culture, Transfection and Drug Treatment

- ts20TG mouse cells were obtained from Dr. Harvey Ozer, New Jersey, USA.
- ts20-CHO cells were obtained from Dr. Ger J Strous, Utrecht, Netherlands.

ts20-TG mouse cells and COS7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) and HeLa cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (JRH). ts20 Chinese Hamster lung cell line, which harbors the temperature-sensitive mutation in E1 ubiquitin-activating enzyme was maintained in α-MEM medium

(Sigma), supplemented with 4.5 g Glucose/L and 10% fetal bovine serum at 30°C. Cells were incubated at 40°C for 24 hours to inactivate the E1 ubiquitin-activating enzyme. Transfection of cultured cell lines has been carried out using Lipofectamine 2000 (Invitrogen). Typically, cells were grown in their respective growth medium without antibiotic and transfected with expression plasmids using lipofectamine 2000 and OPTIMEM according to the manufacturers recommendations. To block the 26S proteasome-mediated protein degradation, cells were treated with 20 μM MG132 for 16 hours. To block the protein synthesis, the ts20 CHO and ts20-TG mouse cells were treated with 50 μg/ml cycloheximide (Sigma) for various indicated times.

Cell cycle synchronization and flow cytometry:

To obtain cells arrested at G1/S and M phase of the cell cycle, asynchronously growing HeLa cells were treated with aphidicolin (1μg/ml) for 24 hours and nocodazole (0.1 μg/ml) for 16 hours respectively. Cells treated similarly with the vehicle (DMSO) were used as the control. The extent of synchronization was assessed by propidium iodide (PI) staining and flow cytometry. Briefly, cells were harvested and fixed with 70% ethanol overnight at 4°C. The fixed cells were washed twice in 0.1% Triton X-100/PBS, resuspended in PI staining solution (50μg/ml Propidium Iodide, 100μg/ml RNAse and 0.1% Triton X-100) and incubated for one hour at room temperature before analysis. DNA content was analyzed using FACSCalibur system (Becton Dickinson) and the data were analyzed using the Modfit software.

Cell lysis and Immunoblotting

Typically, cells were lysed for 15 min on ice in lysis buffer [1X TBS (50mM Tris, 150 mM NaCl pH 7.6); 10% Glycerol and 1% Nonidet P-40] containing protease inhibitor cocktail (Roche molecular Biochemical). The lysates were then cleared by centrifugation at 13000 rpm for 10 min at 4°C. Alternatively cells were also lysed in 1X Laemli Buffer, followed by pulsed sonication on ice and subsequently cleared by centrifugation at 13K rpm at 4°C. The protein concentrations of the lysates were assayed using Bio-Rad Protein Assay Reagent (Pierce). 50-100 μg proteins were separated on a 10 or 12% SDS-PAGE. The proteins were subsequently transferred to the nitrocellulose membrane (Gelman Laboratory). After blocking with 5% non-fat milk in TBS, the blots were incubated with various antibodies at their optimal dilutions overnight at 4°C. The horseradish peroxidase (HRP)-conjugated secondary antibodies [goat anti-rabbit HRP & goat anti-mouse HRP (Pierce)] were diluted at 1: 8000 in blocking buffer and incubated with the blot for 1 hour at room temperature. The conjugated secondary antibodies were detected by SuperSignal Pico or Dura Chemiluminescence (Pierce) detection system.

In vivo ubiquitination Assay

The *in vivo* ubiquitination assays were carried out essentially as described in (17). HeLa cells were co-transfected with HA tagged-Aurora-A, His-tagged wild type or K48R mutant ubiquitin and either pCDNA3 or AURKAIP1. 36 hrs post-transfection, the cells were treated with 20 μM MG132 for 12 hours prior to harvest. The cells were lysed in 1 ml Buffer G (6 M Guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4—pH8.0, 10 mM imidazole) per 60 mm dish. The lysate was sonicated in pulses to reduce viscosity and incubated with 100 μl of 50% slurry of nickel-NTA-agarose (Qiagen) with rotation for 75 min at room temperature. The beads were washed three times with 1 ml Buffer G, twice with 1 ml of Wash Buffer I (Buffer G diluted in [25 mM Tris-HCl, pH 6.8 / 20 mM imidazole] at the ratio of 1:4), and twice with 1 ml of Wash Buffer II (25 mM Tris-HCl, pH 6.8 / 20 mM imidazole). The bound proteins were eluted by boiling the beads in 2X SDS sample buffer supplemented with 100 mM EDTA and analyzed by immunoblotting.

RESULTS

Degradation of Aurora-A by AURKAIP1 is cell cycle-independent

Multiple regulators of Aurora-A kinase stability like cdh1, hcdc4 and chfr have been described recently (18,19), which target Aurora-A through Ub-dependent, proteasome-dependent degradation pathway. Previously, we have described the isolation of AURKAIP1, a novel interacting partner and negative regulator of Aurora-A, which also targets Aurora-A for degradation through proteasome-dependent pathway (16). As a further step towards understanding the role of AURKAIP1 in Aurora-A degradation, we sought to investigate the mechanism by which AURKAIP1 destabilizes Aurora-A. Though the full length AURKAIP1 was capable of interacting and destabilizing Aurora-A, the N-terminal truncated form of AURKAIP1 (TR-AURKAIP1), which was originally isolated in the yeast dosage suppressor screen described in (16), was more stable and potent than the full length AURKAIP1 in the destabilization of Aurora-A (**Fig.1a**). This suggests that the N-terminal 87 amino acids of AURKAIP1 might harbor putative negative elements, which render the full-length protein less stable and thus less effective. To verify the specificity of the truncated AURKAIP1 in destabilizing Aurora-A, the *in vivo* degradation assays were carried out with Aurora-B. The results presented in **Fig.1b** showed that the truncated from of AURKAIP1 did not have any destabilizing effect on Aurora-B while it could destabilize Aurora-A more effectively suggesting

that the truncated AURKAIP1 contain all the necessary elements to specifically destabilize Aurora-A. Hence all the subsequent experiments designed to understand the mechanism of Aurora-A degradation were performed with the truncated form of AURKAIP1. To address the role of AURKAIP1 in cell-cycle dependent turnover of Aurora-A, we carried out the *in vivo* degradation assays in cells arrested at different phases of the cell cycle. Cells were co-transfected with Aurora-A and TR-AURKAIP1 or control empty vector and subsequently synchronized with cell-cycle phase-specific inhibitors before analysis. Monitoring cell cycle distribution by flow cytometry indicated that both aphidicolin and nocodazole arrested more than 80% of the cells in G1/S and G2/M phase of the cell cycle respectively **(Supplementary Fig.S1a)**. TR-AURKAIP1 was able to target Aurora-A for degradation in both aphidicolin and nocodazole treated cells suggesting that the AURKAIP1-mediated degradation of Aurora-A is cell cycle-independent (**Fig.1c)**. To exclude the possibility that the missing N-terminus of AURKAIP1 in the truncated version used above might be involved in the specification of cell cycle-dependent degradation of Aurora-A, identical experiments were performed with the full-length AURKAIP1 also **(supplementary Fig.S1 b-c)**. The results suggested that both full-length and TR-AURKAIP1 could target Aurora-A for degradation independent of the cell cycle stages. The observation that AURKAIP1 targets Aurora-A for degradation independent of cell cycle stages is in contrast to the differential effect of hCdh1 on the steady-state levels of Aurora-A in S and M- phase arrested cells. Human Cdh1 showed a cell cycle-specific differential effect on the steady-state levels of Aurora-A with no significant effect in M phase cells while the control untreated and thymidine treated (S phase) cells showing significant decrease of Aurora-A protein (20).

AURKAIP1 can target cdh1-resistant Aurora-A mutant protein for degradation

To further compare the nature of cdh1-dependent and AURKAIP1-dependent Aurora-A degradation, the effect of AURKAIP1 on the degradation of the A-box mutant, an Aurora-A mutant that cannot be targeted for degradation by cdh1 was studied. Mutation of the serine residue at position 51 located within the A-box of human Aurora-A to aspartic acid leads to stabilization of Aurora-A, through the inhibition of the degradation (6-8). As shown in **Fig. 2a**, wild-type Aurora-A was degraded rapidly upon exit from mitosis into G1, whereas the A-box mutant was stabilized. *In vivo* Aurora-A degradation assays with this A-box mutant showed that the A-box mutant was targeted for degradation with the same efficiency as the wild type Aurora-A in the presence of TR-AURKAIP1 **(Fig.2b)**. These data further reinforced the mechanistic differences in the cdh1-dependent and AURKAIP1-dependent degradation of Aurora-A. However, reversal of this AURKAIP1-mediated degradation of the A-box mutant by proteasome

inhibitors MG132 and lactocystin **(Fig.2c)** confirmed the proteasome-dependent nature of this process.

AURKAIP1 inhibits polyubiquitination of Aurora-A

Ubiquitination represents one of the essential modifications to target the protein(s) for recognition by 26 S proteasome and subsequent degradation. It is known that Aurora-A is poly-ubiquitinated before cell cycle dependent degradation by APC/Cyclosome (17). In order to understand the mechanism of AURKAIP1-mediated degradation, we asked whether AURKAIP1 plays any role in the ubiquitination of Aurora-A. To determine the possibility that AURKAIP1 might potentiate the polyubiquitination of Aurora-A and therefore enhance its degradation similar to chfr, *in vivo* Aurora-A ubiquitination assays were performed as described under experimental methods. HeLa cells were co-transfected with wild type Aurora-A and ubiquitin in the presence of either AURKAIP1 expression constructs or empty vector. As shown in **Fig 3**, wild type Aurora-A can be ubiquitinated readily. However, coexpression of TR-AURKAIP1 totally abolished the polyubiquitination of Aurora-A (**panel 1**). The decreased polyubiquitination of Aurora-A in the presence of TR-AURKAIP1 was not due to the alteration of total cellular polyubiquitination (**panel 2**). Under the experimental condition, which is carried out in the presence of proteasome inhibitor MG132, Aurora-A levels were maintained even in the presence of TR-AURKAIP1 (**panel 3**), suggesting that the decreased polyubiquitination observed in the presence of AURKAIP1 is mainly due to inhibition of the polyubiquitination of Aurora-A *per se* rather than due to decreased Aurora-A protein levels. ΔC198-AURKAIP1 mutant, an Aurora-A non-interactive AURKAIP1 mutant, was also used as a control to verify whether the interaction between AURKAIP1 and Aurora-A is essential for the ubiquitination. Interestingly, ΔC198-AURKAIP1 mutant, that does not interact with Aurora-A and is less efficient in mediating Aurora-A degradation, lacked this inhibitory effect and restored similar level of Aurora-A polyubiquitination as the empty vector control. These observations suggest that AURKAIP1 inhibits polyubiquitination of Aurora-A and its interaction with Aurora-A is essential for the inhibitory effect on polyubiquitination.

To further verify the interaction-dependent inhibition of the ubiquitination of Aurora-A, mapping of the regions of Aurora-A protein essential for the ubiquitination and binding of AURKAIP1 was carried out. Both N-terminal and C-terminal truncated overlapping fragments of Aurora-A were generated **(Fig 4a)** and subjected to *in vivo* ubiquitination and AURKAIP1 interaction assays as described earlier. The results presented in **Fig. 4b** show that both N-terminal deletions

(ΔN300 and ΔN600) of Aurora-A lead to a decreased polyubiquitination, but still were capable of polyubiquitinated. Surprisingly, in contrast to the general belief that the A-box mutant is ubiquitylation-defective, we were able to observe ubiquitylation of A-box mutant to an extent similar to that of wild type Aurora-A under given experimental conditions. The difference in the effect on polyubiquitination by mutations in the A-box and N-terminal deletion mutants of Aurora-A could be due to the nature of the mutations as one is a point mutation compared to deletion in the others. It is worthy of note that despite the prediction that the (S51D) mutation in the A-box of Aurora-A will negatively impact ubiquitination and subsequent degradation of Aurora-A (6,7), a formal demonstration of the effect of Aurora-A ^{S51D} mutation on the ubiquitylation of Aurora-A is still lacking. On the other hand, the C-terminal deletion ΔC300 showed increased polyubiquitination of Aurora-A. This observation is in agreement with the previously published results, which also showed an increase in the polyubiquitination of Aurora-A protein lacking the extreme C-terminal region of the protein (17). However, further deletion of the C-terminus (ΔC600) completely suppressed polyubiquitination, suggesting that this region is essential for the efficient polyubiquitination of Aurora-A. Analysis of the Aurora-A regions necessary for interaction with AURKAIP1 **(Fig.4c)** showed that the ΔN300 could interact with AURKAIP1 with similar efficiency as the full length Aurora-A. The other deletions ΔN600 and ΔC300 were capable of interacting with AURKAIP1 albeit with lower efficiency. Interestingly, the polyubiquitination defective ΔC600 was incapable of binding AURKAIP1, suggesting that there could be an overlap between the regions of Aurora-A protein necessary for polyubiquitination and its interaction with AURKAIP1.

AURKAIP1 targets Aurora-A for degradation in the presence of dominant negative ubiquitin mutants

Destabilization of Aurora-A despite the inhibition of its polyubiquitination by AURKAIP1 prompted us to investigate the ubiquitin-independent degradation of Aurora-A. To this end, a dominant negative ubiquitin mutant (K48R) to suppress cellular polyubiquitination was employed. Ub-dependent degradation involves the attachment of multiple ubiquitins to the lysine residue of the target protein(s), facilitating the substrate recognition by the 26S proteasome. Incorporation of K48R dominant negative mutant ubiquitin has been shown to have chain terminating effect, blocking further ubiquitin chain extension (21). The lysine residue at position 63 (K63) of ubiquitin is also implicated in the polyubiquitination of target proteins. However,

unlike K48, its role in protein degradation is minimal. Regardless, to exclude a role for K63–mediated polyubiquitination in the degradation of Aurora-A, a K48R/K63R double mutant was also generated. To verify whether the AURKAIP1-mediated Aurora-A degradation is affected under the conditions where the cellular polyubiquitination is suppressed, AURKAIP1-mediated *in vivo* degradation of Aurora-A was carried out in the presence of K48R and K48R/K63R dominant negative ubiquitin mutants. It was observed that the AURKAIP1-mediated Aurora-A degradation was unaffected even in the presence of K48R or K48R/K63R mutant ubiquitins and was as efficient as observed with wild type ubiquitin. **(Fig.5a)**. Taken together, efficient degradation of Aurora-A even in the presence of K48R/K63R double mutant and the inhibition of Aurora-A polyubiquitination by AURKAIP1 without compromising its effect on degradation indicated that the Aurora-A could be targeted for degradation even in the absence of polyubiquitination. To verify that the AURKAIP1-mediated degradation of Aurora-A in the presence of mutant ubiquitin involves proteasomal function, a similar experiment as described above was carried out in the presence and absence of proteasomal inhibitors MG132 and lactocystin. The data shown in **Fig.5b** showed that the observed AURKAIP1-mediated degradation of Aurora-A in the presence of dominant negative mutant ubiquitin is also proteasome-dependent.

Lack of polyubiquitination does not completely stabilize Aurora-A

As an alternative approach to verify the existence of an Ub-independent pathway for Aurora-A degradation, the turnover of Aurora-A, p21 and cyclin B1 was assessed in the presence of cycloheximide in ts20 CHO cells harboring a temperature-sensitive mutation in E1-ubiquitin activating enzyme (22). The turnover of these proteins was assessed at either permissive condition (30^{o}C) where both Ub-dependent and independent pathways are functional or non-permissive condition (40^{o}C) where only the Ub-independent pathway is functional. p21 was previously demonstrated to be a target for Ub-independent degradation (23) whereas cyclin B1 has been a prototype target for Ub-dependent degradation. Results presented in **Fig 6a** showed that the temperature shift to 40^{o}C did not completely stabilize the p21, supporting previous findings that p21 can be degraded in the absence of ubiquitination and hence act as the target for Ub-independent degradation. In contrast, Cyclin B1 level was completely stabilized upon temperature shift to 40^{o}C, indicating that cyclin B1 can only be targeted by the Ub-dependent degradation (**Fig.6b**). It was evident that even within 30 minutes of cycloheximide treatment, there was sharp decline in Aurora-A steady state level, indicating that Aurora-A is normally an unstable protein. However, its steady state level was increased but not stabilized when the Ub-

dependent pathway was blocked at 40^{o}C (**Fig.6c**). The lack of complete stabilization implies that Aurora-A can also be targeted for degradation in the absence of ubiquitin similar to p21.

To address the question whether the endogenous Aurora-A also follow the same fate as the exogenous protein with respect to the stabilization in the absence of ubiquitination, we chose to carry out the experiment in the temperature sensitive mouse cell line ts20-TG harboring a mutation in the E1 ubiquitin activating enzyme to facilitate detection of endogenous proteins with the available antibodies. Identical experiments as described previously with ts20-CHO cells to investigate the turnover of exogenous Aurora-A, p21 and cyclin B1 were carried out in this mouse cell line. The results presented in **Fig.6d** demonstrate that the endogenous Aurora-A, p21 and cyclin B1 behave similar to the exogenous counterparts with respect to their turnover in the absence of polybiquitination.

