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Molecular mechanism of Aurora-A kinase in human oncogenesis

Lili He

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Molecular Mechanism of Aurora-A Kinase in Human Oncogenesis

by

Lili He

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Pathology and Cell Biology
College of Medicine
University of South Florida

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This work is dedicated to my mother, Cuiqing Xu, and my father, Chunnian He.
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Molecular Mechanism of Aurora-A Kinase in Human Oncogenesis

Lili He

ABSTRACT

Aurora-A is a mitotic kinase, which regulates cell cycle progression through modulating centrosome function. Aurora-A expression is frequently altered in human malignancies. The discrepancy between overexpression and amplification suggests that elevated Aurora-A level is likely to be regulated also by transcriptional and/or translational modifications. In this study, we have demonstrated: 1) transcriptional regulation of Aurora-A by E2F3; 2) feedback regulation between tumor suppressor CHFR and Aurora-A; 3) CNTD2 as a novel Aurora-A partner and oncogene to activate Aurora-A and induce transformation.

Aurora-A expression and activity are cell cycle regulated. The mechanism of Aurora-A upregulation at onset of mitosis is largely unknown. We demonstrated, for the first time, that transcription factor E2F3 directly binds to Aurora-A promoter and tightly regulates Aurora-A expression during G2/M phase. Moreover, expression of E2F3 considerably correlates with Aurora-A level in human ovarian cancer, indicates that E2F3 is a causal factor for Aurora-A overexpression. Thus, E2F3-Aurora-A axis could be an important target for cancer intervention. The frequent inactivation of prophase checkpoint CHFR caused by DNA methylation or mutation has been reported in human cancers. We showed that CHFR is hypermethylated in ovarian carcinoma. Aurora-A phosphorylates CHFR on Ser-218 and Ser-337 in vivo and in vitro, which inhibits CHFR ubiquitin ligase activity. The feedback regulation loop between Aurora-A and CHFR could play a critical role...
role in regulation of cell cycle progression, imbalance of which may contribute to human oncogenesis. Using yeast 2-hybrid screening, we identified a splicing form of CNTD2 as Aurora-A interacting protein. CNTD2 locates to chromosome 19q13.2 AKT2 amplicon. CNTD2 is amplified and overexpressed in human ovarian, pancreatic and lung cancer cell lines and primary tumors. CNTD2 colocalizes and interacts with Aurora-A in the centrosome. CNTD2 expression induces Aurora-A and cdc2 kinase activity, G2/M progression, and malignant transformation. These data indicate that CNTD2 is an oncogene and could play a pivotal role in Aurora-A activation during the cell cycle and that disruption of CNTD2-Aurora-A axis may represent a potential means to antitumor drug discovery.
CHAPTER I

INTRODUCTION: MOLECULAR MECHANISM OF AURORA-A KINASE IN HUMAN ONCOGENESIS

Aurora Kinases

Homologues of Aurora/Ipl1-related kinase have been reported in various organisms including yeast, nematodes, fruit flies and vertebrates (1-3). In mammals, this subfamily of serine/threonine kinases comprises three members: Aurora-A, -B and -C (4). *Drosophila melanogaster* and *Caenorhabditis elegans* express homologues of Aurora-A and -B, whereas *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have only one Aurora kinase gene (5,6), suggesting that the functions of Auroras have diverged throughout evolution.

Structure

All Aurora kinases share similar structures, with their catalytic domains flanked by very short (15–20 residues) C-terminal tails and N-terminal domains of variable lengths (39–129 residues). The overall homology between these three isoforms in human is about 80% at amino acid level. The central catalytic kinase domain is considered highly conserved. However, it is interesting to note that Aurora-C and -B share 77.6% amino acid sequence identity, while Aurora-C and -A share about 66.5% sequence identity (7), suggesting a functional relationship between Aurora-B and -C. The N-terminal and the C-terminal sequences of the three members are quite different. The C-terminal domain of human Aurora-B shares 53% and 73% sequence similarity to human Auroras-A and -C, respectively. The N-terminals of Auroras-A, -B and -C share low sequence conservation, which may determine the selectivity during protein–protein interactions. Aurora-A N-
terminal domain serves as a localization domain, which targets the protein to the centrosome in a microtubule dependent manner. The unique A-box motif at the N-terminal of Aurora-A is required for proteolysis of the protein. Furthermore, phosphorylation of Ser 51 at N-terminal decides the timing of Aurora-A destruction (8). (see Fig. 1.1)

**Fig. 1.1. Aurora kinase family.** Diagrammatic representation of the domain structure of three Aurora family members. The percentages indicate the degree of the identity between Aurora-A, Aurora-B and Aurora-C.

**Localization and Timing of Activation**
Despite these similarities, the three mammalian Aurora family members differ in their expression patterns, subcellular localization, and timing of activity. (see Fig. 1.2)

   **Aurora-A** is first detected at the centrosome during late S phase. The activation of a small proportion of Aurora-A at centrosomes was first evident before chromosome condensation at late G2 phase (9). Both the amount and activity of Aurora-A rapidly increase in the centrosome, and a fraction of active Aurora-A subsequently translocates into the nucleus coincident with chromatin condensation at prophase. After nuclear-envelope breakdown (NEBD), activated Aurora-A is observed at the spindle poles and bipolar spindles during prometaphase and metaphase. The amount of Aurora-A starts to decrease at the metaphase-anaphase transition, whereas a small fraction of Aurora-A remains on the centrosomes and the spindles at the onset of anaphase and telophase.
Aurora-A is degraded by the cdh1/Fizzy-related form of the anaphase-promoting complex/cyclosome (APC/C). At the final stage of cytokinesis, most of the Aurora-A protein becomes undetectable.

Aurora-B, whose activity appears to reach maximal levels later in mitosis, displays the dynamic properties of a chromosomal passenger protein. It first associates with centromeres/kinetochores — the sites on chromosomes where microtubules attach — then relocalizes to the midzone of the central spindle, and finally concentrates at the midbody between dividing cells (12).

Fig. 1.2. Localization of Aurora-A and -B kinases during the cell cycle. Nature Reviews Cancer 5, 42-50. January 2005.
In line with these distinct localizations, Aurora-A is implicated primarily in centrosome maturation and spindle assembly (5,6), whereas Aurora-B is proposed to regulate chromosome condensation and cohesion, kinetochore assembly and bipolar chromosome attachment, the spindle checkpoint and the coordination between chromosome segregation and cytokinesis (13-16).

Aurora-C has been described only in mammals, where it is expressed in testis and certain tumor cell lines and, like Aurora-B, functions as a chromosomal passenger, which co-localizes with Aurora-B first to centromeres and then to the midzone of mitotic cells. It has been shown recently that Aurora-C cooperates with Aurora-B to regulate mitotic chromosome segregation and cytokinesis (17).

Chromosome Location and Involvement in Human Cancer

Human Aurora-A is located at chromosome 20q13.2, which is commonly amplified in various epithelial malignant tumors, including breast, colon, bladder, ovarian and pancreatic cancers (18-22). In breast cancer in particular, the 20q11-q13 region is amplified in 12-18% of primary tumors. The 20q13 amplification is also detected in 5% (4/72) of gastric cancer from 3.5- to 6.3- fold, and in 52% (41/79) of primary colorectal tumors.

The levels of Aurora-A mRNA and protein are also increased in those tumors. However, alterations of Aurora-A at mRNA and/or protein levels are much common than the gene amplification. For example, amplification of Aurora-A was detected in only 3% of hepatocellular carcinomas (HCCs) while more than 60% of HCCs overexpressed Aurora-A mRNA and protein (23). Discrepancy between amplification and overexpression rates was also reported in breast cancer (24), gastric cancer (25) and ovarian cancer (21). Therefore, Aurora-A overexpression is likely to be regulated not only by gene amplification, but also by other mechanisms such as transcriptional activation and suppression of protein degradation.

Unlike Aurora-A, Aurora-B coding gene, which is mapped to chromosome 17p13.1, is not amplified in human tumors. However, both mRNA and protein levels of Aurora-B are frequently increased in various human tumors, including colorectal cancers
(26,27). Furthermore, exogenous overexpression of Aurora-B in Chinese hamster embryo cells causes chromosome separation defects during mitosis and increased invasiveness of cells in vivo, indicating a role for Aurora-B in tumorigenesis (28).

A gene encoding Aurora-C is localized at chromosome 19q13.43, which is the region frequently deleted or rearranged in several tumor tissues (29). However, the involvement of Aurora-C in cancer development is still unclear.

**Aurora-A, but not Aurora-B and -C, Induces Transformation, Centrosome Amplification and Genetic Instability**

Ectopic expression of Aurora-A, but not Aurora-B and -C, in Rat1 or NIH3T3 cells results in malignant phenotype, including growing in soft agar and forming tumor in nice mouse, demonstrating that Aurora-A is an oncogene. Overexpression of Aurora-A leads to disrupt cell-cycle checkpoint functions. Normal cells have cell-cycle checkpoints that monitor genomic integrity and prevent cells from dividing in the presence of DNA damage. Recent studies have shown that DNA-damage signals inhibit Aurora-A kinase activity to induce cell-cycle arrest at the end of the G2 phase and that, conversely, overexpression of Aurora-A disrupts the DNA-damage-induced G2 checkpoint (30). Additionally, Aurora-A overexpression has been found to override the spindle checkpoint activated by the chemotherapeutic agent paclitaxel (Taxol), allowing cells to inappropriately enter anaphase despite defective spindle formation and to become resistant to paclitaxel-induced apoptosis (31). Many experiments have demonstrated the requirement for Aurora-A in centrosome regulation. In *Drosophila*, Aurora-A mutants show several centrosome defects that were confirmed by RNA-mediated interference (RNAi) (32). Aurora-A overexpression in cell lines leads to centrosome amplification, multipolar spindle and polyploidy. These checkpoint defects and genetic instability, caused by Aurora-A overexpression, might contribute to transformation.

**Aurora-A Kinase Activity is Required for Its Oncogenic Activity**

Transient expression of active Aurora-A in the near diploid human breast epithelial cell line MCF10A leads to aberrant chromosome segregation and an increase in ploidy (24).
In contrast, a C-terminal-deleted mutant of Aurora-A induces no effect in the ploidy state of transfected gastric cancer cells, but inhibits cell proliferation (25). However, surprisingly, an inactive Aurora-A kinase produces virtually identical phenotypes to the wild-type active kinase when overexpressed in Chinese hamster ovary cells (33). Both transfected cells exhibit extra copies of centrosomes. However such inactive Aurora-A is unable to transform Rat1 or NIH3T3 cells, strongly indicating that (1) a gain of Aurora-A kinase activity is necessary to transform cells, and that (2) centrosome amplification is not sufficient to provoke transformation (19). Thus, overexpression of the Aurora-A kinase is able to induce centrosome amplification, but its kinase activity is not necessary to obtain this phenotype. It is possible that the accumulation of the protein leads to a disorganization of the centrosome machinery unrelated to its catalytic function but caused by a steric accumulation that interferes with the centrosomal functions.

In contrast, the kinase activity of Aurora-A is necessary to obtain the transformed phenotype by overexpression in cultured cells. Thus centrosome amplification induced by Aurora-A overexpression is not sufficient for its oncogenic activity. Under overexpression, Aurora-A might phosphorylate substrates unrelated to the centrosome function but involved in oncogenesis. Furthermore, this suggests that kinase activity of Aurora-A is essential for its oncogenic activity. In addition to elevated levels of mRNA and protein, we have demonstrated frequent activation of Aurora-A in human prostate and ovarian cancers, indicating that activation of Aurora-A may be important for development of these subsets of tumors.

**Aurora-A and Mitosis**

In the process of separating replicated genetic materials into two daughter cells during mitosis, cells undergo centrosome maturation, chromosome condensation, nuclear-envelope breakdown, centrosome separation, bipolar-spindle assembly, chromosome segregation and cytokinesis. Studies in lower organisms have shown that these events are controlled by phosphorylation events performed by several serine/threonine kinases, known as mitotic kinases (4). Mitotic kinases include cyclin dependent kinase 1 (CDK1;
also known as p34cdc2), polo-like kinases, NIMA-related kinases, WARTS/LATS1-related kinases and Aurora/Ipl1-related kinases. The structure of these enzymes has been well conserved through evolution. Elucidation of how these mitotic kinases are coordinated will provide a clue to the regulation of cell division.

In human cells, Aurora-A levels and kinase activity increase during late G2 to M phase, and it undergoes dynamic changes in subcellular localization throughout the cell cycle. This dynamic localization pattern indicates that Aurora-A is involved in various mitotic events. In fact, several substrates and/or interacting proteins of Aurora-A identified recently have been shown to have a crucial role in mitotic processes (Table 1.).

Table 1. Aurora substrates/interacting proteins and mitotic processes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Process/Regulation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASAP</td>
<td>bipolar spindle assembly</td>
<td>Venoux et al., 2008</td>
</tr>
<tr>
<td>AURKAIP</td>
<td>complex stabilization</td>
<td>Katayama et al., 2007</td>
</tr>
<tr>
<td>BRCA1</td>
<td>G2-M transition, checkpoint regulation</td>
<td>Ouchi et al., 2004</td>
</tr>
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<td>CDC25B</td>
<td>earliest events inducing mitosis, upstream of CDK1-cyclin B1 activation</td>
<td>Dutertre et al., 2004</td>
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<tr>
<td>CENP-A</td>
<td>chromosome alignment and segregation</td>
<td>Kunitoku et al., 2003</td>
</tr>
<tr>
<td>CPEB</td>
<td>spindle assembly</td>
<td>Takashi et al., 2005</td>
</tr>
<tr>
<td>Eg5</td>
<td>centrosome separation</td>
<td>Giet et al., 1999</td>
</tr>
<tr>
<td>NDEL1</td>
<td>centrosome maturation, separation, and TACC3 recruitment</td>
<td>Mori et al., 2007</td>
</tr>
<tr>
<td>HURP</td>
<td>complex stabilization</td>
<td>Yu et al., 2005</td>
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<tr>
<td>Ilkalpha</td>
<td>activation of NFkB-directed gene expression</td>
<td>Briassouli et al., 2007</td>
</tr>
<tr>
<td>LATS2</td>
<td>centrosome maturation/centrosomal localization of Lats2</td>
<td>Tojii et al., 2004</td>
</tr>
<tr>
<td>p53</td>
<td>abrogation of p53 DNA binding and transactivation activity</td>
<td>Liu et al., 2004</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>microtubule stabilization</td>
<td>Rong et al., 2007</td>
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<td>TACC/Maskin</td>
<td>microtubule polymerization and growth, centrosome maturation</td>
<td>Giet et al., 2002</td>
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<table>
<thead>
<tr>
<th>Interacting protein</th>
<th>Process</th>
<th>Effect on Aurora A</th>
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<tbody>
<tr>
<td>Ajuba*</td>
<td>centrosome maturation, mitotic commitment</td>
<td>Activation</td>
</tr>
<tr>
<td>Bora*</td>
<td>centrosome maturation, asymmetric cell division</td>
<td>Activation</td>
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<td>centrosomin</td>
<td>nucleation, centrosome maturation</td>
<td>Inhibition</td>
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<td>Gadd45a</td>
<td>centrosome stability</td>
<td></td>
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<td>HEF1*</td>
<td>centrosome separation</td>
<td>Inhibition</td>
</tr>
<tr>
<td>NM23-H1</td>
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<td>?</td>
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<tr>
<td>PAK1</td>
<td>centrosome maturation</td>
<td>Activation</td>
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<tr>
<td>PP1</td>
<td>mitosis entry</td>
<td>Inhibition</td>
</tr>
<tr>
<td>PP2A</td>
<td>end of mitosis</td>
<td>Degradation</td>
</tr>
<tr>
<td>TPX2*</td>
<td>bipolar spindle assembly</td>
<td>Activation</td>
</tr>
</tbody>
</table>

* Interacting protein that is also Aurora-A substrate.
Centrosome Maturation

The centrosome is the primary microtubule nucleating centre within most animal cells at all stages of the cell cycle, but on entry into mitosis, microtubule assembly at the centrosome dramatically increases in a process termed maturation. This activation enables microtubules to grow out from the centrosome, interact with chromosomes and form the bipolar spindle required for chromosome segregation. At the same time, many proteins are recruited to centrosomes, including the γ- tubulin ring complex and other microtubule regulators.

Aurora-A has been shown to be essential for the process of centrosome maturation in various organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (32,34,35). Also, in cultured human cells, Aurora-A depletion results in the inhibition of centrosome maturation (9).