AURKAIP1 specifically targets Aurora-A for degradation through Ub-independent pathway

To further confirm the Ub-independent nature of Aurora-A degradation in the presence of AURKAIP1, *in vivo* degradation assays were performed in ts20 CHO cells. In support of the results from the first approach using dominant negative ubiquitin mutant, suppression of poly-ubiquitination by temperature-sensitive mutation of the E1 enzyme increased the levels of Aurora-A but still did not abolish the AURKAIP1-mediated Aurora-A degradation (**Fig.7a**). These data confirm that AURKAIP1 mediates Aurora-A degradation even in the absence of polyubiquitination. Therefore, Aurora-A can be targeted by both Ub-dependent as well as Ub-independent degradation pathways. To verify the specificity of AURKAIP1-mediated Ub-independent Aurora-A degradation, the effect of overexpression of TR-AURKAIP1 on human Aurora-B, p21 and cyclin B1 stability was investigated in ts-20 CHO cells. In contrast to the effect on Aurora-A, overexpression of TR-AURKAIP1 did not influence the stability of either Aurora-B (**Fig.7b**) or p21 (**Fig.7c**) or cyclinB1 (**Fig.7d**). Interestingly, in the absence of AURKAIP1, inhibition of the polyubiquitination leads to a higher basal level of Aurora-A. This implies that under normal conditions, the Ub-dependent degradation is a major pathway operational for Aurora-A turnover and AURKAIP1 promotes Aurora-A degradation through an Ub-independent but proteasome-dependent pathway.

DISCUSSION

The abundance of Aurora-A protein is tightly controlled by synthesis and degradation, as its overexpression leads to disruption of checkpoints (24,25) induction of aneuploidy and transformation (9,10). Cell cycle dependent degradation of Aurora-A is mediated by cdh1 through Ub-dependent proteasomal degradation pathway (6,7). Recently, two other candidate regulators, hcdc4 and chfr, involved in the destabilization of Aurora-A have been described (18,19). All these candidate regulators however, target Aurora-A for proteasome-dependent degradation with prior ubiquitination. Herein we provide the first demonstration that there exists an alternative Ub-independent pathway for Aurora-A degradation and AURKAIP1 facilitates the degradation of Aurora-A through this alternative pathway.

Though ubiquitination is a pre-requisite for majority of the extralysosomal proteolysis by the 26S proteasome, both 20S and 26S proteasome can degrade many proteins in an Ub-independent manner (5). Ornithine decarboxylase was the first example of a protein degraded by the 26S proteasome using this alternative pathway (26). The other proteins, which are degraded by 26S proteasome in an Ub-independent manner, include c-jun (27), p21 (28), p53 (29) and calmodulin (30). The 26S/20S proteasome can degrade proteins in an Ub-independent manner, provided the substrate is targeted to the proteasome machinery by another protein or by a degradation signal present in the substrate itself. For example, Tax, a protein encoded by human T cell leukemic virus promotes binding of IκBα to the HsN3 subunit of 20S proteasome and facilitates the constitutive degradation of IκBα by ubiquitin and phosphorylation-independent manner (31). Similarly, hyperphosphorylated forms of members of retinoblastoma protein family were targeted by the viral protein pp71 for Ub-independent, proteasome-dependent degradation (32). On the other hand, NAD(P)H quinone oxidoreductase 1 (NQO1), which is capable of binding both p53 and 20S proteasome, functions as a gatekeeper of the 20S proteasome and negatively regulates the degradation of p53 (29,33). Dicoumarol, an inhibitor of NQO1, has been shown to induce p53 degradation by 20S proteasome-dependent, Ub-independent pathway (34). p21, a transcriptional target of p53, constitutes an example of a protein, which targets itself for Ub-independent degradation by directly binding to the 20S proteasome (34). It has been shown that p21 directly interacts with C8 subunit of 20S proteasome *in vitro* and the turnover of mutant p21 *in vivo* directly correlates with its affinity for C8 subunit *in vitro* (35). Thus, it is apparent that the interaction of the target proteins with the proteasomal machinery is a prerequisite for Ub-independent degradation.

Using dominant–negative ubiquitin mutant and temperature-sensitive mutant cell lines defective in E1 ubiquitin activating enzyme at the restrictive temperature, we have shown that Aurora-A can be degraded even in the absence of polyubiquitination. We further show that AURKAIP1, which constitutively targets Aurora-A for degradation in proteasome-dependent manner (16), can obviate the need for polyubiquitination. Our results presented herein conform to the trend of a proteins being degraded by both Ub-dependent and independent pathways (27-29). It has been shown recently that MDM2 can target Rb protein for degradation through a similar Ub-independent pathway (36). It is intriguing to note that though MDM2 promotes the degradation of p53 through Ub-dependent pathway with its ubiquitin ligase activity, the degradation of Rb protein by MDM2 is Ub-independent, which accompanies suppression of polyubiquitination. We also observed the suppression of polyubiquitination of Aurora-A in the presence of AURKAIP1 suggesting that the AURKAIP1-mediated suppression of poly-uquitination might be one of the determinants to switch between these alternative pathways. Studies carried out to understand the mechanism by which AURKAIP1 could suppress polyubiquitination of Aurora-A suggest that the binding of AURKAIP1 to Aurora-A might inhibit the interaction of ubiquitination machinery with Aurora-A. This speculation was supported by the observation that the non-interactive AURKAIP1 mutant ΔC-198 restores ubiquitination of Aurora-A. Further studies with Aurora-A deletion mutants showed that there is an overlap of the AURKAIP1 binding region and region essential for proper polyubiqitination of Aurora-A. Thus, the binding of AURKAIP1 to Aurora-A could mask the region essential for ubiquitination thereby inhibiting polyubiquitination.

The next interesting question is how AURKAIP1 targets Aurora-A to the proteasome in the absence of ubiquitination? Generally, marking of the substrates with ubiquitin is thought to serve two functions - unfolding and targeting (31,32,37). It is not yet clear whether binding of AURKAIP1 can unfold Aurora-A so that it can be a better substrate for 20S proteasome. Similarly, it is yet to be shown that AURKAIP1 is capable of targeting Aurora-A directly to the proteasome. In this context, it will be interesting to investigate whether AURKAIP1 can interact directly with proteasome and Aurora-A can be degraded by 26S/20S proteasome *in vitro* in the presence of AURKAIP1. On the other hand, in the AURKAIP1-mediated Aurora-A degradation pathway, other possibilities such as modification of Aurora-A by other small molecules (38) or involvement of other secondary proteins that can cooperate with AURKAIP1 in the targeting of Aurora-A to the proteasome also cannot be ruled out.

In summary, we have shown that Aurora-A can be targeted for degradation even in the absence of ubiquitination and AURKAIP1 facilitates the degradation of Aurora-A through this Ub-independent pathway. At this juncture, it is unclear why there should be two pathways for the degradation of the same protein or what is the cellular context for AURKAIP1-mediated Ub-independent degradation of Aurora-A. The physiological relevance of Aurora-A:AURKAIP1 interaction can be appreciated better when we have adequate information on the biology of AURKAIP1. Preliminary studies carried out on the expression of AURKAIP1 using antibody raised against the C-terminal AURKAIP1 peptide, showed that while transfected and *in vitro* translated AURKAIP1 can be easily detected, the endogenous AURKAIP1 was undetectable even in cells which expressed very high levels of AURKAIP1 transcripts (our own unpublished data). This suggests that AURKAIP1 could be regulated post-transcriptionally and/or the AURKAIP1 protein might be expressed only under specific cellular context. Future studies on the identification of this yet unidentified cellular context as well as the mechanism by which AURKAIP1 promotes Ub-independent degradation of Aurora-A will definitely throw more light on the physiological significance of this alternative pathway.

REFERENCES

1. King, R.W., Deshaies, R.J., Peter, J.M., and Kirschner, M.W. (1996) How proteolysis drives the cell cycle. *Science* **274**, 1652-1659.
2. Peters, J.M. (1998) SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. *Curr. Opin. Cell Biol.* **10**, 759-768.
3. Koepp, D.M., Harper, J.W., and Elledge, S.J. (1999) How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell* **97**, 431-434.
4. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-479.
5. Orlowski, M. and Wilk, S (2003) Ubiquitin-independent proteolytic functions of the proteasome. *Arch. Biochem.Biophys.* **415**, 1-5

6. Castro, A., Arlot-Bonnemains, Y., Vigneron, S., Labbe, J.C., Prigent, C., and Lorca, T. (2002) APC/Fizzy-Related targets Aurora-A kinase for proteolysis. *EMBO Rep.* **3**, 457-462.
7. Littlepage, L.E. and Ruderman, J.V. (2002) Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. *Genes Dev.* **16**, 2274-2285.
8. Castro, A., Vigneron, S., Bernis, C., Labbe, J.C., Prigent, C., and Lorca, T. (2002) The D-Box-activating domain (DAD) is a new proteolysis signal that stimulates the silent D-Box sequence of Aurora-A. *EMBO Rep.* **3**, 1209-1214.
9. Zhou, H., Kuang, J., Zhong, L., Kuo, W.L., Gray, J.W., Sahin, A., Brinkley, B.R., and Sen, S. (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nat. Genet.* **20**, 189-193.
10. Bischoff, J.R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., Chan, C.S., Novotny, M, Slamon, D.J., Plowman, G.D. (1998) A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. *EMBO J.* **17**, 3052-3065.
11. Bischoff, J.R., and Plowman, G.D. (1999) The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. *Trends Cell Biol.* **9**, 454-459.
12. Katayama, H., Brinkley, W.R., Sen, S. (2003). The Aurora kinases: role in cell transformation and tumorigenesis..*Cancer and Metastasis Rev.* **22**, 451-464.
13. Tanaka, T., Kimura, M., Matsunaga, K., Fukada, D., Mori, H., and Okano,Y. (1999) Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast *Cancer Res.* **59**, 2041-2044.
14. Miyoshi, Y., Iwao, K., Egawa, C., Noguchi, S. (2001) Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. *Int. J. Cancer* **92**, 370-373.
15. Goepfert, T.M., Adigun, Y.E., Zhong, L., Gay, J., Medina, D., Brinkley, W.R. (2002). Centrosome amplification and overexpression of aurora A are early events in rat mammary carcinogenesis. *Cancer Res.* **62**, 4115-4122.
16. Kiat, L.S., Hui, K.M., Gopalan, G. (2002) Aurora-A kinase interacting protein (AIP), a novel negative regulator of human Aurora-A kinase. *J. Biol. Chem.* **277**, 45558-45565.

17. Honda, K., Mihara, H., Kato, Y., Yamaguchi, A., Tanaka, H., Yasuda, H., Furukawa, K. and Urano, T. (2000) Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway. *Oncogene* **19**, 2812-2812.
18. Mao, J.H., Perez-Losada, J., Wu, D., Delrosario, R., Tsunematsu, R., Nakayama, K.I., Brown, K., Bryson, S. and Balmain, A. (2004) Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene. *Nature* **432**, 775-779.
19. Yu, X., Minter-Dykhouse, K., Malureanu, L. Zhao, W.M., Zhang, D., Merkle, C.J., Ward, I.M., Saya, H., Fang, G., van Deursen, J. and Chen, J. (2005) *Chfr* is required for tumor suppression and Aurora A regulation. *Nat. Genet*. **37**, 401-406.
20. Taguchi, S., Honda, K., Sugiura, K., Yamaguchi, A., Furukawa, K and Urano, T. (2002) Degradation of human Aurora-A protein kinase is mediated by hCdh1. *FEBS Lett.* **519**: 59-65.
21. Ward, C.L., Omura, S. and Kopito, R.R. (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* **83**, 121-127.
22. Strous G.J., van Kerkhof, P., Govers, R., Ciechanover, A. and Schwartz, A.L. (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *EMBO J.* **15**, 3806-3812.
23. Sheaff, R.J., Singer, J.D., Swanger, J., Smitherman, M., Roberts, J.M., and Clurman, B.E. (2000) Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. *Mol. Cell* **5**, 403-410.
24. Marumoto, T., Hirota, T., Morisaki, T., Kunitoku, N., Zhang, D., Ichikawa, Y., Sasayama, T., Kuninaka, S., Mimori, T., Tamaki, N., Kimura, M., Okano, Y and Saya, H. (2002) Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells. *Genes cells* **2**, 1173-1182.
25. Anand, S., Penrhyn-Lowe, S. and Venkitaraman, A.R. (2003) AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell* **3**, 51-62.
26. Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A. and Kahana, C. (1989) Degradation of ornithine decarboxylase in reticulocyte lysate is ATP-dependent but ubiquitin-independent. *J. Biol. Chem.* **264**, 15949-15952.

27. Jariel-Encontre, I., Pariat, M., Martin, F., Carillo, S., Salvat, C. and Piechaczyk, M. (1995) Ubiquitinylation is not an absolute requirement for degradation of c-Jun protein by the 26 S proteasome. *J. Biol. Chem.* **270**, 11623-11627.
28. Jin, Y., Lee, H., Zeng, S.X., Dai, M.S. and Lu, H. (2003) MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation *EMBO J*. **22**, 6365-6377.
29. Asher, G., Tsevetkov, P., Kahana, C. and Shaul, Y.(2005) A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. .*Genes Dev*. **19**, 316-321.
30. Tarcsa, E., Szymanska, G., Lecker, S., O'Connor, C.M. and Goldberg, A.L. (2000) Ca2+-free calmodulin and calmodulin damaged by in vitro aging are selectively degraded by 26 S proteasomes without ubiquitination. *J. Biol. Chem.* **275**, 20295-20301.
31. Krappmann, D., Wulczyn, F.G. and Scheidereit, C. (1996) Different mechanisms control signal-induced degradation and basal turnover of the NF-kappaB inhibitor IkappaB alpha in vivo. *EMBO J*. **15**, 6716-6726.
32. Kalejta, R.F. and Shenk, T. (2003) Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein. *Proc. Natl. Acad. Sci. USA* **100**, 3263-3268.
33. Asher, G., Lotem, J., Tsvetkov, P., Reiss, V., Sachs, L. and Shaul, Y. (2003) P53 hot-spot mutants are resistant to ubiquitin-independent degradation by increased binding to NAD(P)H:quinone oxidoreductase 1 *Proc. Natl. Acad. Sci. USA* **100**, 15065-15070.
34. Asher, G., Lotem, J., Sachs, L., Kahana, C. and Shaul, Y. (2002) Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1. *Proc. Natl. Acad. Sci. USA* **99**, 13125-13130.
35. Touitou, R., Richardson, J., Bose, S., Nakanishi, M., Rivett, J., Allday, M.J. (2001) A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 alpha-subunit of the 20S proteasome. *EMBO J*. **20**, 2367-2375.
36. Sdek,P., Ying, H., Chang, D.L.F., Qiu. W., Zheng, H., Touitou, R., Allday, M.J.and Xiao, Z.J. (2005) MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein. *Mol. Cell* **20**: 699-708

37. Ghislain, M., Dohmen, R.J., Levy, F. and Varshavsky, A. (1996) Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in Saccharomyces cerevisiae**.** *EMBO J*. **15**, 4884-4899.

38. Jentsch, S. and Pyrowolakis, G. (2000) Ubiquitin and its kin: how close are the family ties. *Trends Cell Biol*. **10**, 335-342.

ACKNOWLEDGEMENT

We thank Dr. Ger. J. Strous and Dr. Harvey Ozer for providing us with the ts20-CHO and ts20TG mouse cell lines respectively. We also thank Dr. Michele Pagano, Dr, Ivan Dikic and Dr. Prochownik for the p21, K48R ubiquitin, cyclin B1 expression plasmids respectively. This work is supported by the National Medical Research Council of Singapore in the form of a research grant (NMRC/0815/2003) to Dr. Gopalan and as Institutional Block Grant to National Cancer Centre, Singapore.

[1] Footnotes

The abbreviations used are: APC/C, anaphase promoting complex/cyclosome; AKIP, Aurora-A interacting protein; TR-AKIP, N-terminal truncated AKIP; TBS. Tris buffered saline: MG132, Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal ; Ub, ubiquitin and CHX, cycloheximide

FIGURE LEGENDS

Figure 1: ***Degradation of Aurora-A by AURKAIP1 is cell cycle-independent***.

a) *N-terminal truncated AURKAIP1 is more effective in destabilizing Aurora-A than the full length AURKAIP1:* COS7 cells were co-transfected with Aurora-A and FLAG-tagged full length AURKAIP1 or TR-AURKAIP1 at 1:9 ratio for 36 hrs before harvested for Western Blot analysis of Aurora-A and AURKAIP1 using IAK1 antibody and anti-FLAG mouse monoclonal antibody, respectively. A vector control has been included in which AURKAIP1 plasmid has been replaced by the vector pCDNA3. β-tubulin was used as the loading control. b) *TR-AURKAIP1 specifically targets Aurora-A for degradation:* ts20-CHO cells were co-transfected with plasmids expressing FLAG-tagged human Aurora-A or Aurora-B and HA-tagged TR-AURKAIP1 at 1:5 ratio respectively. A vector control has been included in which AURKAIP1 plasmid has been replaced by the vector pCDNA3. The effect of TR-AURKAIP1 overexpression on Aurora-A or Aurora-B kinase stability was assessed at 36 hrs post-transfection by immunoblot analysis. Cell extracts were analyzed for Aurora-A, Aurora-B and TR-AURKAIP1 proteins using the FLAG M2 and anti-HA antibodies. The blot was probed with mouse anti-β–tubulin for loading control. c) *TR-AURKAIP1-mediated Aurora-A degradation is not cell cycle-dependent.* Cos 7 cells were co-transfected with HA-tagged human Aurora-A and FLAG-tagged TR-AURKAIP1 at 1:9 ratio respectively. Vector control has been included as described previously. Twenty-four hours post-transfection, the transfected cells were collected at different phases of cell cycle by treatment with either DMSO (Log), Aphidicolin (G1/S) and Nocodazole (M) for another 16 hrs. Cell extracts were analyzed for Aurora-A and TR-AURKAIP1 proteins using the anti-HA and anti-FLAG M2 antibodies, respectively. The blot was probed with anti-β tubulin as loading control.