The discovery of phosphorylation of a conserved centrosomal protein TACC (Transforming Acidic Coiled-Coil) by Aurora-A could partly explain the molecular nature of Aurora-A regulation of centrosomal microtubule assembly (32,36,37). Phosphorylation by Aurora-A recruits TACC, and consequentially its binding partner Msps/XMAP215 to the centrosome in mitosis (38). Targeting of TACC–Msps/XMAP215 to the centrosome may enhance the activity of Msps/XMAP215 in stabilizing microtubule plus ends (1). In another model, the phosphorylation is proposed to allow the complex to stabilize the minus ends of microtubules nucleated at the centrosome (2) (see Fig. 1.3).

Other than for microtubule assembly, C. elegans homologue of Aurora-A kinase AIR-1 is required for centrosome maturation by recruiting centrosomal γ-tubulin and two other PCM (pericentriolar material) components, ZYG-9 and CeGrip, as embryos enter mitosis (34).
Centrosome Separation

Depletion of AIR-1 in *C. elegans* doesn’t affect centrosome separation before nuclear-envelope breakdown (NEBD); however, subsequent to NEBD the two asters collapse back together and remain unseparated, indicating that AIR-1 is not necessary for initial separation of centrosomes but is required to maintain centrosome separation during spindle assembly (34). In human cells (HeLa cells), micro-injection of affinity-purified Aurora-A antibody at late G2 phase inhibits separation of centriole pairs at prophase. Meanwhile, abnormally organized spindles with no centrioles at one pole and two pair of centrioles at the other are frequently noticed (10).

It was previously shown that the *Xenopus* Aurora-related kinase Eg2 phosphorylates the kinesin-like protein XlEg5, which is required for centrosome separation (39). Therefore it is reasonable to speculate that human Aurora-A might also regulate separation of centriole pairs through the phosphorylation of kinesin-like motors during prometaphase.
Mitotic Commitment
Analysis of HeLa cells has revealed that the initial active form of Aurora-A is first detected in late G2 phase at the centrosome. Application of RNA interference (RNAi) to synchronized cells has shown that this activation is required for the recruitment of CDK1–cyclin B1 to the centrosome, where it is fully activated and commits cell to mitosis. A LIM protein called Ajuba was identified as a substrate and an activator for Aurora-A during mitotic commitment. Depletion of Ajuba prevents activation of Aurora-A at centrosomes in late G2 phase and inhibits mitotic entry (9).

Additionally, it has recently been demonstrated that Aurora-A phosphorylates Ser353 of the phosphatase CDC25B — an activator of cyclin-dependent kinases — at centrosomes during late G2 phase, also contributing to the onset of mitosis (40). However, the activation of CDK1–cyclin B1 is in turn required for full activation of Aurora-A at the centrosome and nucleus (9). These findings indicate that the co-activation of Aurora-A and CDK1 at late G2 centrosomes constitutes an essential early event in the progression of cells towards mitosis.

Bipolar Spindle Assembly
The role of Aurora-A in mitotic-spindle assembly was initially revealed through studies in *Xenopus* egg extracts (41), where the phosphorylation and activation of Eg2 (*Xenopus* Aurora-related kinase) stimulated by the Ran-GTP-TPX2 pathway is essential for spindle assembly; a catalytically inactive pEg2 stops the assembly of bipolar mitotic spindles (42-44).

TPX2 (Targeting Protein for XKLP2) was isolated from *Xenopus* as a microtubule-associated protein, which is required for the recruitment of XKLP2 (*Xenopus* kinesin-like protein) to microtubules (45). GTP-binding Ran (the small GTPase) is known to be required for the induction of spindle formation by chromosomes in M phase. And the action of Ran-GTP in spindle formation requires TPX2 (46). Depletion of TPX2 from *Xenopus* egg extracts resulted in the formation of less compact spindles and a range of pole (47). Additionally, in mammalian cells, depletion of TPX2 using small interfering RNA caused the formation of multipolar spindles (48).
Furthermore, TPX2 was found to interact with Aurora-A and to be not only a substrate but also an activator for Aurora-A. Inhibition of the interaction between TPX2 and Aurora-A prevents Aurora-A activation and recruitment to microtubules (49,50). Earlier evidences established that depletion of Aurora-A disrupts the stability of the mitotic spindle in *Xenopus* eggs and causes the formation of multipolar spindles in mammalian cells, similar to depletion of TPX2 (41,51). This indicates that the interaction between TPX2 and Aurora-A could be an important prerequisite for efficient mitotic-spindle formation. (see Fig. 1.4)

Chromosome Alignment on the Metaphase Plate

Studies have shown that Aurora-B is required for the correct kinetochore-microtubule attachment between sister chromatids to spindle poles (52,53). The proper interaction generates the amphitelic attachment state, which is required for accurate chromosome alignment and segregation (12). However, it has been recently shown that Aurora-A is also directly involved in metaphase chromosome alignment, by phosphorylating a kinetochore-specific histone H3 variant CENP-A, which is crucial for kinetochore assembly and function (54). CENP-A is phosphorylated on the amino-terminal serine (Ser7) by Aurora-A, and this phosphorylation is important for not only the proper attachment of microtubules to the kinetochore but also the consequent chromosome alignment and segregation. Given that post-translational modifications of the amino-terminal tail domain of core histones are known to regulate chromatin structure and function (55), phosphorylation of Ser7 of CENP-A by Aurora-A might be a crucial event for kinetochore function. Although Aurora-B is also found to phosphorylate CENP-A at the same site (56), it is believed that Aurora-A phosphorylation is an earlier and more important event. Ser7 of CENP-A is first phosphorylated during prophase in a manner dependent on Aurora-A, and that this reaction is required for the subsequent Aurora-B-dependent phosphorylation of CENP-A as well as the recruitment of Aurora-B to the inner centromere, where Aurora-B maintains the phosphorylation of CENP-A from prometaphase through metaphase (54). The phosphorylation of CENP-A on Ser-7 is required to initiate kinetochore assembly by recruiting several components important for the establishment of kinetochore-microtubule connections. The two Aurora kinases therefore regulate kinetochore functions by phosphorylating this common substrate.

Cytokinesis

Both Aurora-A and Aurora-B seem to be involved in cytokinesis. Inhibition of Aurora-B function results in cytokinesis failure in mammalian cells (13,57-60). Inhibition of Aurora-A by antibody microinjection in metaphase HeLa cells, which have completed centrosome separation, bipolar spindle assembly and chromosome alignment, causes cytokinesis failure; chromosomes are able to segregate, but the daughter cells eventually
fuse to form binucleate cells (10). It is thus possible that Aurora-A has some direct function in cytokinesis. The other possibility is that Aurora-A leads to cytokinesis defect by dysregulation of centriole behavior, which is proposed tightly connected with the completion of cell division. Interestingly, overexpression of Aurora-A also impairs cytokinesis, resulting in formation of multinucleated cells (31,33).

As both the inhibition and the increased activity of Aurora-A lead to multinucleated cell formation, proper timing of activation and subsequent inactivation of this kinase is required for proper cytokinesis. The reduction of Aurora-A levels at late mitosis is mediated by ubiquitin-proteasome-mediated degradation, which is promoted by the anaphase-promoting complex (APC/C) activated by Cdh1 (61,62). This rapid degradation of Aurora-A resulting in Aurora-A inactivation and dephosphorylation of the specific substrates, might be required for completion of cytokinesis.

Aurora-A Signaling

Aurora-A regulates a handful of important signal molecules, including cross-talk with p53 and c-Myc pathways, leading to cell cycle progression, cell proliferation and cell survival.

p53

Aberrations in centrosome numbers have long been implicated in aneuploidy and tumorigenesis. Overexpression of Aurora-A kinase causes centrosome amplification in cultured cells. It does not deregulate centrosome duplication but gives rise to extra centrosomes through defects in cell division and consequent tetraploidization (33). Over expression of other mitotic kinases (Polo-like kinase 1 and Aurora-B) also causes multinucleation and concomitant increases in centrosome numbers (63,64). The accumulation of extra copies of centrosomes is strikingly enhanced in p53–/– cells, consistent with the notion that p53 arrests tetraploid cells as part of a G1 checkpoint (65,66). A study in yeast and human cell lines (HEK293, H1299) showed that Aurora-A interacts with p53 through Aurora-Box in vivo and in vitro (67). And the interaction
resulted in the suppression of Aurora-A oncogenic activity regarding centrosome amplification and cellular transformation by p53 in a transactivation-independent manner. Our lab has demonstrated, on the other hand, that p53 is phosphorylated and regulated by Aurora-A (68). Unlike most identified phosphorylation sites of p53 that positively associate with p53, the phosphorylation of p53 by Aurora-A at Ser-215 abrogates p53 DNA binding and transactivation activity. Downstream target genes of p53, such as p21\(^{Cip/WAF1}\) and PTEN, were inhibited by Aurora-A in a Ser-215 phosphorylation-dependent manner. As a result, Aurora-A overrides the apoptosis and cell cycle arrest induced by cisplatin and \(\gamma\)-irradiation, respectively. However, the effect of Aurora-A on p53 DNA binding and transactivation activity was not affected by phosphorylation of Ser-315, a previously identified Aurora-A phosphorylation site of p53. We have also reported that Aurora-A protects ovarian cancer cells from apoptosis induced by chemotherapeutic agent and activates Akt pathway in a p53-dependent manner (69).

**c-Myc**

Aurora-A plays a pivotal role in transformation, however, the molecular mechanism by which Aurora-A induces ovarian and breast cell transformation remains elusive. We found that Aurora-A induces telomerase activity and human telomerase reverse transcriptase (hTERT) by up-regulation of c-Myc (70). Ectopic expression of Aurora-A activity in human ovarian and breast epithelial cell lines HIOSE118 and MCF-10A induces telomerase activity. The mRNA and promoter activities of hTERT are stimulated by Aurora-A. Furthermore, c-Myc binding sites of hTERT promoter are required for Aurora-A-induced hTERT promoter activity. Ectopic expression of Aurora-A up-regulates c-Myc. Knockdown of c-Myc by RNA interference attenuates Aurora-A-stimulated hTERT expression and telomerase activity.

Regarding the mechanism of Aurora-A upregulation of c-Myc, our data (unpublished) show that Aurora-A inactivates GSK3\(\beta\) through a phosphorylation-dependent manner, which leads to \(\beta\)-catenin cytoplasmic accumulation, nuclear translocation and subsequently activates c-Myc transcription by forming complex with TCF/LEF transcription factors. We believe that knockdown of c-Myc may reduce
Aurora-A-induced transformation and genomic instability, but not necessarily abrogate Aurora-A oncogenic activity. It has been well documented that c-Myc plays a critical role in human oncogenesis by regulation of cell proliferation and differentiation. Thus, Aurora-A up-regulation of c-Myc may play an essential role in Aurora-A-induced malignant transformation in addition to its G2/M cell cycle control.

**AR and ER**

We have recently shown frequent activation/overexpression of Aurora-A kinase in human primary prostate tumors (unpublished data). Constitutively active Aurora-A exhibits more oncogenic activity. Therefore, activation and/or overexpression of Aurora-A could play an important role in prostate cancer development. In addition, our preliminary study shows that Aurora-A phosphorylates androgen receptor (AR) *in vitro* and *in vivo* and activates AR transactivation activity, which might contribute to androgen-independent tumor growth. According to our data, Aurora-A activation of AR could be phosphorylation dependent. Ectopic expression of Aurora-A in prostate cancer cells may lead to more aggressive phenotype and androgen-independent growth; whereas knockdown of Aurora-A could interfere with androgen dependence.

As Aurora-A is frequently altered in breast cancer and induces telomerase activity and tamoxifen resistance in mammary epithelia (unpublished data); we reasoned that Aurora-A could regulate ERα activity. Reporter assay showed that ERE-Luc activity is induced by Aurora-A in a dose-dependent manner in only ERα positive cells. Further, Aurora-A exhibits synergistic effect with estrogen on ERα transactivation activity. In addition, we have demonstrated ERα is phosphorylated by Aurora-A *in vitro* and *in vivo* (data not shown). We have also observed that tamoxifen downregulated Bcl-2 is overridden by Aurora-A, especially constitutively active Aurora-A. These data indicate important function of Aurora-A besides cell cycle control.
Several mechanisms exist by which Aurora-A overexpression might contribute to tumorigenesis. (see Fig. 1.5)


**Centrosome Amplification and Aneuploidy**

Centrosome alterations, including centrosome amplification, are found in brain, breast, lung, colon, prostate, pancreas, bile duct, head and neck tumors and lymphoma (71-77). Overexpression of Aurora-A causes centrosome amplification in both cell culture and rat
mammary models (78,79). However, the phenomenon seems not due to centrosome duplication but a result of cytokinesis failure and consequent multinucleation (33). Since both Aurora-A and Aurora-B are shown to be involved in cytokinesis, it is possible that Aurora-A causes cytokinesis failure directly, or by affecting Aurora-B or other proteins that regulate cytokinesis. The other possibility is that Aurora-A leads to cytokinesis defect by dysregulation of centriole behavior, which is proposed tightly connected with the completion of cell division.

Aneuploidy is thought to be a marker of tumor progression and prognosis. Correlation between Aurora-A overexpression and aneuploidy exists in gastric and colon cancer (25,78). Aurora-A-induced polyploid cells would be arrested at the following G1 phase by a post-mitotic checkpoint dependent on the p53/Rb pathway. The G1 checkpoint is required for the cell to trigger apoptosis. However, when there is no functional p53 present, as in p53/-/- cells, the hyperploidy is not detected and cells go on DNA replication and cell division, which might eventually cause aneuploidy and abnormal centrosome number (33,80).

**Checkpoint Disruption and Chromosome Instability**

Overexpression of Aurora-A tends to disrupt cell-cycle checkpoint functions. Normal cells have cell-cycle checkpoints that monitor genomic integrity and prevent cell dividing in the presence of DNA damage. Recent studies have shown that DNA-damage signals inhibit Aurora-A kinase activity to induce cell-cycle arrest at the end of the G2 phase and that, conversely, overexpression of Aurora-A disrupts the DNA-damage-induced G2 checkpoint (30).

Additionally, Aurora-A overexpression has been found to override the spindle checkpoint, allowing cells to inappropriately enter anaphase despite defective spindle formation. Upon activation of the spindle checkpoint, *Bub* and *Mad* proteins become localized to unattached kinetochores. The concentration of these checkpoint proteins on the kinetochore facilitates the assembly of an active checkpoint complex, which consists of Cdc20, BubR1, Mad2 and Bub3 (81,82). This complex has the ability to inhibit the function of anaphase-promoting complex/cyclosome (APC/C). Overexpression of
Aurora-A leads to a dramatic accumulation in the Cdc20 complex, where BubR1 level is decreased. At the same time, Mad2 and Bub3 can not be efficiently recruited to Cdc20 complex while the expression of both proteins is not changed (83). The dissociation of these checkpoint proteins from Cdc20 results in the prematurely activation of APC/C and bypass of the checkpoint function. Aurora-A plays a critical role in cell cycle checkpoints to ensure the faithful duplication and transmission of genetic materials. Perturbation of this regulatory process will endanger genetic stability and possibly contribute to tumorigenesis.

**Polymorphism**

Aurora-A was recently identified as a candidate low-penetrance tumor-susceptibility gene. The involvement of two polymorphisms of Aurora-A (Phe31Ile and Val57Ile) in human tumor susceptibility was analyzed. The Ile31 allele of Phe31Ile was found to be preferentially amplified in human colon and ovarian cancers (84,85), and the combination of the two polymorphisms (31Ile and 57Val) was related to a significant twofold excess in the risk of breast cancer (86). This genetic evidence also supports the role of *Aurora-A* as a tumor modifier gene.