Figure 2: ***AURKAIP1 can target cdh1-resistant Aurora-A mutant protein for degradation.*** a) *A-box stabilizing mutant of Aurora-A is not degraded at G1:* HeLa cells were transfected with either HA-tagged wild type or A-box mutant of Aurora-A. 24 hrs post-transfection, the cells were treated with 0.1 μg/ml nocodazole for 16 hrs to arrest them at M phase. The floating mitotic cells were collected by shake-off and replated in the presence of 50 μg/ml cycloheximide. The cells were harvested for analysis 4 hrs post-mitotic release. Stability of the wild type and A Box mutant of Aurora-A at the M/G1 transition was detected by immunoblotting using anti-HA antibody. Endogenous cyclin B1 levels were used as the positive control to verify M/G1 transition. b) *TR-AURKAIP1 can degrade mutant Aurora-A:* For *in vivo*

degradation assay, COS7 cells were co-transfected with HA-tagged Aurora-A (wild type and A-box mutant) and FLAG-tagged TR-AURKAIP1 at the 1:9 ratio respectively. A vector control has been included in which TR-AURKAIP1 plasmid has been replaced by the vector pCDNA3. The cells were harvested and analyzed 36 hours post-transfection by immunoblot analysis. Aurora-A and TR-AURKAIP1 proteins were detected with anti-HA and FLAG M2 antibodies, respectively. β tubulin was detected as the loading control as described earlier. *c) AURKAIP1-mediated degradation of A Box Mutant is proteasome-dependent:* HeLa cells were co-transfected with HA-tagged A-Box mutant and FLAG-tagged TR-AURKAIP1 at 1:9 ratio respectively. A vector control has been included in which AURKAIP1 plasmid has been replaced by the vector pCDNA3. Twenty four hours post-transfection, one set was treated with DMSO as control, whereas another set was treated with 20 μM MG132 for 16 hours before harvest for western Blot analysis. A-Box mutant and TR-AURKAIP1 were detected using the anti-HA and FLAG M2 antibodies respectively. β–tubulin was used as the loading control.

Figure 3: ***AURKAIP1 inhibits polyubiquitination of Aurora-A***. HeLa cells were transiently transfected with HA-tagged Aurora-A in combination with either empty vector pCDNA3 or AURKAIP1 constructs (TR-AURKAIP1, AURKAIP1 and ΔC198-AURKAIP1 mutant) at 1:9 ratio, respectively in the presence of an expression construct encoding His-tagged wild type ubiquitin (His-Ub). *In vivo* ubiquitination assays were performed as described in Experimental Procedures. Total ubiquitinated proteins were pulled down with NTA-agarose and the ubiquitinated HA-tagged Aurora-A was detected with anti-HA antibodies. The polyubiquitinated, HA-tagged Aurora-A appears as a ladder (Panel 1). The total cellular polyubiquitination was determined by western blot analysis of the total lysates with anti-His antibodies (Panel 2). The protein levels of Aurora-A in the total lysates under different conditions were analyzed using anti-HA antibodies (Panel 3). The blot used for the experiment described in Panel 3 was reprobed with anti-tubulin for loading control.

Figure 4: ***Mapping of regions of Aurora-A essential for ubiquitination***. a) *Aurora-A Kinase and Its Various Deletion Mutants.* The diagram illustrates the size and location of the deletions of all the Aurora-A deletion mutant proteins with full length Aurora-A protein. All of the Aurora-A mutants contain a FLAG tag at the N-terminus. The locations of KEN, A, and D (D1, D2, D3) boxes are indicated. b) *Domain Mapping for efficient Ubiquitination of Aurora-A.* HeLa cells were transfected with His-tagged wild-type ubiquitin and FLAG-tagged wt-Aurora-A or the A-

box mutant or various deletion mutants of Aurora-A. Twenty-four hours post-transfection, the transfected cells were treated with 20μM MG132 for additional 16 hours before harvesting for immunoprecipitation with anti-His antibody. The ubiquitinated Aurora-A and the deletion mutants were detected using anti-Flag antibody. c) *Mapping of AURKAIP1-Interacting Domain in Aurora-A.* HeLa cells were transfected with HA-tagged TR-AURKAIP1 and FLAG-tagged Aurora-A or its various deletion mutants at 1:1 ratio. 24 hours post-transfection, the transfected cells were harvested for immunoprecipitation with anti-Flag antibody. The interacting TR-AURKAIP1 was detected using the anti-HA antibody.

Figure 5: ***AURKAIP1 targets Aurora-A for degradation in the presence of dominant negative ubiquitin mutants***.

a) Overexpression of *K48R and K48R/K63R dominant negative ubiquitin mutants does not affect AURKAIP1-mediated Aurora-A degradation:* HeLa cells were co-transfected with FLAG-tagged Aurora-A and FLAG-tagged TR-AURKAIP1 at 1:9 ratio, in the presence of either His-tagged wild type or K48R or K48R/K63R mutant ubiquitin expression constructs. A vector control has been included in which TR-AURKAIP1 plasmid has been replaced by the vector pCDNA3. Thirty-six hours post-transfection, the cells were harvested and analyzed for Aurora-A and TR-AURKAIP1 using Flag M2 antibodies. β-tubulin was detected as the loading control as described earlier. *b) AURKAIP1-mediated Ub-independent degradation of Aurora-A is proteasome-dependent:* HeLa cells were co-transfected with HA-tagged Aurora-A and FLAG-tagged TR-AURKAIP1 at 1:9 ratio in the presence of K48R ubiquitin mutant overexpression. A vector control has been included in which AURKAIP1 plasmid has been replaced by the vector pCDNA3. Twenty four hours post-transfection, one set was treated with DMSO as control, whereas another set was treated with 20 μM MG132 for 16 hours before harvest for western blot analysis. Aurora-A and TR-AURKAIP1 were detected using the anti-HA and anti-FLAG antibodies respectively. β– tubulin was used as the loading control.

Figure 6:*Lack of polyubiquitination does not completely stabilize Aurora-A.* To determine the turnover of Aurora-A, cyclin B1 and p21 in ts20-CHO cells in the presence and absence of polyubiquitination, cells were transfected with FLAG-tagged Aurora-A, HA-tagged p21 and cyclin B1 expression plasmids at 30^{o}C. 24 hours post-transfection, the cells were divided into two sets; one set was maintained at 30^{o}C permissive temperature whereas the other set was shifted to non-permissive temperature, 40^{o}C for 16 hrs. After 16 hrs, cells were treated with 50 μg/ml cycloheximide and both sets of cells were harvested at indicated time points. The levels of p21

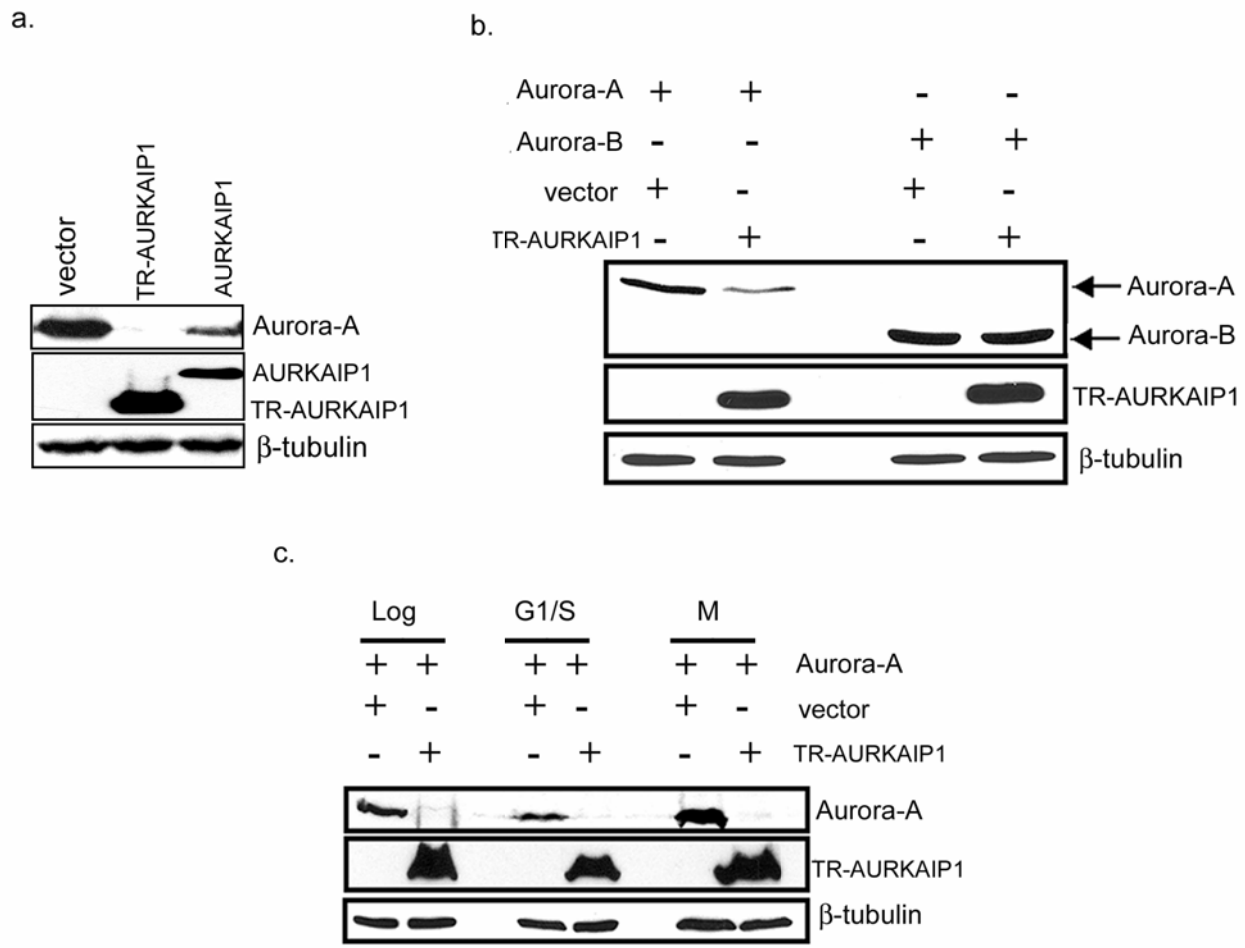
(***a***), cyclin B1 (***b***) and Aurora-A (***c***) were analyzed by immunoblot analysis by FLAG, cyclin B1 and HA-tag antibodies respectively. β tubulin was detected as the loading control as described earlier. ***d.*** *Ub-independent Degradation of Endogenous Aurora-A Kinase:* Mouse ts20b cells were incubated at 32^{o}C or 40^{o}C for 18 hr followed by cycloheximide treatment for the indicated times. The protein levels of endogenous Aurora-A, p21 and cyclin B1 were analyzed by immunoblot analysis using the IAK1, p21 and cyclin B1 antibodies respectively. β–tubulin was detected as the loading control

Figure 7: ***AURKAIP1 specifically targets Aurora-A for degradation in an Ub-independent manner.***

ts20 CHO cells were co-transfected with FLAG-tagged TR-AURKAIP1 and the constructs expressing the targets genes (Aurora-A, Aurora-B, p21 and cyclinB1) at 9:1 ratio. A vector control has been included in which TR-AURKAIP1 plasmid has been replaced by the vector pCDNA3. The transfected cells were divided into two sets; both sets were initially incubated at permissive temperature, 30^{o}C for 24 hrs. One set was maintained at 30^{o}C permissive temperature and the other set was incubated at the non-permissive temperature, 40^{o}C for 16 hrs. The cells were harvested and the steady state levels of Aurora-A (a), Aurora-B (b), p21 (c) and cyclinB1 (d) proteins were analyzed using the respective antibodies. The TR-AURKAIP1 expression was also monitored. The blots were probed with mouse anti-β tubulin for loading control.

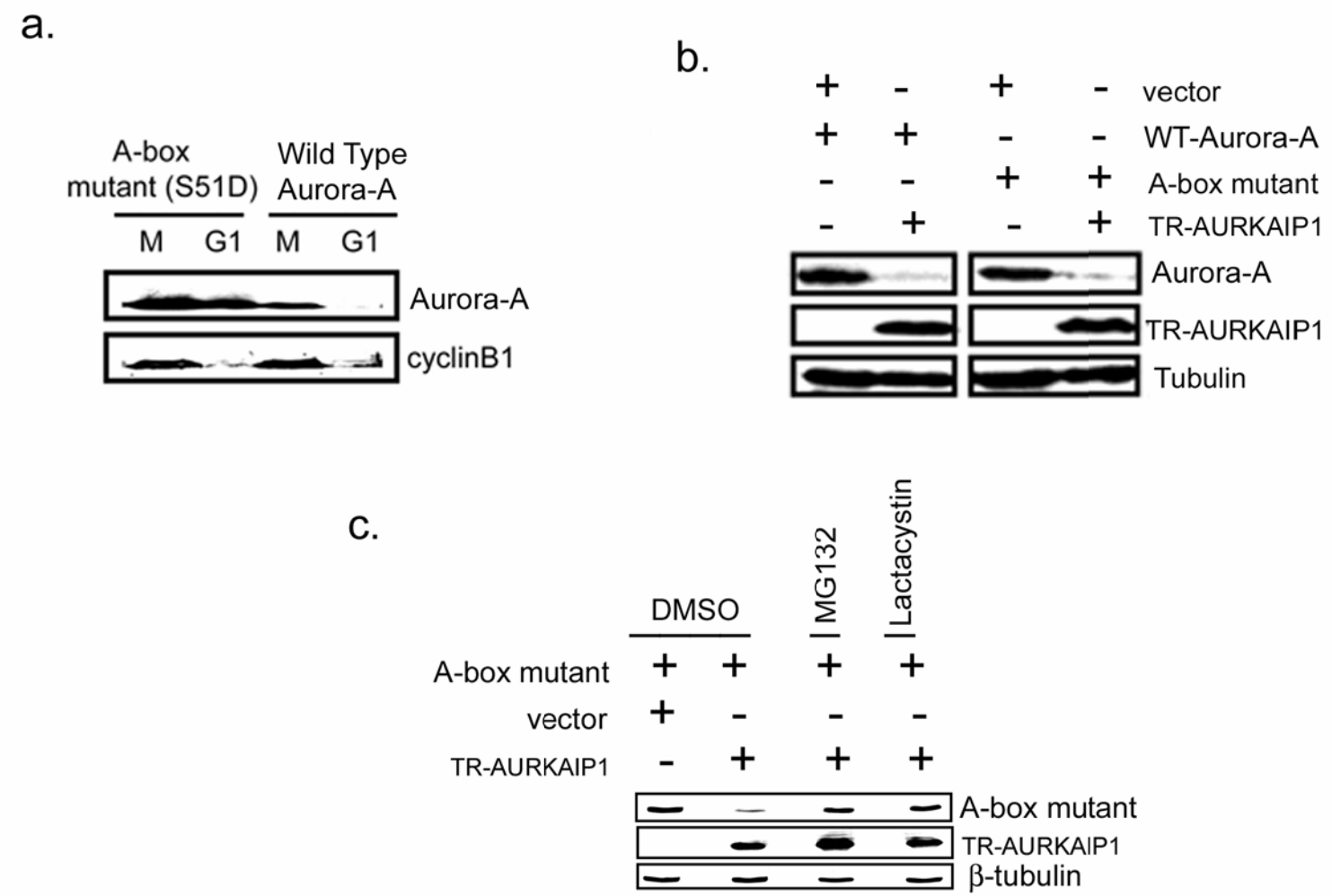
Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

Fig.1



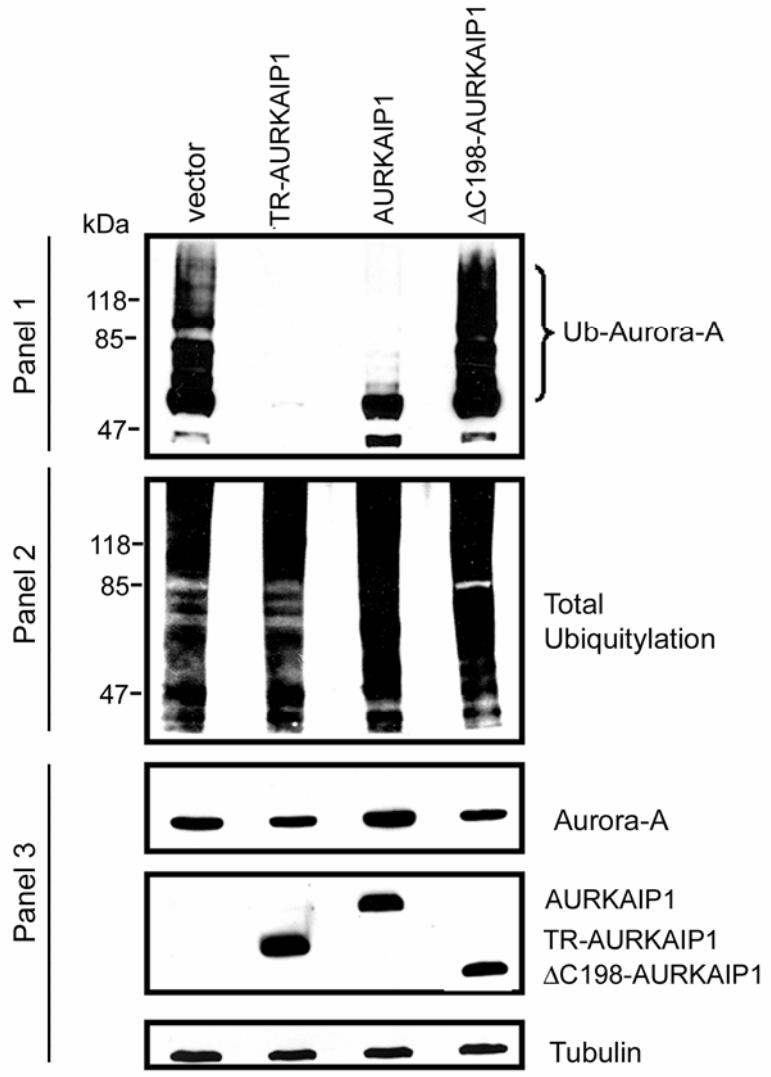
Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

Fig.2



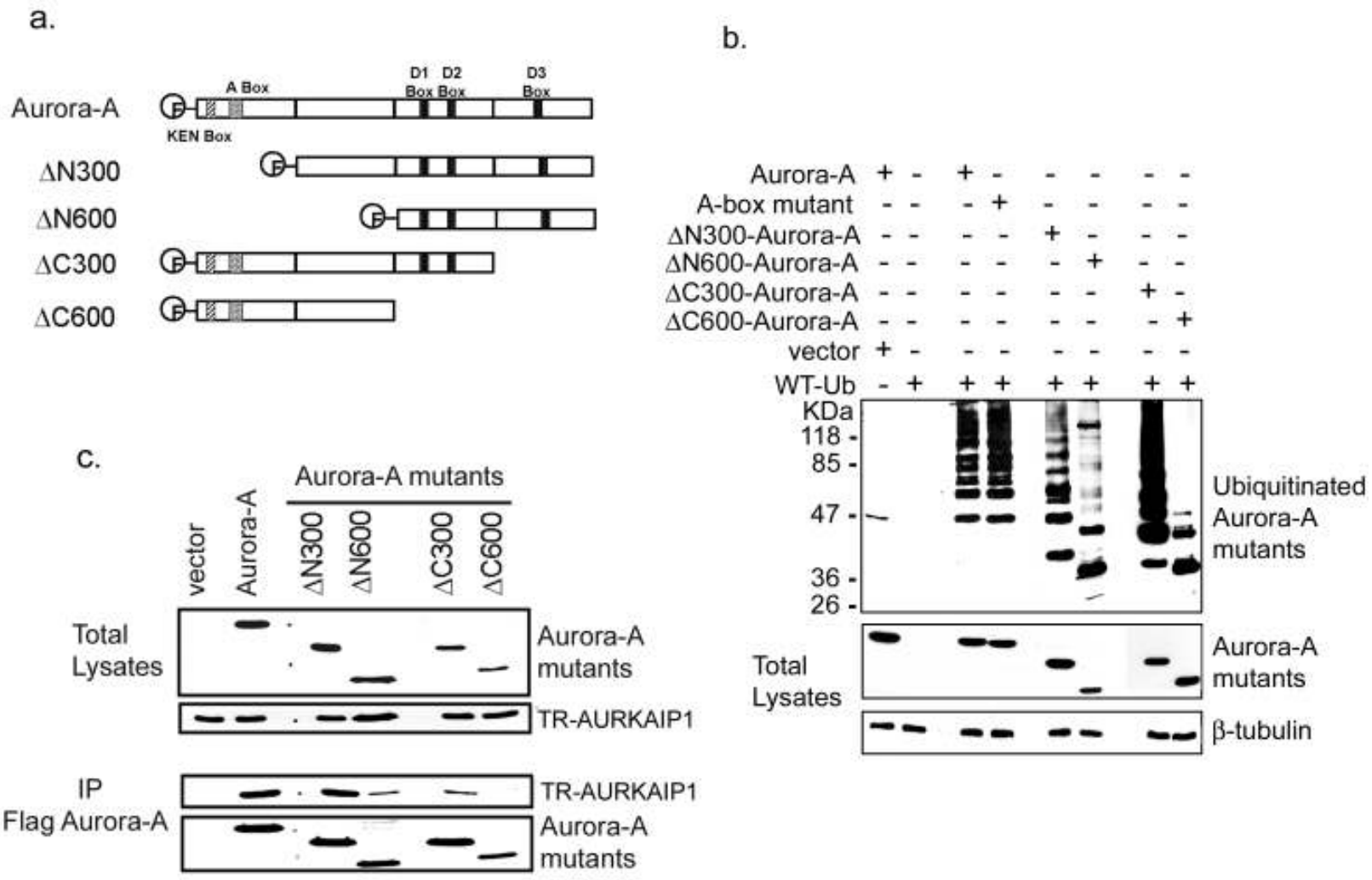
Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

Fig.3



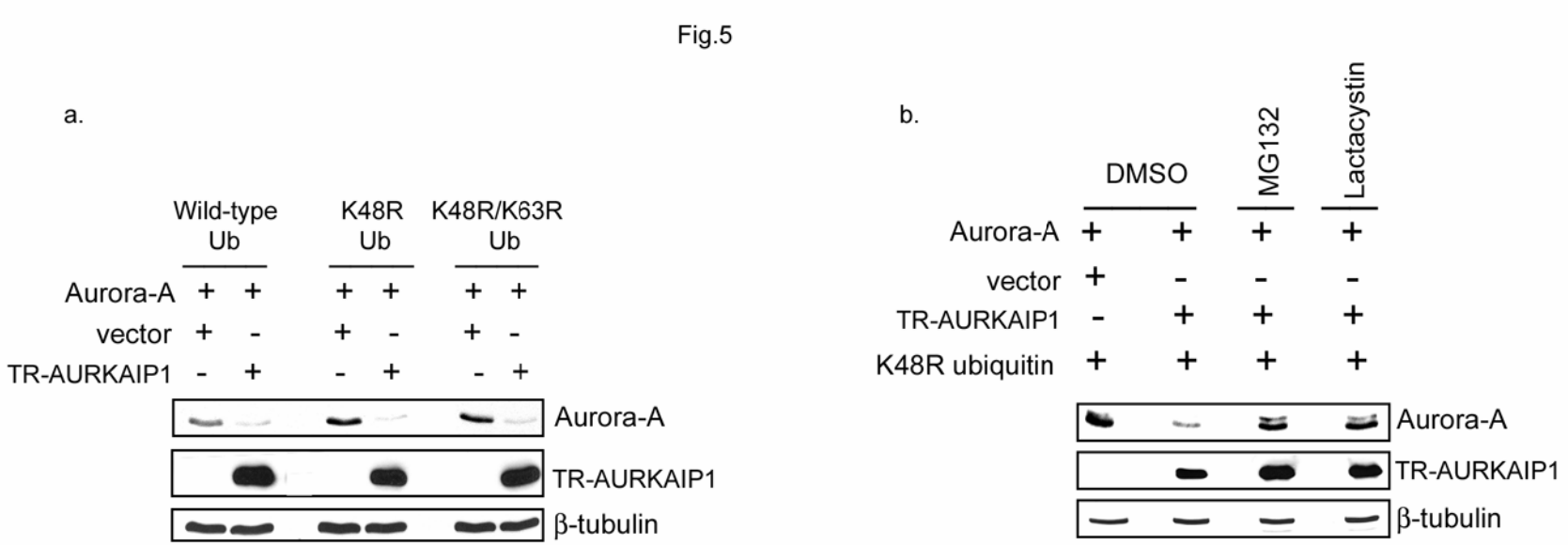
Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

Fig.4



Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

Fig.5



Biochemical Journal Immediate Publication. Published on 27 Nov 2006 as manuscript BJ20061272

Fig.6

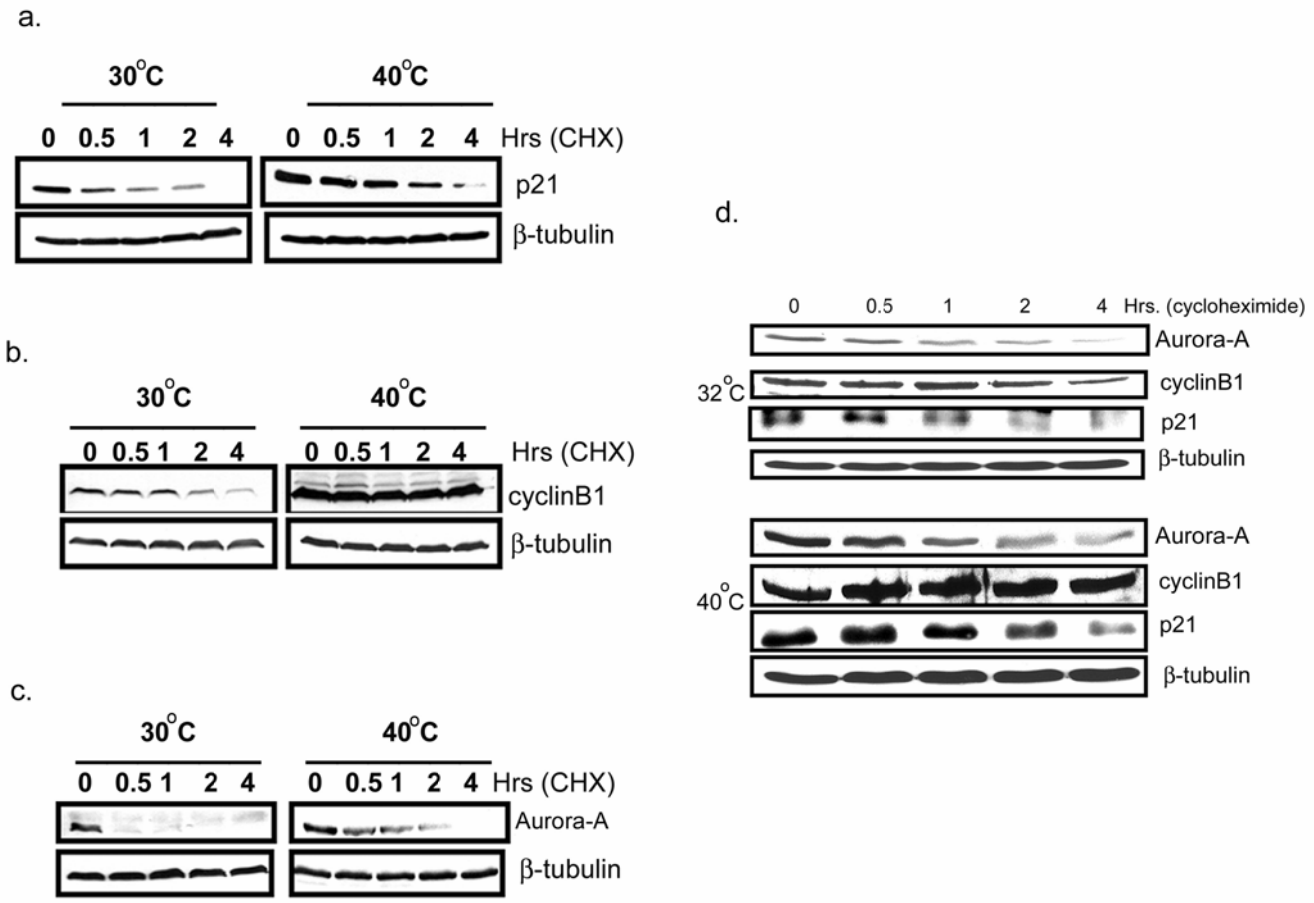
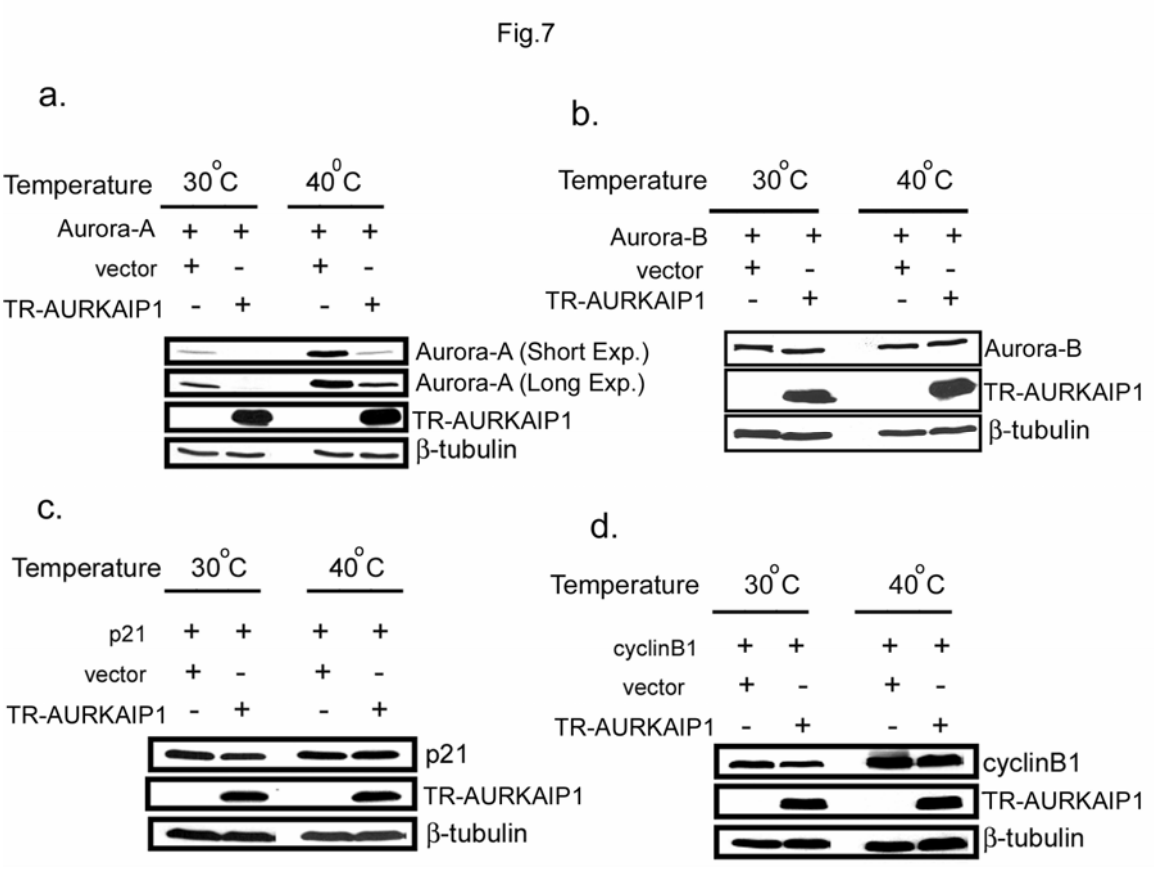


Fig.7



Antizyme1 mediates AURKAIP1-dependent degradation of Aurora-A

Shen Kiat Lim and Ganesan Gopalan*
Laboratory of Gene Structure and Expression
Division of Molecular and Cellular Research
National Cancer Centre, Singapore 16910.

Running Title: Antizyme1-dependent Aurora-A degradation

Keywords: oncogene, degradation, antizyme, Aurora-A, ubiquitin-independent

*To whom correspondence should be addressed: Ganesan Gopalan, Ph.D, # 03-03 Singhealth Research Facilities, Block A, 7 Hospital Drive, Singapore 169611. Phone: (65) 63214075, Fax: (65) 63213606; E mail: cmrgop@nccs.con.sg

ABSTRACT:

Overexpression of Aurora-A oncogene has been shown to induce genomic instability and tumorigenesis. Cellular levels of Aurora-A are regulated by multiple mechanisms including the proteasome-dependent degradation of Aurora-A protein. Cell-cycle-dependent turnover of the Aurora-A is mediated by cdh1 through ubiquitin (Ub) and proteasome-dependent pathway. AURKAIP1, a negative regulator of Aurora-A, also promotes proteasome-dependant Aurora-A destabilization, however, through an Ub-independent mechanism. In an attempt to understand how AURKAIP1 promotes Aurora-A destabilization through Ub-independent pathway, we demonstrate here that antizyme1 (Az1), a well-studied mediator of Ub-independent protein degradation pathway regulates Aurora-A protein stability. We show that ectopic or polyamine-induced expression of Az1 can lower the steady state levels of Aurora-A. The destabilizing effect of Az1 on Aurora-A was shown to be proteasome-dependent, but ubiquitin-independent. Az1 interacts with Aurora-A *in vivo* and that the interaction between Aurora-A and Az1 is essential for the Az1-mediated Aurora-A degradation. Furthermore, we observed that AURKAIP1 could not destabilize Aurora-A mutant, which is defective in Az1 interaction. Co-expression of the antizyme inhibitor (AzI), which downregulates Az1 functions, also abrogated AURKAIP1-mediated destabilization of Aurora-A. We further demonstrated that AURKAIP1, Az1 and Aurora-A could exist as a ternary complex and AURKAIP1 enhances the interaction between Az1 and Aurora-A. We propose that AURKAIP1 might function upstream of the Az1 by enhancing the binding affinity of the Az1 to Aurora-A to promote recognition, targeting to proteasome and subsequent degradation.