**Aurora-A Inhibitors**

The evidence linking Aurora overexpression and malignancy has stimulated interest in developing Aurora inhibitors for cancer therapy. Aurora-A is considered the principal member of the Aurora family with oncogenic activity. Studies have shown a specific role of Aurora-A in cancer development and progression. However, an *in vivo* study of Aurora inhibitors demonstrated that it is not Aurora-A but Aurora-B inhibition induces tumor regression. Dual inhibition of Aurora-A and -B results in phenotypes identical to inactivation of Aurora-B alone (87). Inactivation of Aurora-B bypasses the requirement for Aurora-A and leads to polyploidy, indicating that Aurora-B is responsible for mitotic arrest in the absence of Aurora-A (88). Therefore, it is possible that the better antitumor drug should inhibit all Aurora members instead of only one.
Many Aurora-selective small-molecule inhibitors are currently undergoing preclinical and clinical assessment. VX-680 is a pyrimidine derivative, designed against the ATP-binding site, with affinity for Aurora-A, -B, and -C. VX-680 prevents cytokinesis but allows cells to progress through the other stages of mitosis, which leads to polyploidy and, in some cancer cell lines, massive apoptosis. In preclinical models, VX-680 blocked tumor growth in three xenograft models, including leukemia, colon and pancreatic (87). AZD1152 is a quinazoline prodrug, which is converted in plasma into its active metabolite AZD1152-HQPA, which in turn has high affinity for Aurora-B and -C. The effects of AZD1152-HQPA in cancer cells are comparable with VX-680 (89). In preclinical models, AZD1152 significantly inhibited the growth of human tumor xenografts. PHA-739358 is a pan-Aurora (A, B, and C) inhibitor with documented antitumor activity in multiple tumor xenograft models, which have shown sustained tumor growth inhibition after discontinuation of treatment. MLN8054 was the first orally available Aurora kinase inhibitor and the first Aurora-A–selective inhibitor in clinical trials. This compound induces mitotic accumulation and spindle defects and inhibits proliferation in multiple human cancer cell lines (90). Tumor growth in nude mice was inhibited after oral administration at well-tolerated doses, and the inhibition was sustained after discontinuation of treatment. At least one phase I clinical trial has been completed for each of the inhibitors mentioned above. Although no objective tumor responses were observed, disease stabilization was achieved in some patients. Nonhematologic toxicities were mild. How Aurora kinase targeting interacts with chemotherapy and radiation at the molecular level remains to be determined, but these results are very encouraging and pave the way for clinical studies with Aurora kinase inhibitors in the near future.
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CENTRAL HYPOTHESIS AND OBJECTIVES

Central Hypothesis

Upregulation/activation of Aurora-A by oncogenic factors and inactivation of tumor suppressor by Aurora-A contribute to Aurora-A-induced carcinogenesis.

Objectives

To understand the molecular mechanism of Aurora-A in human cancers:
1). Aurora-A is transcriptionally regulated by E2F3;
2). Identification of tumor suppressor CHFR as an Aurora-A substrate;
CHAPTER II

IDENTIFICATION OF AURORA-A AS A DIRECT TARGET OF E2F3 DURING G2/M CELL CYCLE PROGRESSION

Abstract

Aurora-A is a centrosome kinase and plays a pivotal role in G2/M cell cycle progression. Expression of Aurora-A is cell cycle-dependent. Levels of Aurora-A mRNA and protein are low in G1/S, accumulate during G2/M, and decrease rapidly after mitosis. Previous studies have shown regulation of Aurora-A protein level during the cell cycle through ubiquitin-proteasome pathway. However, transcriptional regulation of Aurora-A remains largely unknown. Here, we demonstrate that E2F3 modulates Aurora-A mRNA expression during the cell cycle. Ectopic expression of E2F3 induces Aurora-A expression. Stable knockdown of E2F3 decreases mRNA and protein levels of Aurora-A and delays G2/M entry. Further, E2F3 directly binds to Aurora-A promoter and stimulates the promoter activity. Deletion and mutation analyses of Aurora-A promoter revealed that a region located 96-bp upstream of transcription initiation site is critical for the activation of the promoter by E2F3. In addition, expression of E2F3 considerably correlates with the protein level of Aurora-A in human ovarian cancer examined. These results indicate for the first time that Aurora-A is transcriptionally regulated by E2F3 during the cell cycle and that E2F3 is a causal factor for upregulation of Aurora-A in a subset of human ovarian cancer. Thus, E2F3-Aurora-A axis could be an important target for cancer intervention.

Key words: cell cycle, G2/M, transcription, promoter activity, ovarian cancer
Introduction

Aurora family of serine/threonine protein kinases is evolutionally conserved from human, Drosophila, to yeast (1,2). In mammals, the Aurora kinase family comprises three members: Aurora-A, Aurora-B and Aurora-C. They share similar structures, with their highly conserved catalytic domains flanked by very short C-terminal tails and N-terminal domains of variable lengths. The N-terminal domains of the three Auroras share low sequence conservation, which may determine selectivity during protein–protein interactions.

As a mitotic kinase, activated Aurora-A is required for mitotic entry, centrosome maturation and chromosome segregation (1,2). Aurora-A protein localizes to centrosomes during interphase and to both spindle poles and spindle microtubules during early mitosis. Ectopic expression of Aurora-A leads to an increase in centrosome number, causes catastrophic loss or gain of chromosomes, and then results in malignant transformation. Aurora-A kinase activity is regulated by phosphorylation, dephosphorylation and by association with a number of proteins such as HEF1, TPX2, or Bora (3-5).

Aurora-A is located at chromosome 20q13.2, which is commonly amplified in various epithelial malignant tumors, including breast, colon, bladder, ovarian and pancreatic cancers, and the levels of Aurora-A mRNA and protein are also increased in those tumors (6-10). However, alterations of Aurora-A at mRNA and/or protein levels are much more common than the gene amplification. Discrepancy between amplification and overexpression rates was also reported in breast cancer, gastric cancer and ovarian cancer (9,11,12). Therefore, Aurora-A overexpression is likely to be regulated not only by gene amplification, but also by other mechanisms such as transcriptional activation and/or suppression of protein degradation. Expression of Aurora-A is cell cycle regulated. Aurora-A mRNA and protein levels begin to accumulate at G2 to M phase. After metaphase, Aurora-A protein is rapidly degraded by the ubiquitin-mediated proteolysis, which is promoted by the hCdh1-activated anaphase-promoting complex/cyclosome
(APC/C). However, transcriptional regulation of Aurora-A during the cell cycle is largely unknown.

It has been well documented that E2F family of transcription factors play important roles in the control of expression of genes at DNA duplication as well as further cell cycle progression (13,14). In addition, previous studies have demonstrated that a substantial number of E2F-induced genes are normally regulated at G2 of the cell cycle, encoding proteins known to function in mitosis (15-18). Moreover, studies in *Drosophila* have provided evidence for a connection between E2F activity and the control of mitotic activities (19). A recent report shows that both E2F1 and E2F3 are required for cells to enter the S phase from a quiescent state, whereas only E2F3 is necessary for the S phase in growing cells (20). The acute loss of E2F3 activity affects the expression of genes encoding DNA replication and mitotic activities, whereas loss of E2F1 affects a limited number of genes that are distinct from those regulated by E2F3 (21). Here we report that E2F3 upregulates Aurora-A at transcriptional level during G2 and the onset of mitosis. Among several E2F3 response elements, the region between -96 to transcriptional initiation site is responsible for E2F3 induction of Aurora-A promoter activity. Knockdown of E2F3 reduces Aurora-A expression and delays mitotic cell cycle progression.
Materials and Methods

Cell Line, Culture and Transfection
HeLa, HEK293, NIH3T3 and ovarian cancer cell lines were obtained as previously described (22,23) and cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum (FBS). Cell transfection was performed with Lipofectamine Plus Reagent (Invitrogen).

Synchronization and Cell Cycle Analyses
Cell synchronization at G1/S phase was performed using double-thymidine blocking (2). Briefly, HeLa cells were grown in 60-mm plates and thymidine (Sigma) was added to the culture medium at a final concentration of 2 mM for 12 h. Following two times washing with serum free medium, cells were released in fresh culture medium containing 24 mM 2-deoxycytidine. After 9 h incubation, the second thymidine block was initiated, and completed after 14 h. Cells were released from the block by washing in warm PBS and replacing with complete culture medium. At different time points, cells were fixed in 70% ethanol. The fixed cells were rinsed with PBS and then stained with the propidium iodide (PI) in a solution containing Triton X-100 and RNase A (Sigma). Cell synchrony at various cell cycle stages was monitored by flow cytometry. To synchronize cells to M-phase, HeLa cells were incubated in DMEM medium containing 50 nM/ml nocodazole for 14 h. Whole cell lysates and total RNA were isolated from parallel experiments for Western and Northern blotting analyses.

Plasmids
pGL3-Aurora-A/-1486/+354, pGL3-Aurora-A/-415, pGL3-Aurora-A/-189 reporters were kindly provided by Dr. Y. Ishigatsubo (Yokohama City University School of Medicine). To create deletion mutants, DNA fragments corresponding to -124/+354, -96/+354 and -49/+354 were amplified by PCR using pGL3-Aurora-A/-1486 as template, respectively. The PCR products were cloned into pGL3-basic vector at Mlu I-Bgl II sites and confirmed by DNA sequencing. The oligonucleotide primers used were as follows: sense
-124 5’-CGACGCGTTGGGACTGCCACAGGTCTGG-3’), -96 (5’-CGACGCGTTGGCCTCCACCACCTCCGG-3’), -49 (5’-CGACGCGTGTGTGCGCCCTAAACACGCGAC-3’); and antisense +354 (5’-GAAGATCTCTCTAGCTGTAATAAGTAAC-3’).

Aurora-A promoter mutant constructs were generated using QuikChange (Multi) Site-Directed Mutagenesis Kit (Stratagene), including pGL3-Aurora-A/-1486mtA, pGL3-Aurora-A/-1486mtB and pGL3-Aurora-A/-1486mtAB pGL3-Aurora-A/-96mtC, pGL3-Aurora-A/-96mtD and pGL3-Aurora-A/-96mtCD. Primers used for mutagenesis reactions were: mutation A, sense 5’-GCGCACGCTGAAAGGGATCCAAGCCGACCGCTGCGCTATCG-3’ and antisense 5’-CGATAGCGCAGCGGTCGGCTTGGATCCCTTTCAGCGTGCGC-3’; mutation B, sense 5’-CACTCTCTCTTGCTTTTCTATCCATCTTACTTACTGGC-3’ and antisense 5’-GCCAGTAAGTAGATGGATAGAAAGCAAGAGAGAGTG-3’; mutation C, sense 5’-GGCTCCACCACTTCATGGTTCTTACTGACGC-3’ and antisense 5’-GCCAGTAAGTAGATGGATAGAAAGCAAGAGAGAGTG-3’; mutation C, sense 5’-GGCTCCACCACTTCATGGTTCTTACTGACGC-3’ and antisense 5’-GCCAGTAAGTAGATGGATAGAAAGCAAGAGAGAGTG-3’; mutation D, sense 5’-GAGCAAGTCGCCTGCGCTGCGCGCGCCCTT-3’ and antisense 5’-AAGGGCGACCCCGCATGCAGGCGACTTGCTC-3’.

**Luciferase Assay**

Cells were cultured in 24-well plates and transfected with pGL3-Aurora-A reporters, β-galactosidase and the plasmids indicated in the figure legend. The amount of DNA in each transfection was kept constant by the addition of empty vector. After 36 h of transfection, luciferase activity was measured using luciferase kit (Promega). The β-galactosidase activity was measured by using Galato-Light (Tropix). Transfection efficiency was normalized by β-galactosidase expression. Luciferase activity was expressed as relative luciferase activity. The experiments were repeated three times.

**Northern and Western Blotting Analyses**

Total RNA was isolated from cells using Trizol Reagent (Invitrogen). Northern blot analysis was performed as previously described (24). Briefly, 20 μg total RNA from each
sample was separated on 1.0% denature agarose gel. After transferring to GeneScreen Plus Membrane (PerkinElmer) and prehybridization, the membrane was hybridized with \[^{32}\text{P}]\text{dCTP}-\text{labeled Aurora-A cDNA probe in Express-Hybridization Solution (Clontech). For Western blot, equal amount of protein was separated by SDS-PAGE gel, and electroblotted onto membranes. Following blocking in TBS-T containing 5% milk, the membranes were hybridized with appropriate antibodies indicated in figure legend.}

**shRNA Lentiviral Infection**

Knockdown of E2F3 was carried out by infection of HeLa cells with lentiviruses (pLKO.1-shRNA, Sigma) expressing five different shRNAs of human E2F3, four of which harmonize coding sequence of E2F3 and the other complements the 3’-untranslation region. Briefly, HeLa cells were plated in 60-mm plates. Following culture for 24 h and addition of polybrene, cells were infected pLKO.1-shRNA/E2F3 viruses at multiplicity of infection (MOI) of 100. After swirling the plate, the cell-viral particle mixture was incubated at 37°C overnight and then replaced with complete culture medium. For transient experiments, the cells were harvested after 48 h of infection and assayed by Western Blot. Stable knockdown of E2F3 cell lines were established by selection with puromycin (5µg/ml).

**Chromatin Immunoprecipitation (ChIP) Assay**

ChIP assay was performed essentially as previously described (25). Solubilized chromatin was prepared from a total of 2 x 10⁷ asynchronously growing HEK293 cells that were transfected with E2F3. The chromatin solution containing cross-linked binding proteins was diluted 10-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1, 0.01% SDS, protease inhibitors), and precleared with protein-A/G-agarose beads blocked with 2 µg of sheared salmon sperm DNA and preimmune serum. The precleared chromatin solution was divided and utilized in immunoprecipitation assays with either an anti-E2F3 antibody or an anti-actin antibody. Following wash, the antibody-protein-DNA complex was eluted from the beads by incubating the pellets in 1% SDS, 0.1 M NaHCO₃ at room temperature for 20 min.
The protein-DNA cross-linking was reversed, and protein and RNA were removed by incubation with 10 µg of Proteinase K and 10 µg of RNase A at 67 °C for 6 h to overnight. Purified DNA was subjected to PCR with primers specific for putative E2F3-binding sites within the Aurora-A promoter. The sequences of the PCR primers used are as follows: site A, sense (5’-GAATCCTGCCCAATCTACCGCTCC-3’) and antisense (5’-GAAAAGCAAGAGAGAGTGGGACCG-3’); site B, sense (5’-GCTATCGATCGGTCCCACTCTCTC-3’) and antisense (5’-CTTGAGTCGCGTTTAAGGGGCAC-3’); site C+D, sense (5’-CGACCGCTTGCTCCACCCTTCGG-3’) and antisense (5’-CCAGGAGCTCAGCCGTTAGAATTCAAGG-3’).
Results

Expression of the Members of E2F Family and Aurora-A during the Cell Cycle
While mRNA and protein levels of Aurora-A are elevated at G2/M phase, the underlying molecular mechanism remains elusive. E2F family members have been shown to play a critical role in cell cycle progression through transcriptional regulation of a number of cell cycle-associated genes. To examine if Aurora-A is regulated by E2F family, we compared the expression patterns of E2F family and Aurora-A proteins during the cell cycle (Fig. 2.1A). HeLa cells were synchronized with double-thymidine blocking and released for various time points. The cell cycle progression was monitored by flow cytometry. Before releasing, over 60% of the total cell population was at S phase. About 6 hours, cells entered G2/M. After 16 hours, the majority of the cells were in G1 phase (Figs. 2.1B and 2.1C). E2F1 and E2F2 protein levels were high in G1/S and decreased at G2/M phase (Fig. 2.1A). E2F4 and E2F5 proteins distributed to the whole cell cycle. Notably, E2F3 protein was elevated in S and maintained through G2/M phases and decreased in G1 phase. Recent studies have shown that E2F3 but not other E2F family members regulates the expression of genes that are involved in G2/M (21), implying that Aurora-A could be transcriptionally regulated by E2F3.

Ectopic Expression of E2F3 Induces and Knockdown of E2F3 Decreases Aurora-A Protein and mRNA Levels
To determine if E2F3 transcriptionally regulates Aurora-A, we transfected E2F3 into three cell lines, which include two human epithelial cell lines Hela and A2780S, and a mouse fibroblast NIH3T3 (Fig. 2.2A). Immunoblotting analysis showed that E2F3 upregulated Aurora-A protein expression in a dose-dependent manner (Fig. 2.2A). Moreover, mRNA levels of Aurora-A was also induced by ectopic expression of E2F3 (Fig. 2.2B). To further demonstrate Aurora-A expression is controlled by E2F3, we knocked down E2F3 by infection of HeLa cells with the lentiviruses (pLKO.1-E2F3/shRNA) expressing 5 different E2F3 shRNAs, 4 of which are corresponding to the coding region and the other matches the 3’-untranslated region. Fig. 2.2C showed that
E2F3 was considerably reduced in the cells infected with three individual shRNA as well as their combination. After selection with puromycin, stable E2F3-knockdown pool cells were obtained. Expression of Aurora-A was significantly reduced in these cells both at interphase and mitosis (Fig. 2.2D).