INTRODUCTION:

Aurora-A kinase is an important member of the family of aurora kinases that play essential roles in mitotic events. Regulation of the steady-state levels of Aurora-A is very important as higher levels of Aurora-A in human and rodent cells induce centrosome amplification, aneuploidy, transformed phenotype and tumor formation in nude mice [Bischoff *et al*., 1998; Zhou *et al*., 1998]. Aurora-A is overexpressed in many cancer types and mapped to chromosome 20q13 region, that is frequently amplified in many human cancers [Gritsco *et al*., 2003; Li *et al*., 2003; Tanaka *et al*., 1999]. Overexpression of Aurora-A significantly correlates with induction of aneuploidy, centrosome anomaly, poor prognosis and invasiveness of the primary human tumors and of experimental tumors in animal model systems [Buschhorn *et al*., 2005; Sakakura *et al*., 2001]. Aurora-A levels and functions are regulated by multiple mechanisms such as gene amplification, transcription, post-translational modifications including phosphorylation/dephosphorylation and protein degradation through proteasome–dependent pathway [Honda *et al*., 2000; Walter *et al*., 2000]. Aurora-A represents one of the many mitotic proteins, whose protein levels are temporally regulated by the Ub-dependent proteolysis at the end of mitosis before cells progress into subsequent G1 phase. Aurora-A is ubiquitinated by the Cdh1-activated APC/C, an E3 ubiquitin ligase through the recognition of C-terminal destruction box (D-box) and N-terminal A-box. It has been proposed that dephosphorylation of the highly conserved S^{51} in A-box during mitotic exit could control the timing of Aurora-A degradation [Honda *et al*., 2000; Littlepage and Ruderman, 2002; Taguchi *et al*., 2002].

Antizyme (Az) represents one of the important classes of evolutionarily conserved proteins that regulate cell growth and metabolism. It is involved in the ubiquitin-independent protein degradation and small molecule transport [Gerner and Meyskens, 2004]. Az expression is induced by an unusual polyamine-dependent mechanism in which a programmed +1 frame-

shift occurs during translation of the Az mRNA leading to the expression of full-length and functional Az protein [Ivanov *et al*., 2000]. Once expressed, Az binds and inhibits ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis and targets it for degradation through ubiquitin-independent pathway [Rom and Kahana, 1994]. Increased polyamines and ODC activities are associated with many human malignancies [Gerner and Meyskens, 2004)]. As a negative regulator of ODC and thus polyamine levels, overexpression of Az leads to cell cycle arrest, apoptosis [Koike et al., 1999; Iwata et al., 1999] and inhibition of tumor growth in *in vivo* mouse models [Feith et al., 2001; Fong et al., 2003]. Az increases the ODC degradation by enhancing ODC association with proteasome, rather than accelerating the rate of proteasomal processing [Zhang *et a*l., 2003]. The attachment of Az causes conformational changes in ODC, thereby exposing its C-terminal degradation signal for recognition by 26S proteasome [Li and Coffino, 1993)]. Unlike ubiquitin, Az is usually spared from destruction and released from the ODC-Az complex at the proteasome [Murakami *et al*., 1992]. Therefore, a single Az molecule can catalyze multiple rounds of ODC degradation. Studies *in vitro* [Bercovich *et al*., 1989] and *in vivo* [Rosenberg-Hasson *et al*., 1989; Glass and Gerner, 1987] have revealed that this Az-mediated protein degradation process is essentially ubiquitin-independent. Recent studies have demonstrated that Az can bind other proteins [Newman *et al*., 2004; Lin *et al*., 2002] besides ODC and facilitate their degradation through ubiquitin-independent pathway.

We identified earlier AURKAIP1 [Kiat *et al*., 2002], an Aurora-A kinase interacting protein, which is involved in the destabilization of Aurora-A through proteasome-dependent pathway. Further studies on the AURKAIP-dependent destabilization of Aurora-A revealed that there exists an alternative Ub-independent pathway for Aurora-A degradation and AURKAIP1 promotes Aurora-A degradation through this Ub-independent yet proteasome-dependent pathway [Lim and Gopalan 2007]. However, the mechanism underlying the targeting of Aurora-A to the proteasome in the absence of ubiquitination remains unexplored. As Az is known to play a role in ubiquitin-independent targeting of a few substrates other than ODC,

we investigated whether Az1, a well-studied member of the antizyme family, could mediate ubiquitin-independent degradation of Aurora-A. Here, we present that Az1 can interact with Aurora-A and target it for degradation through proteasome-dependent but ubiquitin-independent pathway. AURKAIP1 enhances the binding of Az1 to Aurora-A and promotes recognition and targeting of Aurora-A to proteasome in the absence of ubiquitination.

RESULTS:

Az1 targets Aurora-A for proteasomal degradation

Previous studies showed that Aurora-A could be degraded through proteasome-dependent pathway in the absence of ubiquitination [Lim & Gopalan, 2007]. To provide a mechanistic explanation for how Aurora-A is targeted in the absence of ubiquitination, we studied Az1, an established player in ubiquitin-independent proteasomal targeting. To begin with, we investigated whether Az1 can destabilize Aurora-A. Coexpression of Aurora-A and Az1 led to decreased steady state levels of transfected Aurora-A in both HeLa and CHO cells (**Fig.1A**). ODC, a well-documented target of Az1, was also destabilized when coexpressed with Az1, verifying the reliability of the assay (**supplementary Fig.1a).** As the Az1-mediated destabilization was followed with ectopically expressed Aurora-A whose expression is driven by CMV promoter, the involvement of transcriptional mechanisms could be ruled out. Thus, the decrease in the steady-state levels of Aurora-A observed in the presence of Az1 is mainly post-transcriptional. Endogenous Aurora-A protein also was subjected to a similar destabilization effect by ectopic expression of Az1 in HeLa cells. Endogenous CyclinB1 was not affected by the expression of Az1, while cyclinD1, a reported Az1 target was destabilized (**Fig.1B**). Exogenous addition of polyamines has been shown to upregulate the synthesis of functional Az1 through unique translational frame-shift mechanism [Rom and Kahana, 1994]. To verify whether higher endogenous levels of Az1 will have a similar destabilizing effect on Aurora-A, AT2.1 prostate carcinoma cells were treated with 10mM

putrescine to stimulate Az1 expression and Aurora-A levels were monitored. As shown in **Fig.1C**, higher endogenous levels of Az1 could destabilize both Aurora-A and cyclinD1 while cyclin A was spared. To demonstrate that the destabilizing effect of Az1 is indeed the degradation of Aurora-A through the proteasome-dependent pathway, Az1 and Aurora-A were coexpressed in HeLa cells in the presence of proteasome inhibitors MG132 and lactacystin and the destabilization of Aurora-A was followed. Treatment with proteasome inhibitors abolished the Az1-mediated destabilization of both transfected (**Fig.1D**) as well as endogenous (**Fig. 1E**) Aurora-A, suggesting that Az1 destabilize Aurora-A through proteasome-dependent pathway. It has been demonstrated that Az1 is capable of degrading its substrates *in vitro* in reticulocyte lysates, a rich source of proteasome. To demonstrate Az1-mediated destabilization of Aurora-A *in vitro,* degradation assays were performed *in vitro* in reticulocyte lysates as the source of proteasome. Az1 and Aurora-A proteins were synthesized separately in an *in vitro* coupled transcription/translation system and incubated together in the presence of an ATP-regenerating system. Wild type Aurora-A has been shown to be less stable in reticulocyte lysate presumably due to the presence of low levels of cdh1 in the lysates, which could degrade Aurora-A through proteasome-dependent pathway [Crane *et al*, 2004]. Hence, a stable A-box mutant of Aurora-A, which is recalcitrant to cdh1-mediated degradation, was used in the *in vitro* degradation assays. Preliminary studies using this A-box mutant and Az1 proteins showed that both proteins are relatively more stable in the lysates under the assay conditions. However, when combined together, Az1 led to lower levels of Aurora-A in a dose-dependent (**Fig. 1F**) and time-dependent manner (**Fig. 1G**) with faster kinetics (**Fig. 1H**).

Az1 could destabilize Aurora-A in the absence of ubiquitination

Az1 is known to target its substrates like ODC, cyclinD1 and Smad1 for proteasomal degradation in the absence of ubiquitination. To address the nature of Az1-mediated degradation of Aurora-A, we used the A-box mutant of Aurora-A, which is resistant to cdh1-mediated degradation [Littlepage and Ruderman, 2002]. We have earlier shown that

coexpression of Az1 and wild type Aurora-A resulted in the loss of Aurora-A protein. Similarly, when the cdh1-resistant A-box mutant of Aurora-A was coexpressed, Az1 was able to destabilize the A-box mutant of Aurora-A also with similar efficacy (**Fig. 2A**). This suggests that Az1 destabilizes Aurora-A in a manner distinct from cdh1. However, reversal of the destabilizing effect of Az1 on the A-box mutant of Aurora-A by the proteasomal inhibitors MG132 and lactacystin demonstrated that the Az1 effect on A-box mutant is still proteasome-dependent. To demonstrate whether Az1-mediated degradation of Aurora-A can occur in the absence of prior ubiquitination, Az1 and Aurora-A were coexpressed in ts20-CHO, a temperature-sensitive cell line lacking ubiquitin activating enzyme E1 at the restrictive temperature and thus defective in the ubiquitination of proteasomal substrates [Strous *et al.*, 1996]. Az1 was able to destabilize Aurora-A in the ts20-CHO cells even at the restrictive temperature (40^{o}C) as effectively as at the permissive temperature (30^{o}C) or in HeLa cells supporting the ubiquitin-independent nature of degradation (**Fig 2B**). Similar Az1-mediated ubiquitin-independent degradation of the ODC, positive control substrate, could also be demonstrated at the restrictive temperature (**supplementary Fig.1b**). To address whether the Az1-mediated destabilization of Aurora-A is due to increased turnover, a cycloheximide chase experiment was performed. Az1 and Aurora-A were coexpressed in ts20-CHO cells and Aurora-A turnover in the presence of cycloheximide was followed at both permissive and non-permissive temperatures. The results presented in **Fig. 2C** showed that Az1 induced faster turnover of Aurora-A at both permissive and non-permissive temperature suggesting that the destabilizing effect of Az1 could be mainly degradation. Efficient degradation of Aurora-A by Az1 even at restrictive temperature indicated that the Az1-mediated degradation of Aurora-A is ubiquitin-independent. It is noteworthy that the levels of Az1 itself is stabilized at the non-permissive temperature supporting the previous observation that antizyme is rapidly degraded through ubiquitin-dependent proteasomal activity [Gandre *et al*., 2002]. On the other hand, Az1 could not destabilize the CDK inhibitor, p27 or the tumor suppressor p53 at both temperatures (**Fig. 2D**), vouching for the specificity of Az1-mediated Aurora-A degradation through this pathway.

Az1 interacts with Aurora-A in vivo

The results presented in the previous sections suggest that Az1 targets Aurora-A for proteasomal degradation through ubiquitin-independent pathway. To further elucidate the role of Az1 as the targeting molecule, we investigated whether Az1 can interact with Aurora-A. Az1 and HA-tagged Aurora-A were coexpressed in HeLa cells, and pull-down assays were performed with antibodies against anti-HA tag antibody to pull down ectopically expressed Aurora-A. The result presented in **Fig. 3A** show that Az1 could be coprecipitated with Aurora-A suggesting an interaction between Az1 and Aurora-A. To address the specificity of interaction, an interaction domain mapping experiment was performed. To this end, deletion constructs lacking different regions of Aurora-A (**Fig.3B**) were coexpressed with Az1 and pull-down assays as described earlier were performed to map the region of Aurora-A that interacts with Az1. The results presented in **Fig. 3C**, show that a deletion of N-terminal 200 amino acids (ΔN600) and C-terminal 100 amino acids (ΔC300) does not interfere with the binding of Aurora-A with Az1. However, a further deletion of 100 amino acids from the C-terminus (ΔC600) of Aurora-A completely abolished the interaction with Az1 suggesting that the region spanning residues 203-303 of Aurora-A is involved in the interaction with Az1.

Residues R^{131} and G^{145} of Az1 are essential for the destabilization of Aurora-A

It has been demonstrated that N-terminus of ODC interacts with Az1 [Li and Coffino, 1992] and that the element contained within amino acids 130-145 of rat Az1 is essential for the targeting of ODC for proteasomal degradation [Chen et al., 2002]. To define whether this targeting domain of Az1 is involved in the targeting of Aurora-A for degradation, Az1 mutant, which lacks the N-terminal 120 amino acids (ΔN120-Az1) and Az1 which lacks only the amino acids 130-145 (Δ130-145-Az1) (**Fig.4A**) were made and used to study *in vivo* degradation of Aurora-A in HeLa cells as described earlier. ΔN120-Az1 mutant targeted

endogenous Aurora-A for degradation as effectively as the full length Az1. However, the Δ130-145-Az1, which lacked the 16 amino acid region was not competent enough to target Aurora-A protein for degradation (**Fig. 4B**). However, it is possible that the deletion of 16 amino acid residues from the middle of the protein could have hampered the functionality of the protein as predicted before [Hoffman *et al.*, 2005]. To rule out this possibility, a double mutant in which the residues R^{131} and G^{145} of human Az1 changed to aspartic acid (R131D/G145D) as described in [Chen et al., 2002] was also generated and tested for its efficiency of Aurora-A degradation. It has been shown that simultaneous conversion of R^{131} and A^{145} in rat Az1 to aspartic acid totally abolishes its degradative capacity. It should be noted that the arginine residue at 131 has been conserved between rat and human Az1 while the alanine at position 145 of rat Az1 has been replaced with glycine in human Az1. Despite this change, R131D/G145D double mutant of human Az1 showed decreased ability to target endogenous Aurora-A for degradation suggesting that these residues are essential for the degradation of Aurora-A also (**Fig.4B**). Coexpression of Az1 mutants with Aurora-A in HeLa cells followed by immunoblot analysis revealed that the Az1 mutants lacking the element present within amino acids 130-145 of human Az1 are less effective in targeting exogenous Aurora-A also for degradation (**Fig.4C**). These data reiterated the essential nature of the R^{131} and G^{145} residues in targeting Aurora-A for degradation. To further demonstrate that the failure of Δ130-145-Az1 or R131D/G145D-Az1 to degrade Aurora-A protein did not arise due to their inability to interact with Aurora-A, pull-down assays were performed as described earlier. However, the results presented in **Fig. 4D,** show that Δ130-145-Az1 and R131D/G145D-Az1 were as efficient as the full length Az1 in interacting with Aurora-A suggesting that the amino acid residues R^{130} and G^{145} of human Az1 are dispensable for the interaction while essential for the degradation of Aurora-A.

Functional link between Az1 and AURKAIP1

We have shown earlier that the negative regulator AURKAIP1 facilitates destabilization of Aurora-A through proteasome-dependent but ubiquitin-independent pathway [Lim and Gopalan, 2007]. The results presented here suggest that Az1 might play the targeting role in the ubiquitin-independent degradation of Aurora-A. To investigate whether there exists any functional link between AURKAIP1 and Az1, we exploited the antizyme inhibitor (AzI), a physiological regulator of the antizyme family [Mangold, 2006]. High AzI level downregulates antizyme-mediated degradation of ODC as well as polyamine transport [Coffino, 2001]. To investigate whether Az1 plays a targeting role in AURKAIP1-mediated degradation of Aurora-A, AzI was expressed alone or with AURKAIP1 and Aurora-A stability *in vivo* was followed as described earlier. The result presented in **Fig. 5A** show that the expression AzI alone had only a minimal effect on the stability of Aurora-A. However, it was able to counteract the destabilizing effect of AURKAIP1 suggesting the involvement of Az1 in the AURKAIP1-mediated degradation of Aurora-A. To exclude any possibility that reversal of AURKAIP1-dependent Aurora-A degradation by AzI could be Az1-independent or to show a direct involvement of Az1 in this pathway, the Aurora-A deletion mutant ΔC600-Aurora-A, which is defective in interacting with Az1, was employed to study the degradation of Aurora-A *in vivo*. While full-length as well as other deletion constructs such as ΔN300, ΔN600 and ΔC300 could be destabilized by AURKAIP1, the ΔC600 deletion was resistant to AURKAIP1-mediated degradation **Fig. 5B**. Together, the results presented above suggest a role for Az1 in AURKAIP1-mediated degradation of Aurora-A. To define a functional link between Az1 and AURKAIP1, any interaction between AURKAIP1 and Az1 was studied by pull-down assays as described earlier. We could not detect any direct interaction between AURKAIP1 and Az1 by pull down assays (data not shown). However, Az1 was found to coprecipitate with AURKAIP1 when both Az1 and TR-AURKAIP1 [truncated AURKAIP1, a more potent form of AURKAIP1 in interacting and targeting Aurora-A for degradation (Lim and Gopalan, 2007; Kiat et al., 2002)] were coexpressed with Aurora-A (**Fig. 5C**). This raised an intriguing possibility that Az1, AURKAIP1 and Aurora-A

could form a ternary complex. To further explore the existence of such a ternary complex in Aurora-A degradation, we studied the interaction between Az1 and Aurora-A in the presence and absence of AURKAIP1. Az1 showed a basal affinity towards Aurora-A in the absence of AURKAIP1. However, expression of AURKAIP1 enhanced the binding of Az1 to Aurora-A significantly (**Fig. 5D**) suggesting that AURKAIP1 might function upstream of Az1 promoting its interaction with Aurora-A and facilitate the Az1-mediated proteasomal targeting of Aurora-A.