Fig. 2.1. The correlation of expression of E2F family proteins and Aurora-A during the cell cycle. HeLa cells were synchronized with double-thymidine block (DTB) and released for indicated time points. A portion of cells for each time point were lysed and immunoblotted with indicated antibodies (A). The rest were subjected to flow cytometry (B and C).
Fig. 2.2. E2F3 regulates Aurora A expression at protein and mRNA levels. (A and B) Ectopic expression of E2F3 induces Aurora-A expression. HeLa, NIH3T3 and A2780S cells were transfected with increasing amount of E2F3. Following 36 h incubation, cells were subjected to immunoblotting analysis with indicated antibodies (A) and RT-PCR assay for expression of Aurora-A (B). (C and D) Stable knockdown of E2F3 reduces Aurora-A expression. HeLa cells were infected with individual lentivirus expressing 5 shRNAs targeting different regions of E2F3 and all 5 shRNAs together. After selection with puromycin, stable knockdown clonal cells were immunoblotted with anti-E2F3 (top) and -actin (bottom of panel C) antibodies. E2F3-stably knockdown cells were synchronized with and without nocodazole and blotted with indicated antibodies (D). Relative Aurora-A mRNA or protein levels were normalized to actin and quantified by ImageJ software (NIH).
Knockdown of E2F3 Delays G2/M Entry and Reduces Aurora-A Expression during the Cell Cycle

Having demonstrated E2F3 transcriptional regulation of Aurora-A, we then examined the effects of knockdown of E2F3 on G2/M progression and Aurora-A expression during the cell cycle. pLKO.1-shRNA vector infected and E2F3 stable knockdown HeLa cells were synchronized by double-thymidine block. Flow cytometry analysis revealed that cells with knockdown of E2F3 delays to enter G2/M phase as compared to the pLKO.1 vector-infected cells (Fig. 2.3A). In consistence with cell cycle change, elevation of Aurora-A mRNA and protein levels was approximately 2 or 3 h delayed by knockdown of E2F3. Moreover, the mRNA and protein levels of Aurora-A at G2/M phase were also reduced in E2F3-knockdown cells as compared to control shRNA-treated cells (Figs. 2.3B and 2.3C). However, Aurora-A protein underwent degradation at late M phase in both cell lines (Fig. 2.3C). These data further suggest that Aurora-A is transcriptionally regulated by E2F3 during the cell cycle.

E2F3 Binds to and Transactivates Aurora-A Promoter during G2/M Phase

We next examined whether the Aurora-A promoter is transactivated by E2F3. HeLa cells were transfected with pGL3-Aurora-A/-1486/+354-Luc reporter and increasing amounts of E2F3. Luciferase reporter assay revealed that Aurora-A promoter activity was induced by E2F3 in a dose-dependent manner (Fig. 2.4A). Moreover, basal level of Aurora-A promoter activity, especially at mitosis, was reduced in E2F3-knockdown cells as compared to pLKO.1 vector-infected HeLa cells (Fig. 2.4B).

Sequence analysis showed 4 putative E2F-binding elements [TT(C/G)GCGC(C/G)] within the promoter (Fig. 2.4C). To define the response region(s) of the promoter to E2F3, we created a series of deletion mutants of Aurora-A promoter. Reporter assay showed that a mutant with deletion from -1486 to -415 significantly deceased E2F3-induced promoter activity while it still contains all 4 putative E2F-binding elements, implying that this region is of transactivation function. Moreover, the deletion of 2 distal E2F-binding sites (i.e., from -1486 to -189; A and B) further reduced the promoter activity. However, promoter activity of the mutants with additional deletion
of either from -189 to -124 or from -189 to -96, both of which retain the 2 E2F-response elements (C and D) proximal to the transcriptional starting site, was significantly induced by E2F3. The promoter activity was completely abrogated by further deletion of the proximal E2F-response elements (Fig. 2.4C). These results suggest that the region from -189 to -96 contains a repression element(s) and that the all four E2F-binding sites are response to E2F3. However, the two binding elements proximal to the transcriptional start site are sufficient for E2F3 transactivation of the Aurora-A promoter.

Fig. 2.3. Knockdown of E2F3 results in mitotic cell entry delay and decreased Aurora-A expression during the cell cycle. Control shRNA and stable E2F3-shRNA infected HeLa cells were synchronized with double-thymidine block and released for indicated time points. Cells were analyzed by flow cytometry (A), Northern (B) and Western (C) blotting with indicated probe and antibodies.
Fig. 2.4. **Aurora-A promoter is regulated by E2F3.** (A) Ectopic expression of E2F3 induces Aurora-A promoter activity. NIH3T3 cells were transiently transfected with indicated plasmids. Following 48 h incubation, luciferase activity was measured and normalized to β-gal. Results are the mean ± SEM of three independent experiments performed in triplicate (top). Bottom panel shows expression of transfected E2F3. (B) Knockdown of E2F3 reduces basal level of Aurora-A promoter activity. Aurora-A-Luc and β-gal were introduced into pLKO.1 control shRNA- and E2F3-shRNA-infected HeLa cells. After 36h incubation, cells were treated with and without nocodazole for 12 h and then subjected to luciferase assay. (C) Deletion mapping of E2F3 response elements in Aurora-A promoter. NIH3T3 cells were transfected with indicated Aurora-A deletion mutants and different amounts of E2F3 plasmid. E2F3 binding sites were labeled as “A – D”. Luciferase assay was performed after 36 h of transfection.
To determine whether E2F3 could directly bind to the E2F-binding sites of the Aurora-A promoter in vivo and to further define the E2F3 response elements in the promoter, we carried out ChIP assay, which detects specific genomic DNA sequences that are associated with a particular transcription factors in intact cells. HeLa cells were transfected with wild type E2F3. Following 36 h incubation, unsynchronized cells were immunoprecipitated with an anti-E2F3 antibody. The E2F3 bound chromatin was subjected to PCR using 3 pairs of oligonucleotide primers that amplify regions spanning each distal and 2 proximal E2F3-binding sites within the Aurora-A promoter because 2 proximal E2F-binding sites are so close that could not be separated by ChIP assay. As shown in Figs. 2.5A and 2.5B, the anti-E2F3 antibody pulled down all E2F-binding sites. In contrast, immunoprecipitation with an irrelevant antibody (e.g. anti-Actin) resulted in the absence of bands in these sites. These results indicate that E2F3 directly binds to the Aurora-A promoter. By mutation of individual or combinational E2F3 binding sites (CG → AT) in Aurora-A promoter, we further demonstrated that the 2 E2F-response elements proximal to transcriptional start site are required for E2F3 transactivation of the Aurora-A promoter (Figs. 2.5A and 2.5B).

Since mRNA and protein levels of Aurora-A are low at G1 and gradually increase during G2/M phase, we next examined if E2F3 binding to and activation of the Aurora-A promoter is cell cycle dependent. HeLa cells were transfected with pGL3-Aurora-A or pGL3-vector. Following synchronization with double-thymidine block, ChIP and luciferase reporter assays were performed at different phases of the cell cycle. Fig. 2.5C shows that E2F3 barely interacts with Aurora-A promoter during G1/S phase. The binding and transactivation activities of E2F3 toward the Aurora-A promoter were significantly increased upon cell entering G2/M phase. These data further support the notion that E2F3 plays a pivotal role in Aurora-A expression during G2/M phase.
Fig. 2.5. E2F3 binds to Aurora-A promoter in a cell cycle dependent manner. (A and B) E2F3 interacts with Aurora-A promoter in vivo. ChIP assay was performed as
described in Materials and Methods with unsynchronized HeLa cells (left panels). E2F3 binding sites C and D are too close to separate by ChIP assay. Luciferase assay was performed with Aurora-A-Luc reporter plasmids containing E2F3-binding site mutation (right panels). (C) E2F3 binds to Aurora-A promoter during G2/M phase. HeLa cells were synchronized with double-thymidine block and released for indicated times. Cell cycle was monitored with flow cytometry (top) and ChIP assay was performed for each time point (panel 2). Aurora-A RNA levels (pane 3) and promoter activity (bottom) are correlated with E2F3 binding to Aurora-A promoter.

Correlation of Expression of E2F3 and Aurora A
To further demonstrate E2F3 regulation of Aurora-A, we transfected GFP-E2F3 into HeLa cells. After 48 h of transfection, cells were immunostained with anti-Aurora-A antibody. As shown in Fig. 2.6, cells expressing GFP-E2F3 exhibited higher density and clearer centrosome than the cells that did not, further indicating E2F3 upregulation of Aurora-A.

![Image of E2F3-GFP, Aurora A, DAPI, and Merge](image)

**Fig. 2.6. Expression of E2F3 increases Aurora-A density in centrosome.** HeLa cells were transfected with GFP-E2F3 and immunostained with anti-Aurora-A antibody. Two cells are shown in the picture (arrows), one cell expresses E2F3 (green) and the other one does not.
We and others have previously shown amplification and overexpression of Aurora-A in ovarian cancers (9,26). However, the frequency of elevated Aurora-A protein and/or mRNA is much higher than its change at DNA level (e.g. ~15%), suggesting the mechanism of activating transcription and/or translation of Aurora-A in ovarian cancer cells. While overexpression of E2F3 has been detected in different tumors (27-29), the E2F3 status in ovarian cancer has not been well documented. Thus, we reasoned that E2F3 could be elevated in ovarian cancer and might be a causal factor of upregulation of Aurora-A. To this end, we examined protein levels of E2F3 and Aurora-A in human ovarian cancers. Immunoblotting analyses were performed in total 8 ovarian cancer cell lines and 72 microdissected ovarian tumor specimens (Figs. 2.7A and 2.7B). Elevated levels of E2F3 and Aurora-A were detected in 43 of 72 (60%) and 41 of 72 (57%) primary tumors, respectively, as well as majority of ovarian cancer cell lines examined. Notably, 78% (32/41) of tumors with high levels of Aurora-A overexpress E2F3 (Fig. 2.7C). Co-upregulation of E2F3 and Aurora-A seems to be predominantly observed in serous ovarian carcinomas and clear cell tumors although the number of cases is relatively small (Fig. 2.7D). These data indicate that in a large subset of ovarian tumors elevated Aurora-A might result from expression of high level of E2F3 and further support the findings of biochemical and functional links between E2F3 and Aurora-A.
Overexpression of E2F3 correlates with Aurora-A level in human ovarian tumors. (A and B) Western blot analysis. Representative ovarian cancer cell lines (A) tumor and normal ovarian tissue (B) lysates were analyzed by Western blot with indicated antibodies. Intensity of E2F3 and Aurora-A were quantified via ImageJ software. The overexpression of E2F3 and Aurora-A in tumor samples was scored based on the average values of the normal tissues from three independent experiments.

Table 2. Summary of expression of E2F3 and Aurora-A in ovarian cancer.

<table>
<thead>
<tr>
<th>TYPE</th>
<th>Samples</th>
<th>AurA</th>
<th>E2F3</th>
<th>Aurora/E2F3 (Percentage of Samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous</td>
<td>38</td>
<td>27</td>
<td>29</td>
<td>24 (63%)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>9</td>
<td>2</td>
<td>4</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2 (67%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>STAGE</th>
<th>Samples</th>
<th>AurA</th>
<th>E2F3</th>
<th>Aurora/E2F3 (Percentage of Samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-II</td>
<td>20</td>
<td>5</td>
<td>7</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>III-IV</td>
<td>39</td>
<td>28</td>
<td>28</td>
<td>23 (59%)</td>
</tr>
<tr>
<td>NS</td>
<td>13</td>
<td>8</td>
<td>8</td>
<td>7 (54%)</td>
</tr>
</tbody>
</table>

| TOTAL  | 72      | 41   | 43   | 32 (44%)                            |
| Percent (of TOTAL) | 57% | 60% | 44% |

*Correlation between Aurora-A and E2F3 is (32/41) 78%, p<0.05.
NS: unstaged
Discussion

In this report, we investigated the transcriptional regulation of Aurora-A by E2F3. Ectopic expression of E2F3 induced mRNA and protein levels of Aurora-A whereas knockdown of E2F3 decreased Aurora-A expression and resulted in mitotic cell cycle delay, which resembles the mitotic arrest phenotypes in Aurora-A siRNA treated cells (4). Notably, chromatin immunoprecipitation revealed that E2F3 bound to Aurora-A promoter in vivo and the interaction is cell-cycle dependent, i.e., primarily occurred during G2/M phase. Moreover, co-overexpression of Aurora-A and E2F3 was frequently detected in ovarian cancer. These findings are important for several reasons. First, they provide a mechanistic understanding of transcriptional regulation of the Aurora-A expression during the cell cycle. Second, a direct link between Aurora-A and the E2F3 has now been established. Finally, this is the first demonstration of co-alteration of E2F3 and Aurora-A in human ovarian cancer and elevated E2F3 could be a causal factor for deregulation of Aurora-A in this disease.

In mammalian cells, E2F family is composed of 10 distinct gene products encoded by eight independent loci that can be divided into three subfamilies based on their sequence homology – the E2F1–3 genes, the E2F4 and 5 genes and the E2F6–8 genes. The E2F1–3 genes have been shown to be tightly regulated during the cell cycle, whereas the E2F4–8 genes are constitutively expressed. Functionally, E2F1–3 act as positive regulators of transcription whose accumulation is tightly regulated and in most cell types correlates with increased cell proliferation (30-32), whereas E2F4 and E2F5, when bound to p130 or Rb, act as transcriptional repressors (33). The E2F6–8 proteins appear to function as Rb-independent transcriptional repressors (34,35). In addition, the E2F3 locus expresses two distinct transcripts, full-length E2F3a and N-terminal-truncated E2F3b transcribed from an intronic promoter within the E2F3 locus (36). E2F3a expression is cell cycle regulated whereas E2F3b is expressed equivalently in quiescent and proliferating cells, and may have an opposing role to E2F3a in cell cycle control. Accumulated evidence shows that E2F3 (e.g. E2F3a) regulates S and G2/M cell cycle progression (15,16). Gene expression microarray analyses have revealed that E2F3
regulates many of the DNA replication, mitotic and cell cycle regulatory genes (15,21). A previous report has shown that E2F3 regulates cyclin B1, cyclin A2 and cdc2 transcription (18). We demonstrated, in the present study, that E2F3 directly binds to Aurora-A promoter and tightly regulates Aurora-A expression during the G2/M phase.

Previous studies have shown that Aurora-A is transcriptionally regulated by E4TF1, a member of the Ets family, and GABP, the Ets-related transcription factor GABP (37,38). E4TF1 and GABP bind to the same DNA-binding motif (CTTCCGG; -85 to -79) of the human Aurora-A promoter to induce Aurora-A promoter activity and transcription. The transactivation of Aurora-A by GABP is regulated through interaction with TRAP220/MED1, an evolutionarily conserved multisubunit coactivator that plays a central role in regulating transcription from protein-encoding genes (37,38). Tanaka et al. cloned human Aurora-A promoter and identified 2 E2F binding elements which correspond to the binding sites A and B in Fig. 2.4C. The findings presented here show that while E2F3 binds to these 2 sites, their mutations still respond to E2F3 (Fig. 2.5A). This led us to identify 2 more E2F binding motifs proximate to transcriptional start site (Fig. 2.4C). ChIP and reporter assays show that they are required for the transcriptional activation of Aurora-A gene by E2F3 (Figs. 2.5 B and 2.5C).

Previous reports have demonstrated that E2F3 is frequently overexpressed in a variety of types of human malignancy and whose alteration is associated with late stage and high grade tumors. However, alterations of E2F3 in human ovarian cancer have not been well documented while a gene expression array study shows upregulation of E2F3 in ovarian tumor (39). Previously, we have shown frequent overexpression of Aurora-A in ovarian cancer. In the present study, we demonstrated frequent co-existence of elevated levels of E2F3 and Aurora-A in human primary ovarian carcinoma. Strong association of elevated E2F3 expression and Aurora-A in ovarian tumors underscored the clinical significance of the E2F3-Aurora-A signaling axis. It is very likely that elevated E2F3 is one of the major transcriptional factors that contribute to upregulation of Aurora-A in other human primary tumors. As described above, E4TF1 and GABP are known transcriptional regulators of Aurora-A (37,38), however, their role in Aurora-A induction in human cancer are unclear.
Importance of Aurora-A and E2F3 in oncogenesis has been well established by their alterations in human neoplasms and their capacity to induce cell transformation (27-29,40). Based on our current observations, we believe that Aurora-A, as a mitotic E2F3 target gene, could mediate E2F3 oncogenic function. Pharmacological agents that inhibit E2F3 or its downstream mediator Aurora-A may lead to inhibition of tumor development underscoring the significance of the E2F3-Aurora-A axis as an attractive target for cancer therapy.
References


CHAPTER III

AURORA-A FEEDBACK REGULATION OF CHFR, A TUMOR SUPPRESSOR FREQUENTLY DOWNREGULATED IN OVARIAN CANCER

Abstract

CHFR is a tumor suppressor gene which encodes a forkhead-associated (FHA) and ring-finger (RF) domains containing protein and plays an important role in G2/M cell cycle checkpoint. Previous studies show that CHFR exerts tumor suppressor function via its E3 ligase activity by targeting Aurora-A and polo-like kinase degradation. Hypermethylation and mutation of CHFR have been detected in human cancer from different organs including stomach, breast, colon and lung. However, molecule(s) that regulate CHFR function and alteration of CHFR in human ovarian cancer remain largely unknown. Here we demonstrate that Aurora-A phosphorylates CHFR at serine-218 and serine-337 in vitro and in vivo. Phosphorylated CHFR reduces its E3 activity and tumor suppressor function. Further, frequent downregulation of CHFR was detected in human ovarian cancer cell lines and primary tumors. Expression of the CHFR could be restored by treatment with methyltransferase inhibitor 5-aza-2'-deoxycytidine and histone deacetylase inhibitor TSA. Three of 4 cell lines and 9 of 17 tumors with downregulation of CHFR exhibited higher levels of Aurora-A, supporting the notion that CHFR is an E3 ligase of Aurora-A. These findings indicate that CHFR is a substrate of Aurora-A and that epigenetic inactivation of CHFR is a recurrent event in ovarian cancer and could play a pivotal role in ovarian oncogenesis.

Key words: tumor suppressor, E3 ligase, hypermethylation, phosphorylation, ovarian cancer
Introduction

The segregation of chromosomes at mitosis involves several processes, including chromosome condensation in prophase, chromosomal alignment on the spindle in metaphase, and sister-chromatids separation in anaphase (1-3). CHFR (checkpoint with forkhead and ring finger) has been discovered as a prophase checkpoint protein. Activation of CHFR delays chromosome condensation and nuclear envelope breakdown in response to mitosis stress induced by taxol or nocodazole (4). The CHFR protein contains an N-terminal forkhead-associated (FHA) domain, a central ring finger (RF) region, and a C-terminal cysteine-rich (CR) motif. The FHA domain is conserved in several checkpoint genes, which plays a role in recognizing phosphorylated proteins. Deletion of the FHA domain has been shown to attenuate CHFR function; the CR domain is also required for the checkpoint function (4). The RF domain is required for CHFR ubiquitin ligase activity. It has been shown that CHFR mediates the degradation of polo-like kinase 1 (PLK1), Aurora-A and CHFR itself. Ubiquitination of PLK1 by CHFR delays the activation of Cdc25C and the inactivation of Wee1, leading to delay of Cdc2 activation, which provides a molecular mechanism that CHFR executes checkpoint function by ubiquitin-dependent proteolysis (5). However, another study suggests that CHFR delays cells in early prophase by inhibiting entry of cyclin B1 in the nucleus (6).

CHFR localizes to chromosome 12q24.33, where is frequently deleted in human cancers (7-9). The frequent inactivation of CHFR which caused by DNA methylation or mutation has been reported in human primary tumor and cancer cell lines of lung, colon, esophagus, gastric, brain, bone and breast (10-14). CHFR-deficient mice are cancer-prone and develop spontaneous tumors (15).

Aurora-A kinase is required for mitotic entry, centrosome maturation and chromosome segregation (16,17). Aurora-A expression and kinase activity are cell cycle regulated. Aurora-A mRNA and protein levels begin to accumulate at G2/M transition. After executing its mitotic kinase function through metaphase, Aurora-A protein is rapidly degraded by hCdh1-activated anaphase-promoting complex/cyclosome (APC/C) (18). The precise timing of activation and degradation is the key feature of Aurora-A
regulation. A previous report has demonstrated that CHFR serves as the E3 ligase for Aurora-A proteasome degradation (15). In the present study, we show that CHFR is a substrate of Aurora-A and is downregulated in ovarian cancer. Aurora-A phosphorylates CHFR leading to inhibition of its E3 ligase activity. We previous showed frequent upregulation of Aurora-A in human ovarian cancer and demonstrated in this report inverse correlation of Aurora-A and CHFR expression in this malignancy. Thus, these results uncover a feedback regulation loop between Aurora-A and CHFR and suggest potential importance of CHFR-Aurora-A axis in ovarian carcinogenesis.
Materials and Methods

Cell Lines, Transfection and Treatment

The human epithelial ovarian cancer cell lines were cultured in appropriate mediums supplemented with 10% fetal bovine serum: OV3, OV5, OV8, A2780S, IGROV, OV90 and C13 were grown in RPMI 1640 medium; OV2 was cultured in 50:50 medium M199:MCDB105; RMUG-S was grown in F-12 medium; UTOV1, UTOV2, UTOV3A and UTOV4 were cultured in DMEM. All the media were supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 100 IU/ml penicillin and 100 IU/ml streptomycin. Human embryonic kidney (HEK) 293 cells were cultured in DMEM containing 10% FBS. Cells were seeded in 60-mm Petri dishes at a density of 0.5 × 10^6 cells/dish. Cells were grown overnight to reach log growth phase before transfection. Transfection was performed using LipofectAMINE 2000 (Invitrogen).

To restore endogenous CHFR expression, the cells were treated methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza) at concentration of 5 µM for 4 days and HDAC inhibitor Trichostatin-A (TSA, 100 ng/ml) for last 24 hours. Cycloheximide (CHX) was used to block protein synthesis at a concentration of 50 µg/ml. Proteasome inhibitor MG132 was administered to cells at 10µM for 6 hours in ubiquitination assay.

Plasmids

pcDNA3-myc-CHFR was kindly provided by Dr. Junjie Chen. Different truncated forms of GST-CHFR were created by PCR amplification using pcDNA3-myc-CHFR as a template. The PCR products were digested by BamHI and XhoI and cloned into pGEX4T-1 vector. The accuracy of resulting constructs was confirmed by DNA sequencing. The oligonucleotide primers used were as follows: CHFR1 (sense), 5’-CGCGGATCCAGCTTATGGAGCGCCCGAGGAAGGCAAG-3’; CHFR110 (sense), 5’-CGCGGATCCAGCTTAATGAACCGGAACACAACGTGGCA-3’; CHFR124 (antisense), 5’-TGCTCTAGCCCTCGAGACTTAAAGATTCATAGAGGTATGC-3’; CHFR279 (sense), 5’-CGCGGATCCAGCTTAAATGACCAGGAACACAACGTGGCA-3’; CHFR281
(antisense), 5’-TGCTCTAGCCTCGAGGGCATTTCTACGCGGTTGTGCGAC-3’;
CHFR376 (antisense), 5’-
 TGCTCTAGCCTCGAGCACATCTTTCTTCACTGCGACTCTT-3’.

CHFR mutant plasmids were generated by QuikChange (Multi) Site-Directed Mutagenesis Kit (Stratagene), including pCDNA3-myc-CHFR/S218A, pCDNA3-myc-CHFR/S337A, pCDNA3-myc-CHFR/S218S337A; and GST-CHFR-C2/S218A, GST-CHFR-C2/S337A, GST-CHFR-C2/S218S337A. Primers used for mutagenesis reactions were: CHFR-S218A-F, 5’-CCTAAAGGAGCTGGTCCCTCTGTG-3’; CHFR-S218A-R: 5’-CACAGAGGGACCAGCTCCCTCTGTG-3’; CHFR-S337A-F, 5’-
 AGGACACAGGGCCGAGCGCTCCAT-3’; CHFR-S337A-R, 5’-
AGGACACAGGGCCGAGCGCTCCAT-3’.

**RT-PCR**

Total RNA was isolated from cells or tissues using Trizol Reagent (Invitrogen). 1-2 µg RNA was reverse transcribed by M-MLV reverse transcriptase (Promega) at 37°C for 1.5 hour. The sequential PCR reaction to amplify CHFR from the cDNA was carried out using Taq polymerase (Promega) with 30 cycles of denaturation 30 seconds at 95°C; annealing 30 seconds at 60°C; and extension 60 seconds at 72°C. The primers used for PCR were: CHFR-RT-F, 5’-GGCGAGAGCGTTCTCCAGTTG-3’; CHFR-RT-R, 5’-
GCATGTCAGCGTCTCCTCCATCTTG-3’.

**Western Blotting Analysis and Immunoprecipitation**

Western blotting analysis was performed as previously described (19). Briefly, cells and tumor tissues were lysed in RIPA buffer (50 mM NaCl, 0.5% (w/v) DOC, 50 mM Tris-HCl (pH 8.0), 1% (v/v) NP-40, 0.1% (w/v) SDS, 1µg/ml aprotinin, 1µg/ml leupeptin, 0.5mM Na3VO4 and 1mM PMSF). Equal amounts of proteins were separated in SDS-polyacrylamide gel and electroblotted onto Nitrocellulose (Amersham) membranes. Following blocking in TBS-T containing 5% milk, the membranes were hybridized with appropriate antibodies indicated in figure legend. Detection of antigen-bond antibody was carried out using ECL Western Blotting Detection System (Amersham).
For immunoprecipitation (IP), cells were lysed in TNEN buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% (v/v) NP40, 0-0.3% (v/v) Triton X-100, protease and phosphatase inhibitors (1µg/ml aprotinin, 1µg/ml leupeptin, 0.5mM Na₃VO₄ and 1mM PMSF). The lysates were precleared with 25µl protein A-protein G (2:1) agarose beads at 4°C for 30 min. Following removal of the beads by centrifugation, the lysates were incubated with 30µl antibody conjugate protein A:G (2:1) agarose beads at 4°C for 4 hours. The beads were washed 3 times with lysis buffer and subjected to immunoblotting analysis with the antibodies indicated in figure legend.

**In vivo [³²P] Pi Labeling**
HEK293 cells were co-transfected with myc-CHFR and HA-Aurora-A or pcDNA3. After 24 hours incubation, cells was serum starved for overnight and then incubated with phosphate-free and serum-free medium for 2 hours. [³²P] Pi was added to the same medium at final concentration of 0.5 mCi/ml. Following 4 hours incubation, cells were lysed and subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were separated by SDS-PAGE and transferred to membranes. Phosphorylated CHFR band was examined by autoradiography. The expression of transfected CHFR and Aurora-A was detected with anti-Myc and -HA antibodies, respectively.

**In vitro Aurora-A Kinase Assay**
Aurora-A kinase assay was performed as previously described (20). Briefly, reactions were carried out in a kinase buffer in the presence of 10 µCi of [γ-³²P] ATP (Perkin Elmer Life Sciences). Recombinant Histone H3 or purified GST-CHFR protein was used as substrate. After incubation at 30°C for 30 min, the reaction was stopped by adding protein loading buffer. The proteins were separated by SDS-PAGE, and the amounts of incorporated radioactivity were determined by autoradiography.
Following *in vitro* kinase assay, Aurora-A-phosphorylated CHFR was extracted from the SDS-polyacrylamide gel by cutting the protein band and incubating in a buffer containing 50 mM ammonium bicarbonate, 1% 2-mercaptoethanol, and 0.2% SDS for at least 90 min duplicated at 37°C. After chloroform precipitation and oxidation with performic acid, the sample was evaporated and resuspended by ammonium bicarbonate. The protein was digested with 10 ng of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin at 37°C for overnight, followed by an additional overnight incubation at 37°C in the presence of a fresh 10 ng of the same proteinase. The trypsin digests were washed for 4 times, lyophilized, and resuspended in deionized water.

Phosphopeptides of CHFR were resolved at the first-dimension by electrophoresis on the cellulose layer of TLC plate for 30 min at 1.0 kV in 88% formic acid/glacial acetic acid/water (1:3.1:35.9; pH 1.9) and 4.7 (n-butanol:pyridine:glacial acetic acid:water = 2:1:1:36), and then for 20 min in a buffer pH 8.9 (10 g ammonium carbonate in 1 liter water) using the HTLE-7000 system. The phosphopeptides were further separated at second-dimension by chromatography in Phosphochromatography buffer (n-butanol:pyridine:glacial acetic acid:water = 5:3.3:1:4). The phosphopeptides were visualized by autoradiography.

For phosphoamino acid analysis, the phosphopeptides were recovered from the TLC plates and hydrolyzed in 6N HCl at 110°C for 60 min. Following acid hydrolysis, phosphoamino acids were separated by two-dimensional electrophoresis on TLC plates. The electrophoresis was carried out for 20 min at 1.5 kV in pH 1.9 buffer for the first dimension and for 16 min at 1.3 kV in pH 3.5 buffer (pyridine:glacial acetic acid:water = 1:10:189) for the second dimension. Phosphoamino acid standards, which were mixed with each sample, were visualized by staining with 0.25% w/v ninhydrin in acetone.

For Edman degradation, the phosphopeptides were eluted from the cellulose layer of the TLC plates by incubation in deionized water for 30 min at room temperature; second elution may be applied if necessary. The purified phosphopeptides were subjected to manual Edman degradation to determine at which position the phosphorylated amino acid was present in the peptide. At pH 8.0 to 9.0, phenylisothiocyanate reacted with the
free amino group of the most amino-terminal amino acid to form a corresponding phenylthiocarbamyl (PTC) peptide. During each cycle of Edman degradation, treatment of trifluoroacetic acid (TFA) resulted in the cleavage and release of the derivatized amino-terminal amino acid, and a sample from the reaction mixture was withdrawn. If a phosphoserine or phosphothreonine residue is present, a β-elimination during the cyclization released free phosphate. The free phosphate was separated from the peptide by electrophoresis, and the phosphoamino acid was determined.

In vivo Ubiquitination Assay

The cell ubiquitination assay was carried out as previously described (21). HEK293 cells were transfected with combinations of His6-Ubiquitin, HA-PLK1, myc-CHFR and HA-Aurora-A using calcium phosphate. After 36 h of transfection, cells were collected into two aliquots, one of which was used for Western blot analysis to confirm expression of transfected plasmids and the other was subjected to purification of His6-tagged protein using Ni2+-nitrilotriacetic acid (NTA) conjugated agarose beads (Qiagen). Briefly, the cell pellet was lysed in buffer A (6 M guanidinium-HCl, 0.1 M Na2HPO4/NaH2PO4 [pH 8.0], 0.01 M Tris-HCl [pH 8.0], 10 mM imidazole, 10 mM β-mercaptoethanol) and incubated with Ni2+-NTA beads for 6 hours at room temperature. The beads were sequentially washed with buffer A, buffer B [8 M urea, 0.1 M Na2PO4/NaH2PO4 [pH 8.0], 0.01 M Tris-HCl (pH 8.0), 10 mM imidazole, 10 mM β-mercaptoethanol], buffer C [8 M urea, 0.1 M Na2PO4/NaH2PO4 (pH 6.3), 0.01 M Tris-HCl (pH 6.3), 10 mM imidazole, 10 mM β-mercaptoethanol], 0.2% Triton X-100, and then buffer C. The beads bound proteins were eluted with buffer D [200 mM imidazole, 0.15 M Tris-HCl (pH 6.7), 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS] at room temperature. The eluted proteins were analyzed with Western blot in the presence of ubiquitin-conjugated PLK1 using anti-HA antibody.
Results

Downregulation of CHFR in Human Ovarian Cancer and the Correlation between Expression of CHFR and Aurora-A

While epigenetic inactivation of CHFR has been observed in various types of human malignancy (22-26), its alteration in ovarian cancer remains elusive. Since CHFR resides in chromosome 12q24 region, which is frequently deleted in ovarian cancer (27), we examined expression of CHFR in both ovarian cancer cell lines and primary tumors by semi-quantitative RT-PCR. Fig. 3.1A shows that CHFR was downregulated in 8 of 17 ovarian cancer cell lines and 17 of 30 primary ovarian tumors examined. Undetectable CHFR was observed in 4 cell lines and 9 primary tumors. To determine if DNA methylation is responsible for CHFR downregulation in ovarian cancer, we treated 4 ovarian cancer cell lines, which do not express CHFR, with combined 5-aza (methyltransferase inhibitor) and TSA (HDAC inhibitor). CHFR expression was restored in C13 cells (Fig. 3.1B) but not the other 3 cell lines (data not shown), suggesting that DNA methylation is not the only mechanism for downregulation of CHFR. Nevertheless, these results indicate that deregulation of CHFR is a recurrent event in human ovarian cancer.

We and others have previously shown amplification and overexpression of Aurora-A in 15% and 50% ovarian cancers, respectively (28), suggesting that deregulation of Aurora-A at transcriptional or/and translational levels occurs in a large fraction of tumors. Since CHFR is an E3 ligase of Aurora-A (15), downregulation of CHFR could be a causal factor for upregulation of Aurora-A. By comparing expression levels of CHFR and Aurora-A, we found an inverse correlation between Aurora-A and CHFR expression in a subset of ovarian cancer (Fig. 3.1C). For instance, C13 and SW626 cells do not express CHFR, but have high levels of Aurora-A, whereas SKOV3 and OV90 express CHFR but not Aurora-A (Fig. 3.1C). However, it is noted that a subset of ovarian cancers exhibit high levels of both Aurora-A and CHFR, suggesting a feedback regulation between Aurora-A and CHFR.
Fig. 3.1. Frequent downregulation of CHFR in ovarian cancer. (A) RT-PCR analysis of CHFR mRNA levels in ovarian cancer cell lines and primary ovarian tumors. Total RNA was isolated and subjected to RT-PCR analysis for expression of CHFR (top) and β-actin (bottom). (B) Restoration of CHFR in C13 cells by demethylation. C-13 and OV3 cells were treated with 5-Aza for 72 hours and together with TSA for another 24 hours. (C) Correlation between expression of Aurora-A and CHFR. Indicated ovarian cancer cells were immunoblotted with anti-Aurora-A (top panel) and β-actin (panel 2) antibodies. RNAs from these cells were subjected to RT-PCR analysis for expression of CHFR (panel 3) and β-actin control (bottom panel).