DISCUSSION:

Multiple regulators of Aurora-A kinase stability have been described recently [Mao *et al*., 2004; Yu *et al*., 2005]. Most of them target Aurora-A through ubiquitin-dependent and proteasome-dependent degradation pathway. In contrast, we identified a negative regulator Aurora-A, AURKAIP1, which destabilizes Aurora-A in a proteasome-dependent but ubiquitin-independent pathway [Lim and Gopalan; 2007] and demonstrated for the first time that an ubiquitin-independent pathway exists for Aurora-A degradation. To provide a mechanistic explanation regarding how AURKAIP1 targets Aurora-A to the proteasome machinery in the absence of ubiquitination, we investigated antizyme, an established proteasome-targeting molecule in the ubiquitin-independent pathway. We have shown here that antizyme1 can also target Aurora-A for degradation through proteasome-dependent but ubiquitin-independent pathway. Both ectopic and polyamine-induced expression of Az1 can regulate the steady state levels of Aurora-A. Increased turnover of Aurora-A in the presence of Az1 under both *in vivo* and *in vitro* conditions and the restoration of the steady state levels of Aurora-A by proteasomal inhibitors supported the conclusion that Az1-mediated loss of Aurora-A is not an indirect effect of Az1 expression on Aurora-A levels but is proteasome-dependent degradation of Aurora-A. This is further supported by the direct interaction between Az1 and Aurora-A. Studies carried out using the A-box mutant of Aurora-A and temperature-sensitive cell line defective in ubiquitination at the restrictive temperature,

suggest that Az1-mediated degradation of Aurora-A is mechanistically different from the cdh1-dependent degradation of Aurora-A and is ubiquitin-independent.

Despite the dismal knowledge we have on the significance and the physiological relevance of this alternative pathway, the growing list of protein substrates [Asher *et al.*, 2005; Jin *et al.*, 2003; Sdek *et al.*, 2005] targeted to the proteasome in the absence of ubiquitination suggests that the ubiquitin-independent route to the proteasome is as important as the ubiquitin-dependent pathway. Antizyme-mediated degradation of ornithine decarboxylase, the first demonstrated prototype example of ubiquitin-independent degradation, is essential for the maintenance of polyamine homeostasis in cells [Li and Coffino, 1992]. Antizyme is capable of interacting with β subunit HsN3 of proteasome and this interaction might facilitate targeting of its substrates to the proteasome [Lin *et al.*, 2002]. It has been shown that the amino acid residues $R^{131}$and A^{145} of rat Az1 are essential for the degradation of ODC [Chen *et al.*, 2002]. Mutating similar residues in human Az1 downregulated the ability of Az1 to target Aurora-A also for degradation suggesting an important role for these residues in substrate degradation. This observation also increased the likelihood that these residues might be involved in the targeting of other substrates such as cyclinD1 and Smad1 [Lin *et al.*, 2002; Newman *et al.*, 2004]. It is yet to be shown whether these residues are indispensable for its interaction with the proteasome.

Higher levels of antizyme have been shown to be associated with decreased cell proliferation and hence antizyme has been envisaged as a tumor-suppressor [Fong et al., 2003; Iwata *et al.*, 1999]. It is tempting to speculate that the negative regulation of the oncogenic Aurora-A could be one of the manifestations of its tumor-suppressor functions. Recently, cyclinD1, a crucial regulator of cell cycle progression, has been described as the target of antizyme1. Antizyme-mediated downregulation of cyclinD1 by proteasome was proposed as a mean to cease cell proliferation with G1 arrest following antizyme up-regulation [Newman *et al.*, 2004]. Current knowledge on the expression and functions of Aurora-A does not support a

role as a mediator of polyamine-induced G1 arrest. However, antizymes have evolved as a family with different functions other than polyamine metabolism and transport. It has been shown that treatment of cells with the rare polyamine, agmatine, suppresses proliferation by frame-shift induction of antizyme and attenuation of cellular polyamine levels [Satriano *et al.*, 1998]. Moreover, HTC cells treated with agmatine showed a progressive accumulation of cells in G2/M phase of the cell cycle with no evident signs of apoptosis or necrosis [Gardini *et al.*, 2003]. Against this background, it is tempting to speculate that antizyme-mediated Aurora-A destabilization might be one of the multiple functions of antizyme on cell growth.

.

The identification of the Aurora-A binding proteins AURKAIP1 and Az1 as the regulators of the ubiquitin-independent degradation of Aurora-A, poses the obvious question, whether there are any functional link between these two proteins. The results presented in this communication **(Fig.5)** suggest that AURKAIP1 might act upstream of Az1 and promote its interaction with Aurora-A. Inhibition of Az1 functions by antizyme inhibitor or use of Aurora-A mutant defective in interacting with Az1 ameliorated AURKAIP1-dependent degradation of Aurora-A, predicting a mediator role for Az1 in this pathway. However, it is yet to be investigated whether antizyme could function independent of AURKAIP1 in the degradation of Aurora-A or despite its ability to target Aurora-A, Az1 should await for cue from AURKAIP1. In other words, the cellular context under which these pathways are operative is an intriguing future direction of investigation, which has the potential to unravel the mysteries of the physiology of AURKAIP as well as the significance of this alternative pathway of Aurora-A degradation. Based on the current knowledge on the mechanism of antizyme-mediated degradation where binding of antizyme to its protein substrate enhances the recognition and targeting of the substrate to the proteasome, we propose that AURKAIP1 functions upstream of Az1 promoting its interaction with Aurora-A and facilitates the Az1-mediated proteasomal targeting.

MATERIALS AND METHODS

Plasmids

$p27^{Kip1}$ wt-pFLAG-N3 was obtained from Christoph Geisen, The Burnham Institute, USA. FLAG-tagged mouse ODC-pCDNA3 was obtained from Phillip Coffino, University of California, USA.

The cDNA encoding the wild type human antizyme1 was obtained by RT-PCR (Promega) of the RNA prepared from putrescine-treated HeLa cells using the primers: Az1-Forward (5'gaggaattcatggtgaaatcctccctgcagcg3') and Az1-Rev (5'gcactcgagctactcctcctcctcccgaa gactctctc3'). The human antizyme inhibitor (AzI) cDNA was also obtained by RT-PCR of normal HeLa cell RNA using the primers: AzI-Forward (5'gaattcatgaaaggatttattgatgatgc3') and AzI-Rev (5'ctcgagttaagcttcagcggaaaagctg3'). Various epitope tags (His, HA, FLAG) were added to the cDNAs by PCR and cloned into the pCDNA3 (Invitrogen) and pIRES (Clontech) vectors. The frame-shift mutant of Az1 was generated by site-directed mutagenesis using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) according to the manufacturer's protocol. The mutagenic oligonucleotide used is 5'gtggtgctccgatgcccctc3'. All Az1 deletion mutants were constructed by PCR. ΔN120-AZ1 mutant cDNA was amplified using the designed primer pairs spanning the region of Az1 from amino acid residue 121 to 229 (stop codon). FLAG tag was subsequently added to the N terminus by second PCR. The Δ130-145-Az1 mutant, an internal deletion of 16 amino acids from residue 130 to 145 was generated by nested PCR amplification. During this amplification, the 48 bp sequences corresponding to the region between amino acid residues from 130 –145 were replaced with sequences containing *Sma*1 and *EcoR*1 restriction sites without compromising the reading frame. FLAG-Az1 in pCDNA3 (full length, frameshifted mutant) was used as the PCR template. Two individual primary PCRs were set up; one reaction amplifying from 5' end of FLAG-Az1 cDNA (T7 primer) till amino acid residue 129 (gene-specific primer with *Sma*I and *EcoR*I sites at the 5' end) and the second reaction amplifying from amino acid residue 146 (gene-specific primer with *Sma*I and *EcoR*I sites at the 5' end) till 3' end of FLAG-Az1 cDNA (SP6 primer). The two overlapping (*Sma*I and *EcoR* I regions) primary PCR products

were gel purified and mixed in equal ratio and the Δ130-145 Az1 mutant cDNA was amplified by secondary PCR using the primer pair flanking the FLAG tag at the 5' end to the stop codon of Az1. The double mutant R131D/G145D was also generated using a two-step PCR. Desired mutations have been incorporated into the gene-specific primers used for the primary PCR amplification. The sequence of the gene-specific primers used in the primary PCR are R131D-R: 5'gtagaggctgccgccactcagcactgtgtcccagttaatgcg3' and G145D-F: 5'ggcgg cagcctctacatcgagatcccggacggcgcgctgc3'. FLAG tagged wild type Az1 in pCDNA3 was used as the template in two PCR reactions: one with T7 and R131D-R primers and the other with SP6 and G145D-F primers. The primary PCR products were gel purified, mixed and subjected to second PCR using the primer pair flanking the FLAG tag at the 5' end to the stop codon of Az1.

Antibodies

The specific antibodies used in this study are as follows: Anti-β-tubulin (mouse monoclonal, 1:1000, Sigma), Anti-Cyclin A (rabbit polyclonal, 1:500, Santa Cruz), Anti-Cyclin D1 (mouse monoclonal, 1: 200, Santa Cruz), Anti-Cyclin B1 (rabbit polyclonal, 1: 3000, Santa Cruz), Anti-Az1 (rabbit polyclonal, 1: 2000, a gift from John Mitchell, Northern Illinois University), Anti-FLAG M2 (mouse monoclonal, 1: 2000, Stratagene), Anti-FLAG (rabbit polyclonal, 1:3000, Sigma), Anti-HA (mouse monoclonal, 1:2000, Sigma), Anti-HA (rabbit polyclonal, 1:400, Santa Cruz), Anti-His (mouse monoclonal, 1:1000, Sigma), Aurora-A (anti-IAK1, mouse monoclonal, 1:2000, Pharmingen) and anti-p53 (mouse monoclonal,1:2000, Santa Cruz). All HRP-conjugated secondary antibodies (Pierce) were used at 1:8000 dilution.

Cell Culture, Transfection and Drug Treatment

The AT2.1 Dunning rat prostate carcinoma cells were obtained from Dr. John T Isaacs, Baltimore, USA and maintained at 37^{o}C in RPMI 1640 medium supplemented with 250 nM Dexamethasone (Sigma). ts20 Chinese Hamster cell line, which harbors the temperature-sensitive mutation in E1 ubiquitin-activating enzyme, were obtained from Dr. Ger J Strous,

Utrecht, Netherlands and maintained at 30^{o}C in α-MEM medium supplemented with 4.5g/L glucose. HeLa cells were maintained in RPMI 1640 medium. All mediums were supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids.

For transient transfection, HeLa and ts20-CHO cells were plated at the density of 1 x 10^6 and 2.5 x 10^6 per 60 mm dish one day prior to transfection. On the day of transfection, the cells were transfected with 3 μg of plasmid DNA using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Due to very high level of transgene expression, the p27 ^{kip1}-expressing construct was trasfected with only 0.3 μg (1/10^{th} of 3 μg normally used) of plasmid DNA .

For proteasomal inhibition, the cells were treated with 20 μM of MG132 (Sigma) or 20 μM lactacystin (Calbiochem). For induction of endogenous antizyme expression, the AT2.1 cells were treated with 10 mM putrescine (Sigma) for 24 hours.

Cell Lysis, Immunoblotting and Immunoprecipitation

Harvested cells were washed twice with ice cold PBS and lysed in 1x Laemmli Buffer (25mM Tris Base, 192 mM Glycine, 0.1% SDS), followed by pulsed sonication (Vibra Cell, Sonics & Materials Inc.; 5 x 5 secs with 10 sec interval) on ice and subsequently cleared by centrifugation at 16000 x g for 10 min at 4^{o}C. The protein concentrations of the lysates were assayed using Bio-Rad Protein Assay Reagent (Pierce). 50-100 μg proteins were separated on a 10 or 12% SDS-PAGE. The proteins were subsequently transferred to the nitrocellulose membrane (Gelman Laboratory). After blocking with 5% non-fat milk in TBS, the blots were incubated with various antibodies at their optimal dilutions overnight at 4^{o}C. The horseradish peroxidase (HRP)-conjugated secondary antibodies [goat anti-rabbit HRP & goat anti-mouse HRP (Pierce)] were diluted in blocking buffer and incubated with the blot for 1 hour at room temperature. The conjugated secondary antibodies were detected by SuperSignal Pico or Dura Chemiluminescence (Pierce) detection system. To quantify the signal intensities in the

immunoblots, ImageJ (version 1.36b) software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006) was used.

For immunoprecipitation, cells were lysed for 15 min on ice in lysis buffer (1 X TBS, 10% glycerol, 1% Nonidet P-40) containing protease inhibitors cocktail (Roche). The lysates were cleared by centrifugation at 16000 x g for 10 min at 4^{o}C. After measuring the protein concentration of the lysates as described above, the lysates were precleared by incubation with 50 μl of 50% slurry of protein G-agarose (Sigma) for one hour at 4^{o}C. Antibodies were coupled to Protein G-agarose by incubation for one hour at 4^{o}C and the precleared lysates were mixed with antibody-coupled protein G-agarose and rotated for two hours at 4^{o}C. Immune complexes were washed twice with buffer I (1xTBS, 10% glycerol, 0.5% Nonidet P-40, 1% bovine serum albumin) and twice with buffer II (1xTBS, 10% glycerol, 0.5% Nonidet P-40). The immune complexes were solubilized with the sample buffer and subjected to immunoblot analysis. Typically, a total of 500 μg of lysate protein were used for immunoprecipitation experiments while 50 μg of the total lysates were used for immunoblot analysis of the total lysates.

In vitro Protein Degradation Assay

Aurora-A and Az1 were translated separately *in vitro* using reticulocyte-based TNT T7 Quick-Coupled Transcription/Translation system (Promega). The Aurora-A expressing lysates were mixed with antizyme expressing lysates at 1:3 ratio and incubated in a assay buffer containing 50 mM Tris-HCl, pH 7.5; 2 mM DTT with 1X Energy Regeneration Solution (Boston Biochem, USA) for up to 4 hours at 37^{o}C. The reactions were stopped at different time points by addition of an equal volume of 2X SDS-PAGE sample buffer (0.24M Tris, pH 6.8, 2.5% SDS, 20% glycerol, 8% β-mercaptoethanol) and boiled prior to analysis by SDS-PAGE.

ACKNOWLEDGEMENT:

We thank Dr. Ger. J. Strous and Dr. Issacs for providing us with the ts20-CHO and AT2.1 rat prostate carcinoma cell lines respectively. We also thank Dr.Geisen, Dr. Coffino and Dr. Prochownik for the p27kip1, mouse ODC and cyclin B1 expression plasmids respectively. We would like to acknowledge the generosity of Dr. John Mitchell for the anti-antizyme antibody. This work was supported by the National Medical Research Council of Singapore in the form of a research grant (NMRC/0815/2003) to G.G and as Institutional Block Grant to National Cancer Centre, Singapore.

REFERENCES:

Asher, G., Tsevetkov, P., Kahana, C. and Shaul, Y (2005) A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73.*Genes Dev*. **19**, 316-321.

Bercovich Z., Rosenberg-Hasson Y., Ciechanover A., Kahana C (1989) Degradation of ornithine decarboxylase in reticulocyte lysate is ATP-dependent but ubiquitin-independent *J. Biol. Chem.* **264**, 15949-15952

Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B, Schryver B, Flanagan P, *et.al.*.(1998) A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. *EMBO J*. **17**, 3052-3065.

Buschhorn HM, Klein RR, Chambers SM, Hardy MC, Green S, Bearss D, Nagle RB (2005) Aurora-A over-expression in high-grade PIN lesions and prostate cancer *Prostate* **64**, 341-346.

Chen, H., MacDonald, A. and Coffino, P (2002) Structural elements of antizymes 1 and 2 are required for proteasomal degradation of ornithine decarboxylase.*J. Biol. Chem*. **277**, 45957-45961.

Coffino, P (2001) Regulation of cellular polyamines by antizyme. *Nature Rev.* **2**, 188-194

Crane, R., Klofper, A and Ruderman, J.V (2004) Requirements for the destruction of human Aurora-A. *J. Cell Sci.* **117**, 5975-5983.

Feith, D.J., Shantz, L.M. and Pegg, A.E (2001). Targeted antizyme expression in the skin of transgenic mice reduces tumor promoter induction of ornithine decarboxylase and decreases sensitivity to chemical carcinogenesis. *Cancer Res.* **61**, 6073-6081.

Fong, L.Y., Feith, D.J. and Pegg, A.E (2003) Antizyme overexpression in transgenic mice reduces cell proliferation, increases apoptosis, and reduces N-nitroso methyl-benzylamine-induced forestomach carcinogenesis. *Cancer Res*. **63**, 3945-3954.

Gandre, S., Bercovich, Z. and Kahana, C (2002) Ornithine decarboxylase-antizyme is rapidly degraded through a mechanism that requires functional ubiquitin-dependent proteolytic activity *Eur. J. Biochem.* **269**, 1316-1322.

Gardini, G., Cravanzola, C., Autelli, R., Testore, G., Cesa, R., Morando, L., S *et.al.* (2003) Agmatine inhibits the proliferation of rat hepatoma cells by modulation of polyamine metabolism. *J. Hepatol*. **39**, 793-799.

Gerner, E.W. and Meyskens, F.L Jr. (2004). Polyamines and cancer: old molecules, new understanding. *Nat. Rev. Cancer*. **4**, 781-792.

Glass J.R., and Gerner E.W (1987) Spermidine mediates degradation of ornithine decarboxylase by a non-lysosomal, ubiquitin-independent mechanism *J. Cell Physiol* .**130**, 133-141.

Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, *et al.*(2003) Activation and overexpression of centrosome kinase BTAK/Aurora-A in human ovarian cancer *Clin Cancer Res.* **9**, 1420-1426.

Hoffman, D.W., Carroll, D., Martinez, N. and Hackert, M.L (2005) Solution structure of a conserved domain of antizyme: A protein regulator of polyamines *Biochemistry*, **44**, 11777-11785.

Honda K, Mihara H, Kato Y, Yamaguchi A, Tanaka H, Yasuda H, Furukawa K, Urano T (2000) Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway *Oncogene* **19**, 2812-2819.

Ivanov, I.P., Gesteland, R.F. and Atkins. J.F (2000) Antizyme expression: a subversion of triplet decoding, which is remarkably conserved by evolution, is a sensor for an autoregulatory circuit. *Nucleic. Acid. Res*. **28**, 3185-3196.