Aurora-A Phosphorylates CHFR in vivo and in vitro

To determine if Aurora-A phosphorylates CHFR, we carried out in vivo labeling experiment. HEK293 cells were co-transfected with Myc-CHFR together with/without HA-Aurora-A. Following 48 h incubation, cells were labeled with orthophosphate [$^{32}$P] Pi and immunoprecipitated with anti-Myc antibody. The immunoprecipitated Myc-CHFR
was separated by SDS-PAGE and the phosphorylated CHFR protein was revealed by autoradiography. Fig. 3.2A shows that phosphorylation level of CHFR was significantly increased in cells cotransfected with Aurora-A/CHFR as compared to cells treated with CHFR alone. We next carried out *in vitro* kinase assay. Immunoprecipitated CHFR was incubated with or without recombinant Aurora-A protein (Upstate). As shown in Fig. 3.2B, CHFR phosphorylation was detected only in the presence of Aurora-A kinase. These findings demonstrate that CHFR is phosphorylated by Aurora-A *in vitro* and *in vivo*.

<table>
<thead>
<tr>
<th>A</th>
<th>myc-CHFR</th>
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<td>HA-AurA</td>
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\[ {^{32}P} \text{CHFR} \]

\[ \text{CHFR} \text{ (fold)} \]

\[ 1 \quad 2.2 \]

\[ \text{HA} \]

\[ \text{myc} \]

\[ \text{Actin} \]

\[ \text{in vivo Labeling} \]

<table>
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<tr>
<th>B</th>
<th>myc-CHFR</th>
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<td>rAurora A</td>
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\[ {^{32}P} \text{CHFR} \]

\[ \text{IP: anti-myc} \]

\[ 1 \quad 1.6 \text{ (fold)} \]

\[ \text{CBS} \]

\[ \text{in vitro Kinase Assay} \]

**Fig. 3.2. CHFR is phosphorylated by Aurora-A.** (A) *In vivo* labeling. HEK293 cells were transfected with myc-CHFR and HA-Aurora-A or vector, labeled by \[^{32}P\] Pi and immunoprecipitated with anti-Myc antibody. The immunoprecipitates were separated in SDS-PAGE and the phosphorylated CHFR was revealed by autoradiography (top panel). Expression of Aurora-A and CHFR as well as even loading were confirmed by Western blot analysis with anti-HA (panel 2), -Myc (panel 3) and -actin (bottom panel) antibodies. (B) *In vitro* kinase assay. Myc-CHFR was introduced to HEK293 cells and immunoprecipitated with anti-myc antibody. The immunoprecipitated Myc-CHFR was incubated with recombinant Aurora-A protein in kinase buffer for 30 min. The reactions were stopped by protein loading buffer and resolved by SDS-PAGE (top panel). Bottom panel is the gel stained with Coomassie blue. Above phosphorylation levels of CHFR were quantified by ImageJ.
Mapping Phosphorylation Sites of CHFR by Aurora-A

To define the Aurora-A phosphorylation site(s) of CHFR, we generated a series of glutathione S-transferase (GST)-CHFR truncated fusion proteins (Fig. 3.3A). *In vitro* kinase assay revealed that CHFR fragments C2 (aa1-376) and C4 (aa110-376) were highly phosphorylated by Aurora-A; but minimal or no phosphorylation was observed in C1 (aa1-124), C3 (aa110-281) and C5 (aa279-376). This indicates that Aurora-A phosphorylation of CHFR site(s) must reside in a region between aa110 to aa376.

To map the phosphorylation sites of CHFR by Aurora-A, we performed 2-Dimensional Phosphopeptide Mapping analysis. *In vitro* kinase assay was carried out by incubation of GST-CHFR-C2 protein with recombinant Aurora-A. The phosphorylated GST-CHFR-C2 was extracted from SDS-PAGE gel. After digestion with trypsin, which cleaves the peptides after R/K, the phosphopeptides were separated on TLC plates. As shown in Fig. 3.3B, we identified 2 major phosphopeptides A and B. To determine whether the phosphorylated residue is serine or threonine, we performed Phosphoamino Acid Analysis. The phosphopeptides were recovered from the TLC plates. Following acid hydrolysis, phosphoamino acids were separated by two-dimensional thin-layer electrophoresis on TLC plates. The plates were dried in 80°C for 30 minutes, sprayed with ninhydrin in acetone and reheated for 5 minutes to visualize the phosphoamino acid standards. Following autoradiography, align the film with the plate and both the residues were identified to be serine (Fig. 3.3C). The phosphopeptides isolated from TLC plates were subjected to manual Edman degradation to determine the position of the phosphorylated amino acid in the peptide. Basically, the labeled peptides were cleaved one by one from the very amino-terminal of the peptide without disrupting the peptide bonds between other residues. The [\(^{32}\text{P}\)]-labeled phosphate group was released where the phospho-residue was cleaved, and form a separate spot from the peptide and closer to the anode, because the phosphate is smaller and negatively charged. As shown in Fig. 3.3D, both the phosphopeptides released the phosphate at the second cycle. Judging from the amino acid sequence of CHFR-C2, the endoproteinase trypsin-digested peptides that contain serine or threonine at position 2 are serine-218 and serine-337, respectively (Fig. 3.3D).
Fig. 3.3. Define Aurora-A phosphorylation sites of CHFR. (A) Different CHFR fragments were fused to GST (top). In vitro Aurora-A kinase assay was carried out by incubation of GST-CHFR fusion proteins with recombinant Aurora-A in a kinase buffer containing 10 μCi of [γ-32P] ATP. After 30 min incubation, the reactions were separated in SDS-PAGE and exposed to X-ray film. Histone H3 was used as a positive control and GST-protein as a negative control (middle). The gel was subsequently stained with Coomassie blue (bottom). (B) Aurora-A phosphorylates CHFR at 2 sites identified by two-dimensional phosphopeptide mapping. The phosphorylated GST-CHFR-C2 protein, which was extracted from SDS-PAGE mentioned in A, was digested by trypsin and separated on TLC plate by electrophoresis at first-dimension and chromatography at second-dimension. Each spot on the plate represents a phosphopeptide. (C) Aurora-A phosphorylation of CHFR on serine residues. Phosphopeptides were recovered from TLC plates. Following acid hydrolysis, phosphoamino acids were separated by two-dimensional electrophoresis on TLC plates. Phosphoamino acid standards, which were mixed with each sample, were visualized by staining with 0.25% w/v ninhydrin in acetone. (D) The phosphopeptides were eluted from TLC plates and purified for manual Edman degradation. During each cycle of Edman degradation, the most amino-terminal amino acid was cleaved, and a sample from the reaction mixture is withdrawn. The free phosphate, which can be separated from the peptide by electrophoresis, was released where a phosphoserine or phosphothreonine residue is present.

To confirm this result, we converted Ser218 and Ser337 to alanine. In vitro kinase assay, using wild-type GST-CHFR-C2, C2-S218A, C2-S337A and C2-S218/337A as substrates, shows that mutation of either site or both significantly reduces Aurora-A phosphorylation of CHFR (Fig. 3.4A). Further, immunoprecipitation and immunoblotting analysis with anti-pS/T antibody revealed that Aurora-A phosphorylation of CHFR also decreased in myc-CHFR single or double A-form mutants as compared to wild-type CHFR (Fig. 3.4B). Therefore, Ser-218 and Ser-337 of CHFR are major residues targeted by Aurora-A.
**Fig. 3.4. Confirm Aurora-A phosphorylation sites of CHFR.** (A) *In vitro* kinase assay using GST fused wild type CHFR, CHFR-S218A, -S337A and -S218/337A as substrates. Recombinant Histone H3 was used as positive control (top). Bottom panel is Coomassie blue staining showing equal amount of GST-CHFR protein used in the reaction. (B) Western blot analysis. HEK293 cells were transfected with Myc-tagged wild type, S218A, S337A and S218/337A CHFR expressing constructs together with HA-Aurora-A or control vector. Following 48 h incubation, the cells were lysed and immunoprecipitated using anti-Myc antibody. The immunoprecipitates were immunoblotted with anti-phosphor-Ser/Thr antibody (top). Expression of the transfected CHFR and Aurora-A was shown in panels 2 and 3. Relative phosphorylation levels of CHFR were normalized to expression and quantified by ImageJ.

**Mutation of the Phosphorylation Sites Abolishes CHFR E3 Activity**

Since serine-218 is close to RF domain and seine-337 locates within the RF domain, which is required for E3 activity (29), we reasoned that Aurora-A phosphorylation of these 2 sites will decrease E3 activity of CHFR. *In vivo* ubiquitination assay was performed using PLK1 as readout. HEK293 cells were transfected with HA-PLK1, His-Ubiquitin, Myc-CHFR and HA-Aurora-A. Following treatment with a proteasome
inhibitor MG132 for 6 hours, cells were lysed and subjected to Ni\(^{+}\)-NTA-agarose beads to pull-down His-tagged ubiquitin. The ubiquitin conjugated PLK protein was detected by western blotting with anti-HA antibody. The result showed that a portion of PLK1 was conjugated to ubiquitin in vivo. Ectopic expression of CHFR stimulated PLK1 polyubiquitination. Addition of Aurora-A reduced CHFR-induced PLK1 degradation (Fig. 3.5A), suggesting that Aurora-A negatively regulates CHFR ubiquitin ligase function.

We next examined if Aurora-A inhibition of CHFR E3 activity depends on phosphorylation of serine-218 and serine-337. We created CHFR-S218/337D and CHFR-S218/337A forms by converting serine residues to aspartic acids and alanines, respectively. HEK293 cells were transfected CHFR, CHFR-S218/337D or CHFR-S218/337A together with/without Aurora-A and then analyzed by Western blot for PLK1 expression. As expected, wild type CHFR reduced PLK1 protein level, which was abrogated by expression of Aurora-A. Further, nonphosphorylatable CHFR-S218/337A decreased PLK1 expression and Aurora-A failed to rescue it, whereas expression of phospho-mimic CHFR was unable to reduce PLK1 protein (Fig. 3.5B). Thus, we conclude that Aurora-A inhibits CHFR function through a phosphorylation-dependent mechanism.
Fig. 3.5. Aurora-A inhibits CHFR E3 activity through phosphorylation of Ser218 and Ser337. (A) *In vivo* ubiquitination assay. HEK293 cells were transfected with indicated plasmids and treated with MG132. The cells were lysed and incubated with Ni\[^{2+}\]-NTA beads to pull-down His\[^{6}\]-tagged protein. The ubiquitin conjugated PLK1 was evaluated by Western blot analysis with anti-HA antibody. (B) Western blot. HEK293 cells were transfected with indicated expression constructs. Following 48 h incubation, cells were lysed and immunoblotted with indicated antibodies. The relative PLK1 level was normalized to actin and quantified by ImageJ.
Discussion

In this study, we demonstrated that CHFR is a substrate of Aurora-A kinase. Aurora-A phosphorylates CHFR at Ser218 and Ser337 and inhibits CHFR E3 activity. As a result, its downstream targets, such as PLK1, become stable. Further, we also showed frequent downregulation of CHFR and inverse correlation of expression of Aurora-A and CHFR in human ovarian cancer, which supports the notion that CHFR is an E3 ligase of Aurora-A (15). However, co-expression of CHFR and elevated Aurora-A was observed in a subset of ovarian cancers, suggesting that Aurora-A might phosphorylate CHFR and inhibit its tumor suppressor function in these tumors. Taken together, these findings indicate a feedback regulation loop between Aurora-A and CHFR.

Previous studies have primarily focused on genetic alteration and normal cellular function of CHFR. Posttranslational regulation of CHFR remains largely unknown. A previous report shows that Akt/PKB phosphorylates CHFR at Thr-39 in FHA domain after DNA damage, which led to inhibition of E3 activity and shortening G2 arrest induced by DNA damage (30). In this study, we showed that Aurora-A inhibits E3 activity of CHFR by phosphorylation of Ser218 and Ser337, which are adjacent and within RF region, respectively. Taken together, these data indicate that posttranslational modification of CHFR plays a critical role in regulation of its function.

It has been suggested that R/K/N-R-X-S/T-B or R/KXT/S-I/L/V is Aurora kinase phosphorylation consensus sequence, X represents any amino acid and B represents hydrophobic residues with the exception of Pro (31). However, a number of Aurora-A substrates identified so far do not contain this motif. While the serine-218 [PKGS(218)G] of CHFR is not perfect match either sequence and the serine-337 [ERSS(337)L] does not comply with R/K/N-R-X-S/T-B, they are phosphorylated by Aurora-A in vitro and in vivo. Mutation of these 2 sites to aspartic acids, which mimic Aurora-A phosphorylation, reduces E3 activity of CHFR and resemble the effect of Aurora-A phosphorylation of CHFR, whereas CHFR-S218/377A retains its E3 activity that was not inhibited by Aurora-A. Therefore, CHFR is a physiological substrate of Aurora-A and the Aurora-A consensus phosphorylation motif could be more variable than originally expected.
Aurora-A exerts its mitotic function through regulation of a number of proteins (32-36), which include histone H3, TACC3, Eg5, CPEB and TPX2. We and others have previously shown Aurora-A phosphorylation of p53 which leads to inactivation of p53 and G2/M cell cycle progression (20). CHFR has been shown to mediate a delay in cell cycle progression early in mitosis in response to microtubule stress. Identification of Aurora-A inhibition of CHFR provides additional molecular mechanism for Aurora-A function in control of G2/M cell cycle progression.

Several groups have shown that CHFR mRNA expression is lost or decreased in primary lung, colon, esophageal, gastric, brain, and breast tumors as well as cancer cell lines. The best characterized means of expression loss is promoter hypermethylation, which occurs in a subset of tumors and cell lines and the frequency of which seems to be dependent on the tissue of origin (10-14). A recent report performed DNA sequence and methylation specific PCR analyses and showed no mutation and hypermethylation in 48 ovarian cancers examined (37). However, we found frequent downregulation of CHFR mRNA in both ovarian cancer cell lines and primary tumors by RT-PCR (Fig. 3.1). The hypermethylation was only detected in 1 of 4 cell lines, which is consistent with the previous findings that only a fraction of tumors with loss of CHFR expression are due to promoter hypermethylation. Further investigation is required for determining the mechanism of downregulation of CHFR in human cancer including ovarian carcinoma.
References

CHAPTER IV

CYCLIN N-TERMINAL DOMAIN CONTAINING 2, CNTD2, IS AN ONCOGENE THAT INTERACTS WITH AND ACTIVATES AURORA-A

Abstract

Cyclin N-terminal domain containing 2, CNTD2, is an uncharacterized cyclin. Here, we identify CNTD2 as an Aurora-A interacting protein. CNTD2 colocalizes with Aurora-A in the centrosome. Interaction between CNTD2 and Aurora-A leads to activation of Aurora-A and cdc2 kinase, which promotes G2/M cell cycle progression. Further, CNTD2 resides at chromosome 19q13.2 and is amplified and overexpressed in human cancer cell lines and primary tumors of ovary, pancreas, breast, prostate and lung. Alterations of CNTD2 appear to be associated with poor clinic outcome. Ectopic expression of CNTD2 murine fibroblasts results in centrosome amplification, genomic instability and malignant phenotype. These data suggest that CNTD2 is a key regulator of Aurora-A and could play a pivotal role in cell cycle progression and oncogenesis.

Key words: amplicon, cyclin, cell cycle, cdc2, oncogenesis
Introduction

Aurora-A is a mitotic kinase, which localizes to centrosome and regulates cell cycle progression through modulating centrosome function. Aurora-A is amplified and/or overexpressed in a range of human cancers, including breast, ovarian, colon, bladder and pancreatic cancers (1-5). Aurora-A overexpression in cultured cells leads to centrosome amplification, multipolar spindle and polyploidy, resulting in cell-cycle checkpoint defects and genetic instability, which contributes to malignant transformation. Aurora-A executes regulatory and transforming function through its kinase activity, which is regulated by phosphorylation and dephosphorylation as well as association with a number of proteins such as HEF1, TPX2, or Bora (6-8).

Genetic and chromosomal abnormalities, including amplification, deletion and aneuploidy in chromosomes, are common features associated with cancer development and progression (9,10). Amplified chromosomal regions, known as amplicons, have been implicated in a wide variety of cancers. Well-known amplicons in cancer include chromosome 17q12 containing ErbB-2 (11,12), chromosome 7q11.2 containing EGFR (13), 20q13 containing Aurora-A (14), chromosome 19q13.1-q13.2 containing AKT2 (15), chromosome 11q13 containing CCND1 and EMS1 (16,17), chromosome 12q13–14 containing MDM2 (18,19), N-Myc amplification on chromosome 2p24 (20-22), newly discovered co-amplification of PI3KCA and ZASC1 on 3q26.3 (23) and chromosome 19q12 containing CCNE1 (24). Studies on these genes not only have provided new insights on how cancer develops but also have significant translational implications. For example, ErbB-2 and EGFR are the molecular targets for the humanized antibodies Trastuzumab (Herceptin) and Matuzumab, which are used for the treatment of breast and lung cancer, respectively (25,26).

The two major processes common to all cell cycles are S phase, when chromosomes are replicated, and M phase, when the replicated chromosomes are segregated into two daughter cells. In most cell cycles, an interval of time, G1 phase, separates the previous cell division from the beginning of the next S phase. It is now firmly established that progression of the cell cycle — that is, transitions between one
phase of the cycle and the next — are controlled by cyclin-dependent kinases (CDKs). The CDK regulating G2/M transition is CDK1/Cdc2, which is the major mitotic kinase. Cdc2 interacts with B-type cyclins to form a stoichiometric complex, known as mitosis-promoting factor (MPF). Binding of the cyclin subunit is required for the phosphorylation and activation of the Cdc2 subunit by cdk-activating kinase (CAK). Two B-type cyclins, B1 and B2, have been identified in mammals. Cyclin B1 was first cloned at 1989, and found predominantly expressed in the G2/M phase of cell division.

In this study, we identified hypothetical protein FLJ13625 as an Aurora-A interacting protein by yeast two-hybrid screening. The coding region of this protein maps to chromosome 19q13 amplicon. Sequence analysis showed that this protein contains a cyclin domain (CD) at C-terminus, and it is homologous to yeast B-type cyclin 6 (clb6). The protein is identical to human cyclin N-terminus Domain containing protein 2 (CNTD2), only with a cyclin domain that is 30 amino acids longer than CNTD2. Therefore, we consider it as an alternative splice form of CNTD2. Like Aurora-A, CNTD2 localizes to the centrosome and activates Aurora-A and cdc2. Further, the CNTD2 gene is amplified and overexpressed in human cancer. Ectopic expression of CNTD2 is able to cause centrosome amplification, genomic instability and oncogenic transformation in 3Y1 cells.
Materials and Methods

Cell Culture, Transfection and Treatment
Human cancer cell lines (MDA-MB-231, MDA-MB-435s, MDA-MB-453, MDA-MB-468, T47D, UTOV2, UTOV3A, UTOV5, UTOV7, DU145, D32, U118, PANC-1, Colo357, SW480 and HeLa) and HEK293 cells were maintained in Dulbecco modified Eagle medium (DMEM). Cells cultured in RPMI 1640 medium are OV3, OV8, OV2008 C13, MCF7, LNCaP, PC3, AsPC-1 and A549 cells. Ovarian cancer SW626 cells were cultured in 50:50 medium M199:MCDB105; A2780S, A2780CP and RMUG-S were grown in DMEM/F-12 medium. MCF10A cells were maintained in Mammary Epithelial Growth Medium (MEGM). HCT116 was cultured in McCoy’s 5A medium. All culture mediums were supplemented with 10% fetal bovine serum (FBS). Rat fibroblast cell line 3Y1 was cultured in DMEM containing 10% calf serum. Transfection was performed with LipofectAMINE 2000 reagent (Invitrogen). For protein degradation assay, cells were treated with cycloheximide (CHX) at a concentration of 50 µg/ml for 6 hours.

Plasmid Constructions
For yeast two-hybrid screening, the N-terminal portion of Aurora-A encoding amino acids 1 to 110 was cloned into the EcoR1 and BamH1 sites of pJK202 to create the bait pNLexA-Aurora-AN. The oligonucleotide primers used were as follows: forward, 5’-ATGGACCGATCTAAAGAAAACTGC-3’, and reverse 5’-AGGATTATTTTCAGGTGCCGATG.

The coding sequence of CNTD2 was ligated to pGEX4T-1 vector to generate GST-CNTD2 fusion protein (Pharmacia). HA-CNTD2 was constructed by cloning CNTD2 into EcoR1/Not1 of pHM6 vector; Flag-CNTD2 was cloned by insertion of EcoR1/BamH1 digested CNTD2 into p3×FLAG/CMV10 and/or pIRESflagEGFP2 vectors, respectively. Inducible CNTD2 was obtained by cutting out flag-CNTD2 sequence from pIRES2-flag-CNTD2 and ligated into pTRE2hyg2-HA vector through PvuII site. The primers were: H_CNTD2-1 (forward), 5’-
CGCGGATCCGCGGAATTCCATGCTGGTGAGAGGCAGGGACCAG-3', and H_CNTD2-2 (reverse), 5’-CGCGGATCCGCGGCCGCTTACTCCCTGCCCTGAAAG-3’.

Yeast Two-Hybrid Screen

A genetic screen using the yeast two-hybrid system was performed as previously described (27). Briefly, yeast strain EGY191, which harbors the LexAop-Leu2 reporter gene, was transformed with bait plasmid pNLexA-Aurora-AN and reporter plasmid pSH18-34 and subsequently transformed with a human brain interaction library. Approximately 2 × 10^6 primary library transformants were obtained and plated on Ura− His− Trp− Leu− galactose-raffinose plates. Candidate clones were identified by their ability to grow on Ura− His− Trp− Leu− galactose-raffinose plates, but not on Ura− His− Trp− Leu− glucose plates, and their ability to yield blue colonies on Ura− His− Trp− X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-galactose-raffinose plates, but not on Ura− His− Trp− glucose plates. KC8 cells were transformed with plasmids isolated from positive colonies. The specificity of interaction of candidate plasmids with pNLexA-Aurora-AN was tested by retransformation of positive clones into yeast harboring either the aurora-A bait or several unrelated bait plasmids. Nucleotide sequence analysis of cDNA inserts was performed using an Applied Biosystems automated sequencer.

Western Blotting and Immunoprecipitation

Cells were lysed for Western blotting analysis in RIPA buffer (50 mM NaCl, 0.5% (w/v) DOC, 50 mM Tris-HCl (pH 8.0), 1% (v/v) NP-40, 0.1% (w/v) SDS, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM Na3VO4 and 1 mM PMSF). The proteins were resolved under denaturing condition by SDS-polyacrylamide gel and transferred onto Nitrocellulose (Amersham) membranes. The membranes were blocked and then incubated with appropriate antibodies indicated in figure legends.

For immunoprecipitation (IP), cells were lysed in TNEN buffer containing 50 mM Tris-HCl (pH 7.5), 50-150 mM NaCl, 2 mM EDTA, 0.5% (v/v) NP40, 0-0.3% (v/v)
Triton X-100, protease and phosphatase inhibitors (1µg/ml aprotinin, 1µg/ml leupeptin, 0.5mM Na$_3$VO$_4$ and 1mM PMSF). Precleared-lysates were incubated with 30µl antibody conjugate protein A:G (2:1) agarose beads at 4°C overnight. Following 3 times washing, the precipitates were denatured and subjected to Immunoblotting with the antibodies indicated in figure legends.

**Northern and Southern Blot**

Total RNA was isolated from cells using Trizol Reagent (Invitrogen). Northern blot analysis was performed as previously described (15). Briefly, 20 µg total RNA from each sample was separated on 1.0% denature agarose gel. After transferring to GeneScreen Plus Membrane (PerkinElmer) and prehybridization, the membrane was hybridized with [$^{32}$P]dCTP-labeled Aurora-A cDNA probe in Express-Hybridization Solution (Clontech).

For Southern blot, 10 µg of genomic DNA extracted from cancer cell lines was digested by EcoR I in 100 µl volume overnight, then precipitated with 2.5 folds of ice-cold 100% ethanol. The digested and purified DNA was subjected to 0.8% agarose gel electrophoresis at 40 volts for 16 hours. After transfer, the membrane was hybridized with radioactive labeled CNTD2 probe. The probe was obtained from PHM6-CNTD2 by restriction enzyme digestion with EcoR I and Not I. The cDNA fragment was gel purified and resuspended in TE buffer, then labeled with [$^{32}$P]-dCTP using Prime It® II Random Primer Labeling Kit (Stratagene).

**Indirect Immunofluorescence (IIF)**

Cells were seeded on 2 cm × 2 cm coverslips in 6-well plates, the coverslips were pre-treated with poly-L-Lysine as needed. After expression of transfected proteins or appropriate treatment, cells were fixed and penetrated by 4% paraformaldehyde + 0.2% Triton X-100 in PBS/Mg (0.5 mM MgCl$_2$) 10 min at room temperature. Following 4 times wash in PBS/Mg, cells were stored at 4°C or proceeding to blocking. Non-related antigens were blocked by incubating in Knudsen Modified Blocking buffer (0.5% BSA, 0.5% NP-40, 1 mM MgCl$_2$, 1 mM NaN$_3$ in 1×PBS) 1 hour at room temperature. After removing the blocking buffer, cells were incubated with diluted primary antibodies for 2
hour at room temperature or 4°C overnight. Multiple primary antibodies of different origins were combined. Fluorescence conjugated secondary antibodies were applied at appropriate dilutions after washing off the primary antibodies. Following final washes by PBS/Mg, coverslips were mounted onto glass slides using VECTASHIELD mounting medium (with DAPI) (VECTOR), which stains DNA while mounting. All the steps were performed in humidified chamber and in dark if fluorescence was present. The slides were kept in dark at 4°C before viewed by ZEISS automated fluorescent scope.

**In vitro Aurora-A, p34cdc2 Kinase Assay**

Aurora-A kinase assay was performed as previously described (28). Briefly, reactions were carried out in the presence of 10 µCi of [γ-32P] ATP (Perkin Elmer Life Sciences) and 1 nM cold ATP (Invitrogen) in 30 µl kinase buffer containing 16.5 mM MOPS (pH 7.0), 0.375 mM EDTA, 2.25 mM EGTA, 33.75 mM MgCl2, 225 µM ATP (cold), 11.25 mM β-glycerophosphate, 0.45 mM Na3VO4, 0.5 mM DTT, 0.0125% (v/v) 2-mercaptoethanol, and 0.625% (v/v) glycerol. 4 µg recombinant Histone H3 used as substrate. After incubation at 30°C for 30 min, the reaction was stopped by adding protein loading buffer and denaturing. The proteins were separated by SDS-PAGE, and the amounts of incorporated radioactivity were determined by autoradiography.

p34cdc2 kinase activity was measured as previously described (29). Briefly, transfected Hela cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 2 mM EDTA, 137 mM NaCl, 10% glycerol, 2 mM sodium vanadate, 100 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. 500 µg of protein were incubated with anti-cyclin B antibody conjugated protein A/G-agarose beads in 1 ml lysis buffer at 4 °C for at least 4 hours. Followed 3 times washing, beads-bond cdc2/cyclin B complexes were incubated at 30 °C with [γ-32P]-ATP and Histone H1 in a buffer containing 100 mM NaCl, 10% Triton X-100, 50 mM ATP and 10 mM MgCl2. The reaction product was resolved by SDS-PAGE and exposed to autoradiography.
Soft Agar Assay and Tumorigenicity in Nude Mice

Soft agar assay was performed as previously described (30). $2 \times 10^5$ of CNTD2-transfected 3Y1 cells were suspended in 5 ml of 0.33% (w/v) agar containing DMEM/20% fetal calf serum, overlaid onto a 10 ml volume 0.66% agar solution in 10 cm plates. The cultures were fed twice a week for 4 weeks.

Tumor formation in 6-week old NOD SCID mice (Jackson Laboratory) was tested by subcutaneous inoculation and assessed for 6 weeks. 3Y1 cells transfected with CNTD2 or control vector were each injected into 8 animals at $5 \times 10^6$ cells per mouse. Tumor measurements were made with linear calipers in two orthogonal directions by the same observer.
Results

Identification of CNTD2 as an Aurora-A Interacting Protein

In order to understand Aurora-A function and its involvement in molecular pathways, we performed yeast two-hybrid screening using N-terminal region of Aurora-A as bait. Two overlapping clones were isolated from a human brain library. Sequence analysis revealed that it encodes a longer splicing form of human cyclin N-terminal domain containing 2 (CNTD2), with extra 30 amino acids that insert between residues 89 and 90 of CNTD2. CNTD2 is an uncharacterized B-type cyclin which contain a C-terminal cyclin domain and an N-terminal CDK5 activator homology region (Fig. 4.1A). The amino acid sequence differs substantially from those of the well characterized cyclins but is similar to hypothetical protein LOC779739 in Xenopus tropicalis (similarity 70%; identity 50%) and clb6 in Saccharomyces cerevisiae [similarity, 60%; identity, 42%; (Fig. 4.1B)].

To confirm the interaction between Aurora-A and CNTD2, we performed coimmunoprecipitation experiment in HEK293 cells that were transfected with HA-tagged-CNTD2 and/or GFP-Aurora-A. Antibody against GFP was able to immunoprecipitate CNTD2, which is detected by HA antibody, from cells co-transfected with both Aurora-A and CNTD2, but not cells transfected with either Aurora-A or CNTD2 alone. In addition, HA-CNTD2 also co-precipitated with GFP-Aurora-A (Fig. 4.2A). Furthermore, antibodies against endogenous Aurora-A or CNTD2 identified the physical interaction of between Aurora-A and CNTD2 (Fig. 4.2B).

CNTD2 Colocalizes with Aurora-A in Centrosome

Since Aurora-A is a centrosome kinase and interacts with CNTD2, we next examined if CNTD2 confine to centrosome and colocalizes with Aurora-A. HeLa cells were transfected with GFP-tagged-CNTD2 and then immunostained with antibody against γ-tubulin, a centrosome marker. As shown in Fig. 4.2C, CNTD2 localizes to centrosome during the cell cycle. Co-immunofluorescence staining of HeLa cells with anit-CNTD2 and -Aurora-A antibodies revealed that CNTD2 and Aurora-A co-localize in the centrosome. These observations further indicate CNTD2 interaction with Aurora-A.
**Fig. 4.1. CNTD2 sequences.** (A) Domain structure of human CNTD2 (longer splicing form). CDK5\_a, CDK5 activator homology region; CYCLIN, cyclin domain. (B) Sequence alignment of human CNTD2 and yeast clb6 by ClustalW2 program. Cyclin domains of CNTD2 and clb6 were highlighted in purple and grey color, respectively.
Fig. 4.2. Interaction and colocalization of CNTD2 and Aurora-A. (A and B) Co-immunoprecipitation. HEK293 cells were co-transfected with GFP-Aurora-A and HA-CNTD2, and immunoprecipitated with anti-HA and detected with anti-GFP antibody (top panel) or vise versa (second panel). Panels 3 and 4 show expression of transfected plasmids (A). PANC1 cells were lysed and immunoprecipitated with anti-AurA or anti-CNTD2 antibody and immunoblotted with anti-CNTD2 or anti-AurA antibody, respectively (B). (C) Immunofluorescence staining. As shown in upper panel, GFP-CNTD2 (green) transfected Hela cells were synchronized to mitosis and centrosomes was stained with anti-γ-tubulin antibody followed by TRITC-conjugated secondary antibody (red); in lower panel, mitotic arrested HeLa cells were stained with anti-CNTD2 and anti-
Aurora-A antibodies. CNTD2 was shown in red (TRITC), Aurora-A was shown in green (FITC), and the nucleus was stained by DAPI.

**CNTD2 Stabilizes Aurora-A Protein and Stimulates Aurora-A Kinase Activity**

We next investigated the regulation between CNTD2 and Aurora-A. As Aurora-A is a serine/threonine protein kinase, we first examined if CNTD2 is a substrate of Aurora-A. *In vivo* phosphorylation and *in vitro* kinase assays exhibited no phosphorylation of CNTD2 by Aurora-A (data not shown). However, ectopic expression of CNTD2 increases Aurora-A protein but not mRNA levels in a dose-dependent manner. In Tet-On inducible CNTD2 HeLa cells, Aurora-A protein level was upregulated upon CNTD2 expression induced by doxycycline (Fig. 4.3A). To determine if CNTD2 inhibits Aurora-A degradation, HeLa cells were treated with CHX to block de-novo protein synthesis. As shown in Fig. 4.3B, without induction of CNTD2, Aurora-A protein degraded rapidly with 20% decrease in an hour. Once CNTD2 was expressed, Aurora-A protein became more stable and reached the same degradation level (20%) until 6 hours after CHX treatment.