Iwata, S., Sato, Y., Asada, M., Takagi, M., Tsujimoto, A., Inaba, T., Yamada, T., Sakamoto, S., Yata, J., Shimogori, T., Igarashi,K. and Mizutani, S (1999) Anti-tumor activity of antizyme which targets the ornithine decarboxylase (ODC) required for cell growth and transformation *Oncogene* **18**, 165-172.

Jin, Y., Lee, H., Zeng, S.X., Dai, M.S. and Lu, H (2003) MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation. *EMBO J*. **22**, 6365-6377.

Kiat, L.S., Hui, K.M. and Gopalan, G (2002) Aurora-A kinase interacting protein (AIP), a novel negative regulator of human Aurora-A kinase. *J. Biol. Chem.* **277**, 45558-45565.

Koike, C., Chao, D.T. and Zetter, B.R (1999) Sensitivity to polyamine-induced growth arrest correlates with antizyme induction in prostate carcinoma cells *Cancer Res.* **59**, 6109-6112.

Li D, Zhu J, Firozi PF, Abbruzzese JL, Evans DB, Cleary K, Friess H, and Sen S (2003) Overexpression of oncogenic STK15/BTAK/Aurora A kinase in human pancreatic cancer *Clin Cancer Res.***9**, 991-997.

Li X., and Coffino, P (1993) Degradation of ornithine decarboxylase: exposure of the C-terminal target by a polyamine-inducible inhibitory protein *Mol. Cell. Biol* **13**, 2377-2383.

Lim, S.K. and Gopalan, G. (2007) Aurora-a kinase interacting protein 1 (aurkaip1) promotes Aurora-a degradation through alternative ubiquitin-independent pathway. *Biochem. J*. In press

Lin, Y., Martin, J., Gruendler, C., Farley, J., Meng, X., Li, B.Y., Lechleider,R., Huff, C., Kim, R.H., Grasser, W.A., Paralkar, V. and Wang, T(2002) A novel link between the proteasome pathway and the signal transduction pathway of the bone morphogenetic proteins (BMPs). *BMC cell Biol.* **3**, 15

Littlepage LE, Ruderman JV (2002) Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit *Genes Dev.* **16**, 2274-85.

Mangold, U (2006) Antizyme inhibitor: mysterious modulator of cell proliferation *Cell Mol. Life Sci.* **63**, 2095-2101.

Mao, J.H., Perez-Losada, J., Wu, D., Delrosario, R., Tsunematsu, R., Nakayama, K.I., Brown, K, Bryson, S. and Balmain,A (2004) Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene *Nature* **432**, 775-779.

Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., Ichihara, A (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination *Nature* **360**, 597-599.

Newman, R.M., Mobascher, A., Mangold, U., Koike, C., Diah, S., Schmidt, M., Finley, D. and Zetter, B.R (2004) Antizyme targets cyclin D1 for degradation. A novel mechanism for cell growth repression *J.Biol. Chem.* **279**, 41504-41511.

Rom, E. and Kahana, C (1994) Polyamines regulate the expression of ornithine decarboxylase antizyme in vitro by inducing ribosomal frame-shifting *Proc. Natl. Acad. Sci*.USA **91**, 3959-3963.

Rosenberg-Hasson Y., Bercovich Z. , Ciechanover A., Kahana, C (1989) Degradation of ornithine decarboxylase in mammalian cells is ATP dependent but ubiquitin independent *Eur. J. Biochem*. **185**, 469-474.

Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, Masuda K, Shimomura K, Nakamura Y, InAz1awa J, Abe T, Yamagishi H (2001) Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. *Br J Cancer* **84**, 824-831.

Satriano, J., Matsufuji, S., Murakami, Y., Lortie, M.J., Schwartz, D. and Kelly, C.J (1998) Agmatine suppresses proliferation by frameshift induction of antizyme and attenuation of cellular polyamine levels *J. Bio. Chem*. **273**, 15313-15315.

Sdek,P., Ying, H., Chang, D.L.F., Qiu. W., Zheng, H., Touitou, R., Allday, M.J.and Xiao, Z.J (2005) MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein *Mol. Cell* **20**: 699-708

Strous G.J., van Kerkhof, P., Govers, R., Ciechanover, A. and Schwartz, A.L (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor *EMBO J.* **15**, 3806-3812.

Taguchi S, Honda K, Sugiura K, Yamaguchi A, Furukawa K, Urano T.(2002) Degradation of human Aurora-A protein kinase is mediated by hCdh1 *FEBS Lett*. **519**, 59-65.

Tanaka T, Kimura M, Matsunaga K, Fukada D, Mori H, Okano Y (1999) Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast *Cancer Res.* **59**, 2041-2044.

Tsuji, T., Usui, S., Aida, T., Tachikawa, T., Hu, G. F., Sasaki, A., Matsumura, T., Todd, R., and Wong, D. T (2001) Induction of epithelial differentiation and DNA demethylation in hamster malignant oral keratinocyte by ornithine decarboxylase antizyme *Oncogene* **20,** 24–33.

Walter AO, Seghezzi W, Korver W, Sheung J, Lees E (2000) The mitotic serine/threonine kinase Aurora2/AIK is regulated by phosphorylation and degradation *Oncogene* **19**, 4906-4916.

Yu, X., Minter-Dykhouse, K., Malureanu, L. Zhao, W.M., Zhang, D., Merkle, C.J., Ward, I.M., Saya, H., Fang, G., van Deursen, J. and Chen, J (2005) Chfr is required for tumor suppression and Aurora A regulation *Nat. Genet*. **37**, 401-406.

Zhang, M., Pickart, C.M., and Coffino, P (2003) Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate *EMBO J*. **22**, 1488-1496

Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR, Sen S (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation *Nat Genet.* **20**, 189-193.

TITLES AND LEGENDS TO FIGURES:

Figure 1: Az1 targets Aurora-A for proteasomal degradation. ***A.*** HeLa or CHO cells were co-transfected with HA-tagged Aurora-A and His-tagged Az1 at 1:9 ratios. A negative control has been included in which Az1 plasmid was replaced with the empty vector. Thirty-six hours post-transfection, the cells were harvested for western blot analysis of exogenous Aurora-A protein stability. Aurora-A and Az1 were detected using anti-HA and anti-Az1 antibodies, respectively. β tubulin was used as the loading control. ***B.*** HeLa cells were transfected with either empty vector or His-tagged Az1. Thirty-six hours post-transfection, cell lysates were prepared for western blot analysis of the endogenous Aurora-A protein. Aurora-A and Az1 was detected using anti-IAK1 and anti-Az1 antibodies, respectively. Cyclin D1 and cyclin B1 were detected as the positive and negative control respectively. β tubulin was used as the loading control. ***C.*** AT 2.1 cells were treated with 10 mM of putrescine for 24 hours prior to harvest for western blot analysis of endogenous Aurora-A with anti-IAK1 antibody. Cyclin D1 and cyclin A were detected with their respective antibodies. Induction of endogenous Az1 was followed with anti-Az1 antibody. β -tubulin was used as the loading control. ***D.*** HeLa cells were co-transfected with HA-tagged wild type Aurora-A and His-tagged Az1 at 1:9 ratios. A negative control has been included in which Az1 plasmid was replaced with the empty vector. Twenty-four hours post-transfection, cells were treated with either DMSO or 20 μM MG132 or lactacystin for 16 hours. The cells were harvested and analyzed for Aurora-A and Az1 proteins. β-tubulin was used as the loading control. ***E***. HeLa cells were transfected with His-tagged Az1 or empty vector. Twenty-four hours after transfection, cells were treated with either DMSO or 20 μM MG132 or lactacystin for another 16 hours before analyzing for the levels of endogenous Aurora-A and expression of Az1. β-tubulin was detected as the loading control. ***F***. HA-tagged A-box mutant of Aurora-A and Az1 were individually synthesized by coupled *in vitro* transcription/translation in rabbit reticulocyte extracts. Aurora-A and Az1-containing lysates

were mixed at different ratios in the presence of ATP-regenerating system and incubated for two hours at 37ºC. Stability of Aurora-A was assessed by immunoblot analysis. Aurora-A and Az1 were detected using the anti-HA and anti-Az1 antibodies. ***G.*** HA-tagged, A Box Mutant of Aurora-A and Az1 were individually synthesized by coupled *in vitro* transcription/translation in rabbit reticulocyte extracts. Aurora-A and Az1-containing extracts were mixed at 1:3 ratios and incubated with an ATP-regenerating system at 37ºC. Samples were withdrawn at defined intervals and the stability of Aurora-A at different time points was assessed by immunoblot analysis. Az1 was detected using anti-Az1 antibody. ***H.*** The Aurora-A levels (signal intensities) in the presence and absence of Az1 at different time points (immunoblot from Fig. 1G) were quantified using ImageJ software and plotted against time. The intensities at zero time points were assumed as 100 percent. Control (□) and Az1 (•).

Figure 2: Az1 destabilizes Aurora-A in the absence of ubiquitination. ***A.*** HeLa cells were co-transfected with HA-tagged A-box mutant of Aurora-A and His-tagged Az1 at 1:9 ratios. Vector control has been included in which Az1 plasmid has been replaced with pCDNA3. Twenty-four hours post-transfection, cells were treated with either DMSO or 20 μM MG132 or 20 μM lactacystin for 16 hours. The cells were harvested and analyzed for Aurora-A and Az1 using anti-HA and anti-Az1 antibodies. β–tubulin was used as the loading control. ***B.*** ts20-CHO cells were co-transfected with HA-tagged Aurora-A and either empty vector pCDNA3 or His-tagged Az1 at 1:9 ratios. Twenty-four hours post-transfection, cells were divided into two sets; one set was maintained at 30ºC, while the other set was incubated at 40ºC for 16 hours. The cells were harvested and analyzed for Aurora-A and Az1 using the anti-HA and anti-Az1 antibodies. β-tubulin was used as the loading control. ***C.*** ts20-CHO cells were co-transfected with HA-tagged Aurora-A and either pCDNA3 (vector) or His-tagged Az1 at 1:5 ratios. Eight hours post-transfection, one set of transfected cells was maintained at 30ºC while the other set was shifted to 40ºC. At 24 hours post-transfection, both sets were treated with 50 μg/ml cycloheximide for the indicated times and harvested to

assess Aurora-A protein turnover by immunoblot analysis. β–tubulin was detected as the loading control. ***D***. ts20-CHO cells were co-transfected with FLAG-tagged p27 or mouse p53 and His-tagged Az1 at 1:9 ratios. Vector control has been included in which Az1 plasmid has been replaced with pCDNA3. Twenty-four hours post-transfection, cells were divided into two sets; one set was maintained at 30^{o}C while the other set was incubated at 40^{o}C for 16 hours. The cells were harvested and analyzed for p27, p53 and Az1 expression using anti-FLAG M2, anti-p53 and anti-Az1 antibodies, respectively. β-tubulin was used as the loading control.

Figure 3: Az1 interacts with Aurora-A in vivo. A. HeLa cells were co-transfected with His-tagged Az1 and either empty vector or FLAG-tagged Aurora-A at 1:1 ratio. Twenty-four hours post-transfection, cells were harvested for pull down with anti-FLAG antibody. The interacting Az1 was detected using the anti-Az1 antibody. Aurora-A was detected using the rabbit anti-FLAG antibody. ***B.*** Comparison of the size and location of the deletions of all the Aurora-A deletion mutant proteins with full length Aurora-A protein. All of the Aurora-A variants contain a FLAG tag at the N-terminus. The *numbers* within the *parentheses* denote the nucleotides of Aurora-A cDNA, and number 1 corresponds to the nucleotide A of the translational start ATG. The locations of KEN, A, and D (D1,D2,D3) boxes are indicated. ***C.*** HeLa cells were co-transfected with His-tagged Az1 and FLAG-tagged wild type or various deletion mutants of Aurora-A at 1:1 ratio. A negative vector control has been included in which Aurora-A plasmid was replaced with the empty vector. Twenty-four hours post-transfection, cells were harvested for pull down with mouse anti-Flag antibody. The interacting Az1 was detected using the rabbit anti-Az1 antibody and Aurora-A was detected using the rabbit anti-FLAG antibody.

Figure 4: Amino acid residues R^{131} and G^{145} of human Az1 are essential for the destabilization of Aurora-A. A. Comparison of the size and location of the deletions of the Az1 deletion mutant proteins with full length Az1 protein is presented. All of the Az1 variants

contain a FLAG tag at the N-terminus. The numbers within parentheses denote the amino acids of the Az1 protein, and number 1 corresponds to the translation start methionine. ***B***. HeLa cells were transfected with either empty vector or wild type/mutants of Az1. Thirty-six hours post-transfection, cells were harvested for western blot analysis of the endogenous Aurora-A in the presence of overexpressed wild type or mutant Az1. The Aurora-A and Az1 were detected using rabbit anti-IAK1 antibody and mouse anti-FLAG M2 antibody, respectively. β–tubulin was used as the loading control. ***C.*** HeLa cells were co-transfected with HA-tagged Aurora-A and either empty vector or wild type or mutants of Az1 at 1:9 ratios. Thirty-six hours post-transfection, the transfected cells were harvested for western blot analysis of the protein stability of Aurora-A in the presence of overexpressed wild type or mutant Az1. The Aurora-A and Az1 was detected using rabbit polyclonal anti-HA antibody and mouse anti-FLAG M2 antibody, respectively. β–tubulin was used as the loading control. ***D.*** HeLa cells were co-transfected with HA-tagged Aurora-A and either empty vector or FLAG-tagged wild type/deletion mutant of Az1 at 1:1 ratio. Twenty-four hours post-transfection, the transfected cells were harvested for pull down with mouse anti-FLAG antibody. The interacting Aurora-A was detected using rabbit polyclonal anti-HA antibody. Both wild type and deletion mutants of Az1 were detected using the rabbit anti-FLAG antibody.

Figure 5: Functional link between Az1 and AURKAIP1. ***A.*** ts20-CHO cells were co-transfected with HA-tagged Aurora-A and (i) pIRES (Lane 1); (ii) pIRES- [FLAG-TR-AURKAIP1] (Lane2); (iii) [HA-AzI]-pIRES (Lane3); (iv) [HA-AzI]-pIRES- [Flag TR-AURKAIP1] (Lane 4) at 1:9 ratio. Thirty-six hours post-transfection, transfected cells were harvested for western blot analysis to assess the stability of Aurora-A kinase in the presence of TR-AURKAIP1 or AzI or both. Both Aurora-A and AzI were detected using the anti-HA antibody and TR-AURKAIP1 was detected using the anti-FLAG M2 antibody. β tubulin was used as the loading control. ***B.*** ts20-CHO cells were co-transfected with HA-tagged TR-AURKAIP1 and FLAG-tagged wild-type or various deletion mutants of Aurora-A at 5:1 ratio. Thirty-six hours post-transfection, the transfected cells were harvested for western blot

analysis to assess the stability of both wild type and mutant Aurora-A, in the presence of TR-AURKAIP1. The Aurora-A and TR-AURKAIP1 was detected using the anti-FLAG M2 and anti-HA mouse monoclonal antibodies, respectively. β tubulin was used as the loading control. ***C.*** HeLa cells were co-transfected with His-tagged Az1, HA-tagged Aurora-A and FLAG-tagged TR-AURKAIP1 at 1:1:1 ratio. A negative control has been included in which TR-AURKAIP1 plasmid was replaced with the empty vector. Twenty-four hours post-transfection, the transfected cells were harvested for immunoprecipitation with mouse anti-FLAG antibody. The interacting Az1 and Aurora-A were detected using the anti-Az1 and anti-HA antibodies, respectively. TR-AURKAIP1 was detected using the rabbit anti-FLAG antibody. ***D.*** HeLa cells were co-transfected with His-tagged Az1 and FLAG-tagged Aurora-A at 1:1 ratio in the absence or presence of HA-tagged TR-AURKAIP1. Twenty-four hours post-transfection, the transfected cells were harvested for immunoprecipitation with mouse anti-FLAG M2 antibody. The interacting Az1 was detected using the anti-Az1 antibody. Aurora-A and TR-AURKAIP1 were detected using the anti-FLAG and anti-HA antibodies respectively.

Fig.1

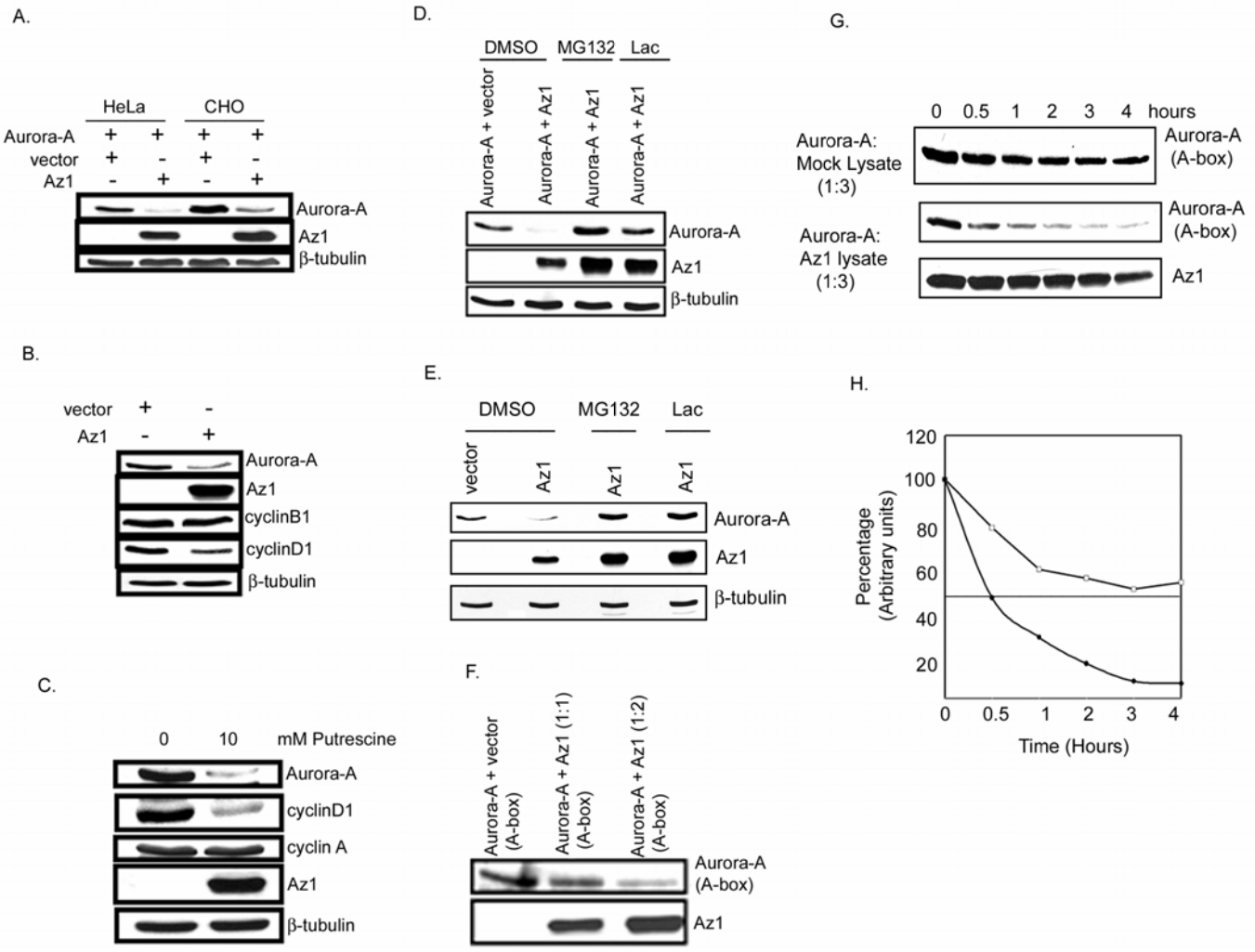

A.
HeLa
CHO
Aurora-A + + + +
vector + - + -
Az1 - + - +
Aurora-A
Az1
β-tubulin
B.
vector + -
Az1 - +
Aurora-A
Az1
cyclinB1
cyclinD1
β-tubulin
C.
0 10 mM Putrescine
Aurora-A
cyclinD1
cyclin A
Az1
β-tubulin
D.
DMSO MG132 Lac
Aurora-A + vector
Aurora-A + Az1
Aurora-A + Az1
Aurora-A + Az1
Aurora-A
Az1
β-tubulin
E.
DMSO MG132 Lac
vector
Az1
Az1
Az1
Aurora-A
Az1
β-tubulin
F.
Aurora-A + vector (A-box)
Aurora-A + Az1 (1:1) (A-box)
Aurora-A + Az1 (1:2) (A-box)
Aurora-A (A-box)
Az1
G.
0 0.5 1 2 3 4 hours
Aurora-A: Mock Lysate (1:3)
Aurora-A (A-box)
Aurora-A: Az1 lysate (1:3)
Aurora-A (A-box)
Az1
H.
Percentage (Arbitrary units)
120
100
80
60
40
20
0 0.5 1 2 3 4
Time (Hours)

Fig.2

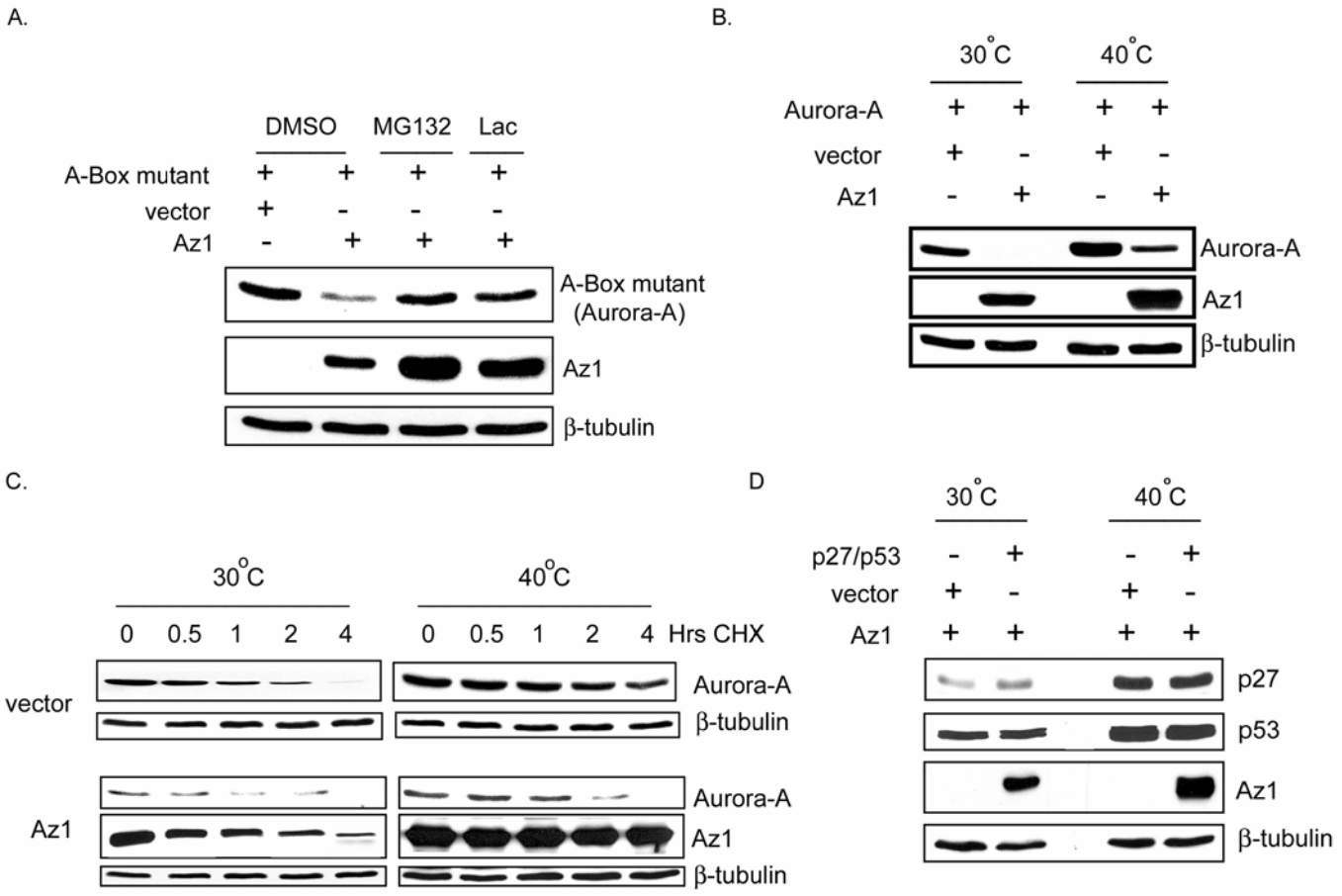
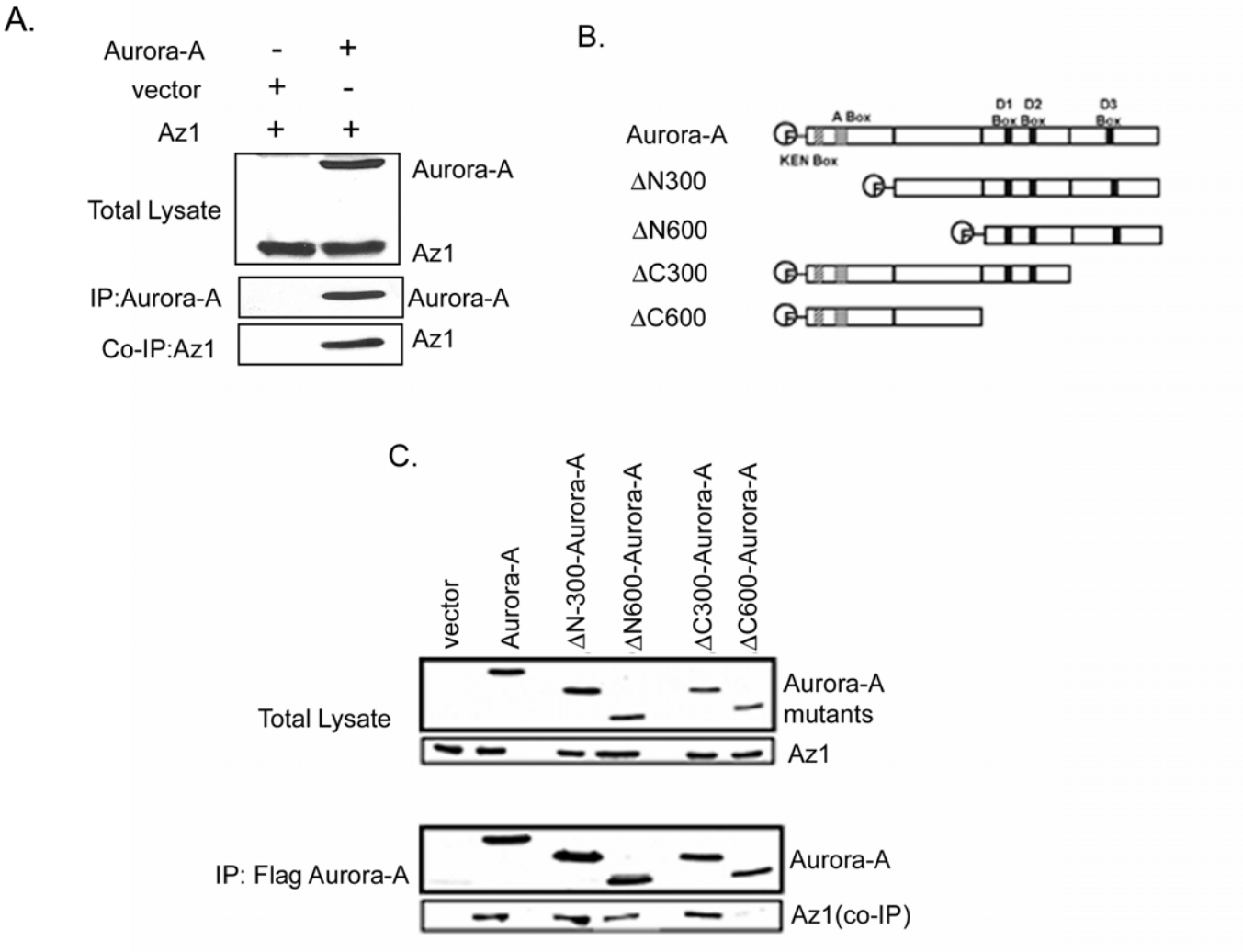

A.
DMSO
MG132
Lac
A-Box mutant
vector
Az1
A-Box mutant (Aurora-A)
Az1
β-tubulin
B.
30°C
40°C
Aurora-A
vector
Az1
Aurora-A
Az1
β-tubulin
C.
30°C
40°C
0 0.5 1 2 4 0 0.5 1 2 4 Hrs CHX
vector
Aurora-A
β-tubulin
Az1
Aurora-A
Az1
β-tubulin
D
30°C
40°C
p27/p53
vector
Az1
p27
p53
Az1
β-tubulin

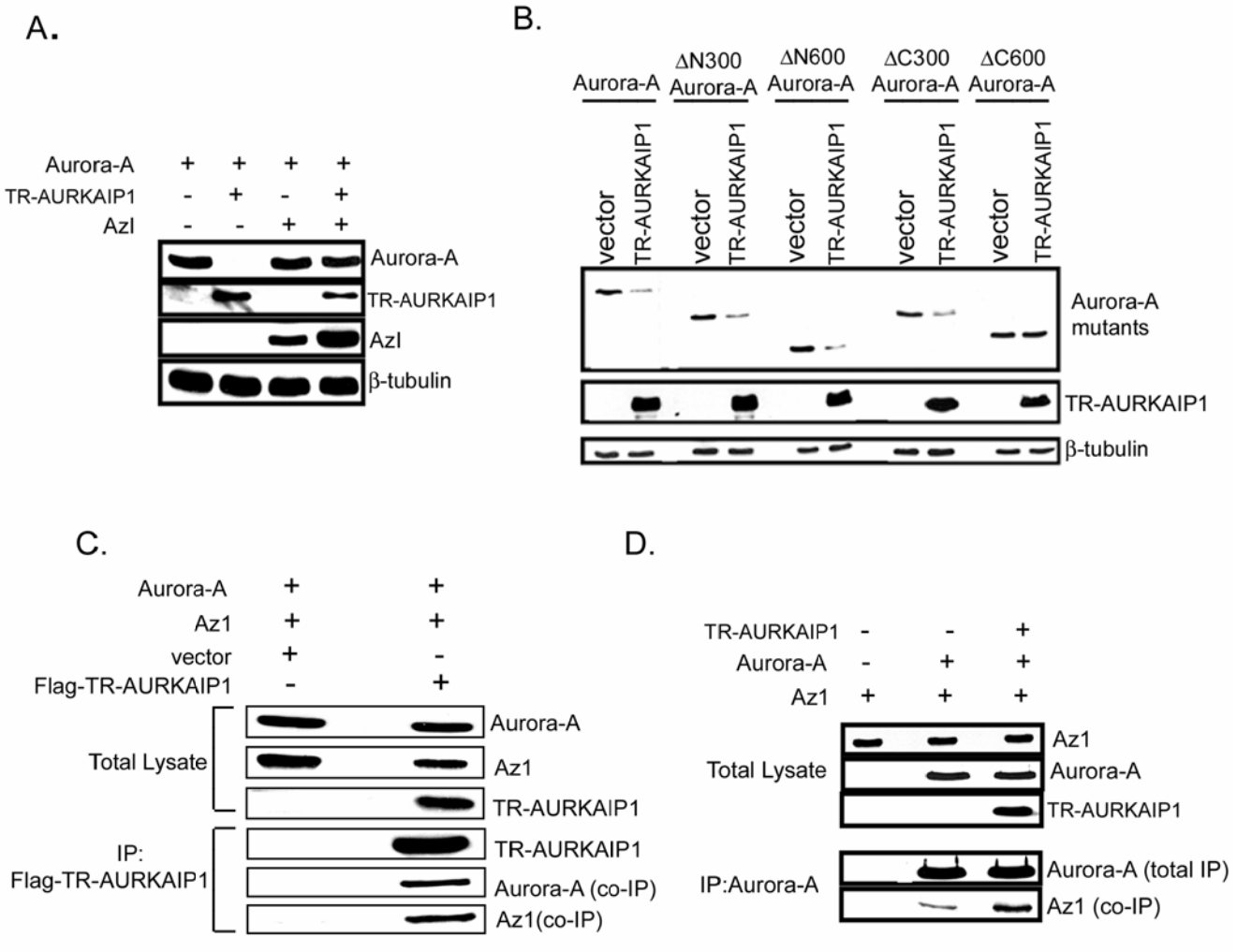
Fig.3


A.
Aurora-A - +
vector + -
Az1 + +
Total Lysate
Aurora-A
Az1
IP:Aurora-A
Aurora-A
Co-IP:Az1
Az1
B.
A Box
D1 Box
D2 Box
D3 Box
KEN Box
Aurora-A
ΔN300
ΔN600
ΔC300
ΔC600
C.
vector
Aurora-A
ΔN-300-Aurora-A
ΔN600-Aurora-A
ΔC300-Aurora-A
ΔC600-Aurora-A
Total Lysate
Aurora-A mutants
Az1
IP: Flag Aurora-A
Aurora-A
Az1(co-IP)

A.
F
Az1(1-228 aa)
ΔN120-Az1(121-228 aa)
Δ130-145-Az1
B.
vector
Az1
ΔN130-145-Az1
R131D/G145D-Az1
ΔN120-Az1
Aurora-A
Az1 mutants
β-tubulin
C.
vector
Az1
R131D/G145D-Az1
ΔN130-145-Az1
ΔN120-Az1
Aurora-A
Az1 mutants
β-tubulin
D.
Total Lysate
IP: Flag Az1 mutants
vector
Az1
ΔN120-Az1
ΔN130-145-Az1
R131D/G145D-Az1
Aurora-A
Az1 mutants
Aurora-A (co-IP)

Fig.4

Fig.5


A.
Aurora-A + + + +
TR-AURKAIP1 - + - +
AzI - - + +
Aurora-A
TR-AURKAIP1
AzI
β-tubulin
B.
Aurora-A
ΔN300 Aurora-A
ΔN600 Aurora-A
ΔC300 Aurora-A
ΔC600 Aurora-A
vector
TR-AURKAIP1
Aurora-A mutants
TR-AURKAIP1
β-tubulin
C.
Aurora-A + +
Az1 + +
vector + -
Flag-TR-AURKAIP1 - +
Total Lysate
Aurora-A
Az1
TR-AURKAIP1
IP: Flag-TR-AURKAIP1
TR-AURKAIP1
Aurora-A (co-IP)
Az1(co-IP)
D.
TR-AURKAIP1 - - +
Aurora-A - + +
Az1 + + +
Total Lysate
Az1
Aurora-A
TR-AURKAIP1
IP:Aurora-A
Aurora-A (total IP)
Az1 (co-IP)