Aurora-A kinase is activated during G2/M phase due to the fact that Aurora-A is accumulated and interacts with TPX2 through its C-terminal hydrophobic region (7). In addition, we have previously shown that Aurora-A kinase activity is closely associated with Aurora-A protein levels in human cancers (4). Thus, CNTD2 could activate Aurora-A through upregulation or/and interaction with Aurora-A. To this end, Aurora-A kinase activity was examined in both CNTD2-inducible and -knockdown cells. Following treatment of Tet-On CNTD2 HeLa cells with doxycycline for 12 h and 24 h, Aurora-A was immunoprecipitated with anti-Aurora-A antibody. *In vitro* kinase assay showed an increase in Aurora-A kinase activity in both time points (Fig. 4.3C), when the Aurora-A protein expression was induced (Fig. 4.3A). Further, knockdown of CNTD2 in PNAC1 cells, in which CNTD2 is amplified (Fig. 4.5B), reduced Aurora-A kinase activity, especially in mitotic cells in which Aurora-A protein and activity reach the peak (Fig. 4.3D).
Fig. 4.3. CNTD2 stabilizes Aurora-A protein and induces Aurora-A kinase activity. (A) Aurora-A is upregulated by CNTD2. HeLa-TetOn-CNTD2 cells were treated with doxycycline for indicated times and immunoblotted with indicated antibodies. (B) CNTD2 inhibits Aurora-A degradation. HeLa-Tet-on-CNTD2 cells were treated with or without doxycycline overnight. Following addition of CHX for indicated times, cells were subjected to Western blot analysis with indicated antibodies. (C) CNTD2 induces Aurora-A kinase activity. Following treatment with and without doxycycline, HeLa-TetOn-CNTD2 cells were lysed and the endogenous Aurora-A was immunoprecipitated by anti-Aurora-A antibody and subjected to in vitro kinase assay using Histone H3 as substrate. (D) Knockdown of CNTD2 decreases Aurora-A kinase activity. PANC1 cells were transfected with scramble or CNTD2 siRNA, and selected by puromycin. Endogenous CNTD2 protein was analyzed by Western blot (right). Following treatment with or without nocodazole, cells were immunoprecipitated with anti-Aurora-A antibody.
and subjected to \textit{in vitro} Aurora-A kinase assay. The Aurora-A protein and activity were normalized to expression and quantified by ImageJ software.

\textit{CNTD2 Promotes G2/M Progression and Activates cdc2 Activity}

Since Aurora-A activates cdc2 activity and promotes G2/M progression by regulation of mRNA polyadenylation of cyclin B1 through phosphorylation of cytoplasmic polyadenylation element binding protein (CPEB) (31), we next examined the effects of CNTD2 on cell cycle progression and cdc2 activity. CNTD2 inducible HeLa cells were synchronized by double-thymidine block and simultaneously treated with doxycycline or DMSO at the second thymidine block. Cell cycle analysis showed that CNTD2-cells reached G2/M peak at 6 hours after release from the double-thymidine block as compared to control cells, in which G2/M accumulation appeared at 9 hours of release (Fig. 4.4A). Further, knockdown of CNTD2 delayed G2/M cell cycle progression (Fig. 4.4B).

To investigate the effect of CNTD2 on cdc2 kinase activity, \textit{in vitro} cdc2 kinase assay was performed in CNTD2-inducible and control-TetOn Hela cells. After treatment with doxycycline for 12 h and 24 h, cells were immunoprecipitated with anti-cyclin B1 antibody and cdc2 activity was measured by \textit{in vitro} kinase assay using Histone H1 as substrate. Induced expression of CNTD2 in Hela-TetOn-CNTD2 cells significantly induced cdc2 kinase activity; whereas in control cells where there is no CNTD2 expression, cdc2 activity was not affected by doxycycline treatment (Fig. 4.4C).
Fig. 4.4. CNTD2 promotes G2/M progression and stimulates cdc2 kinase activity. (A and B) CNTD2 promotes G2/M progression. Following induction of CNTD2 by doxycycline, HeLa-TetOn-CNTD2 cells were synchronized to G1/S with double-thymidine block. After release for indicated times, cells were assayed with flow cytometry (A). PANC1 cells were stably transfected with siRNA of CNTD2 and control
siRNA. After treatment with hydroxyurea for 16, cells were released for indicated times and subjected to flow cytometry analysis (B). (C) CNTD2 induces cdc2 activity. HeLa-TetOn-CNTD2 and control cells were treated with doxycycline and immunoprecipitated with anti-cyclin B antibody. The cdc2 kinase activity was assayed using Histone H1 as substrate and quantified by ImageJ.

**CNTD2 is Ubiquitously Expressed in Normal Tissues and is Frequently Altered in Human Cancers**

Northern and Western blot analyses revealed ubiquitous expression of CNTD2 in different normal mouse tissues (Fig. 4.5A) and human cancer cell lines. Notably, two and three different transcripts were observed in the cell lines examined with elevated levels of CNTD2 in HL60 and Raji cells (Fig. 4.5B). However, expression levels of CNTD2, unlike Aurora-A, is not changed during the cell cycle (Fig. 4.5C).

Genomic database analysis shows that CNTD localizes to chromosome 19q13.2 within the 19q13.1-13.2 amplicon (Fig. 4.6A). Amplification of chromosome 19q13 has been linked to different types of cancers including pancreatic carcinoma (32-34), ovarian carcinoma (35-37), breast cancer (38) and other cancers (39-41). Interestingly, CNTD2 is 32-kb centromeric to AKT2, an oncogene frequently amplified in human cancer. To investigate if CNTD2 is also altered in human cancer, we performed Southern blot analysis in 18 cancer cell lines and found that CNTD2 was amplified in PANC1, ASPC1 and OV3 cells (Fig. 4.6B), in which AKT2 is also amplified (15,32).
Fig. 4.5. CNTD2 expression in normal tissues and human cancer cell lines. (A) CNTD2 protein expression in normal mouse tissues shown by Western blot. (B) Northern blot membrane from Clontech was hybridized with CNTD2 probes. Human cancer cell lines examined: 1. HL60, 2. Hela S3, 3. K562, 4. MOLT-4, 5. Raji, 6. SW480, 7. A549, 8. G361. (C) Hela cells were synchronized and collected at different time points for Northern, Western blot and cell cycle analyses.
**Fig. 4.6. CNTD2 is amplified in human cancer cell lines.** (A) CNTD2 locates to chromosome 19q13.2 close to AKT2. (B) Southern blot analysis. DNAs from indicated cell lines were digested with EcoRI and electrophoresed in 0.8% agarose gel. After transferring, a membrane was hybridized with $^{32}$P-dCTP labeled CNTD2 (top) and β-actin (bottom) probes.
We further examined the expression of CNTD2 protein in human cancer cell lines. Immunoblotting analysis revealed an elevated CNTD2 protein level in 3 of 8 breast cancer, 5 of 10 ovarian cancer and 2 of 4 prostate cancer cell lines examined (Fig. 4.7A). The majority of cell lines with overexpression of CNTD2 protein did not have change at DNA level, suggesting that amplification of CNTD2 is responsible for increase in its protein expression in a small subset of cell lines.

To determine the expression level of CNTD2 in human primary tumors, we analyzed 18 pairs of lung cancer and normal samples for CNTD2 protein expression. Fifteen (83%) tumor samples showed increased CNTD2 level compared to the adjacent normal tissues (Fig. 4.7B). We further investigated the alteration of CNTD2 in ovarian cancer due to the fact that chromosome 19q13 is frequently amplified in this malignancy. Western blotting and immunohistochemistry analyses revealed overexpression of CNTD2 in 35 of 65 (54%) ovarian tumors, whereas only 3 of 10 normal ovary samples expressed moderate to low levels of CNTD2 (Figs. 4.8A and 4.8B; Table 3). No significant correlation was found between CNTD2 protein levels and histological types (Table 4). However, more frequent overexpression of CNTD2 was observed in late stage and, more significantly, high grade tumors (Table 5). We also analyzed the association of the expression of CNTD2 with the ovarian cancer patients’ survival. Kaplan-Meier curves analysis of 65 patients with ovarian tumors demonstrated a statistically significant negative correlation between patient overall survival time and CNTD2 expression level \((P = 0.05; \text{Fig. 4.8C})\). These results suggest that CNTD2 is a candidate oncogene in chromosome 19q13 amplicon and could play a role in human oncogenesis and that its alterations are recurrent events in human cancer and associated with tumor progression and poor clinical outcome in ovarian carcinoma.
Fig. 4.7. **CNTD2 is overexpressed in human cancer.** (A) Elevated levels of CNTD2 in human cancer cell lines. Cell lysates from indicated cell lines were immunoblotted with anti-CNTD2 (top) and -actin (bottom) antibodies. (B) Overexpression of CNTD2 in primary lung cancer. Tissue lysates from paired normal lung and tumor specimens were assayed by Western blot analysis with indicated antibodies.
Fig. 4.8. Overexpression of CNTD2 in ovarian tumors is associated with poor prognosis. (A) Western blot. Human primary ovarian tumors were immunoblotted with anti-CNTD2 and -actin antibodies. (B) Immunohistochemical staining. Representative tumors were immunostained with anti-CNTD2 antibody. (C) Overall survival in patients with high CNTD2 (n = 35) versus the remaining patients (n = 30) was plotted by the Kaplan-Meier method. Statistical comparison of survival between groups with the log-rank statistic suggests that patients with overexpression of CNTD2 in the tumor had poor survival compared with low and no expression (P = 0.018).
Table 3. CNTD2 expression in ovarian tumors and normal tissues.

<table>
<thead>
<tr>
<th></th>
<th>CNTD2 protein level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>normal</td>
<td>10</td>
</tr>
<tr>
<td>tumor</td>
<td>65</td>
</tr>
</tbody>
</table>

Table 4. Association of CNTD2 expression with tumor histological types.

<table>
<thead>
<tr>
<th>TYPE</th>
<th>Samples</th>
<th>CNTD2 (Percentage of Samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous</td>
<td>40</td>
<td>21 (53%)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>8</td>
<td>3 (38%)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>7</td>
<td>4 (57%)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>3</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>other</td>
<td>7</td>
<td>4 (57%)</td>
</tr>
</tbody>
</table>

Table 5. Correlation of CNTD2 expression level with tumor grade and stage.

<table>
<thead>
<tr>
<th>GRADE</th>
<th>Samples</th>
<th>Low/No</th>
<th>High/Mod</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>I-II</td>
<td>18</td>
<td>12</td>
<td>6 (33%)</td>
<td></td>
</tr>
<tr>
<td>III-IV</td>
<td>42</td>
<td>17</td>
<td>25 (60%)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>STAGE</th>
<th>Samples</th>
<th>Low/No</th>
<th>High/Mod</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-II</td>
<td>18</td>
<td>10</td>
<td>8 (44%)</td>
</tr>
<tr>
<td>III-IV</td>
<td>35</td>
<td>18</td>
<td>17 (49%)</td>
</tr>
</tbody>
</table>

Ectopic Expression of CNTD2 Induces Malignant Transformation

To determine CNTD2 oncogenic activity, we stably transfected 3Y1 cells with pHM6-HA-CNTD2 and vector alone. pHM6 vector-transfected 3Y1 grew in monolayer with fibroblast feature, whereas CNTD2 stably-transfected cells exhibited epithelial morphology, larger nucleus and lost their contact inhibition (Fig. 4.9A). Further, CNTD2 cells grew much faster than control cells, suggesting the role of CNTD2 in regulating cell proliferation (Fig. 4.9B).
Furthermore, soft agar assay was carried out to investigate anchorage-independent growth. Colonies of CNTD2-transfected cells appeared microscopically after 10 days and became visible to the naked eye after 20 days of incubation. Colonies were not observed in soft agar cultures of parental 3Y1 cells or of cells transfected with pHM6 vector (Fig. 4.9C). To investigate the tumorigenic activity of CNTD2, clonal cell lines were injected subcutaneously into nude mice. All mice inoculated with CNTD2 transfected cells formed tumors within 14-21 days following injection, whereas vector-transfected 3Y1 cells did not develop tumors until over 2 months (Fig. 4.9D and Table 6). Western blot analysis revealed expression of CNTD2 protein only in pHM6-HA-CNTD2-transfected cells and tumors.

**Fig. 4.9. Ectopic expression of CNTD2 transforms 3Y1 cells.** (A) Cell morphology. CNTD2- and vector-transfected 3Y1 cells were observed under microscope. (B) Cell growth curve. CNTD2-transfected and control 3Y1 cells were plated in 96-well plates and cell number was calculated daily for 6 days. (C) CNTD2-transfected but not control 3Y1 cells grew in soft agar. (D) Tumor formation in nude mice. CNTD2-transfected and control 3Y1 cells were subcutaneously injected into nude mice (5 × 10⁶ cells/mouse; 8
mice/group). Tumor formation was observed in all the mice injected with CNTD2-transfected cells and in 1 of 8 mice received mock cells. Western blot showed CNTD2 expression in dissected tumors.

Table 6. Tumorigenicity of CNTD2 and control transfected 3Y1 cells.

<table>
<thead>
<tr>
<th>Clonal cell lines</th>
<th>Soft agar growth*</th>
<th>Tumorigenicity in nude mice</th>
<th>Latency (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Y1/CLB6</td>
<td>+++</td>
<td>8/8</td>
<td>14</td>
</tr>
<tr>
<td>3Y1/mock</td>
<td>-</td>
<td>1/8</td>
<td>60</td>
</tr>
</tbody>
</table>

*Number of colony/10 cm plate: 1-10+, 11-30 ++, >30 +++

**CNTD2 Expression Induces Centrosome Amplification and Genomic Instability**

Since CNTD2 localizes to centrosome and induces transformation, we hypothesized that overexpression of CNTD2 causes centrosome amplification and aberrations in chromosome partitioning at mitosis, leading to catastrophic loss or gain of chromosomes and resulting in either cell death or survival through malignant transformation. To test this hypothesis, we immunofluorescence-stained CNTD2-transformed 3Y1 and control cells with anti-\(\gamma\)-tubulin and -HA (e.g., CNTD2) antibodies (Fig. 4.10A). Fig. 4.10 shows that control 3Y1 cells have either 1 (interphase) or 2 centrosomes (dividing). However, the number of centrosomes is significantly increased in CNTD2-transformed cells. Approximately 28% of CNTD2 transfectants revealed more than two centrosomes, compared with less than 3% of the vector-transfected cells showing a similar phenotype (Fig. 4.10B).

Centrosome amplification is related to abnormal cell division, chromosome instability and cancer development (42). Chromosomal analysis showed that most (42 of 50) vector-transfected 3Y1 cells were near-diploid, whereas 39 of 50 (78%) metaphases from CNTD2-transfected cells were polyploid. Among these 39 metaphase spreads, 23 were near-tetraploid and contained one or two small marker chromosomes, and the remaining 16 metaphases had chromosome counts ranging from 132 to more than 600
(Fig. 4.10C). One metaphase contained a ring chromosome and numerous (>50) small markers, fragments and double minutes. Thus, overexpression of CNTD2 induces genomic instability.

Fig. 4.10. CNTD2 induces centrosome amplification and genomic instability. (A) HeLa cells were immunostained with anti-γ-tubulin (left) and anti-CNTD2 (middle) antibodies. CNTD2 colocalizes with γ-tubulin (right panel). (B) CNTD2-transfected and control 3Y1 cells were immunostained with anti-γ-tubulin antibody (left). The number of centrosome was counted in both CNTD2-transfected and control cells, 200 events each (right). (C) Karyotype analysis of CNTD2-transfected and control 3Y1 cells.
Discussion

Using the amino-terminal region of Aurora-A as bait in a yeast two-hybrid screen, we have identified a novel B-type cyclin, CNTD2. CNTD2 colocalizes and immunoprecipitates with Aurora-A. The interaction results in stabilization Aurora-A protein and activation of Aurora-A kinase. CNTD2 is conserved between yeast, *Xenopus* and human cells. CNTD2 locates to chromosome 19q13 amplicon. Amplification and/or overexpression of CNTD2 were detected in human cancer cell lines and primary tumors. Ectopic expression of CNTD2 induces transformation, centrosome amplification and genomic instability. These findings indicate that CNTD2 functions as an oncogene and is associated with and regulates Aurora-A.

In mammalian cells, the Aurora kinase family has three members: Aurora-A, -B, and -C. Aurora-A expression up-regulates at mitosis and it localizes at centrosome and mitotic spindles. Aurora-A T288 phosphorylation activates its kinase activity in late G2 phase at centrosomes, which is prior to and required for the recruitment of cdc2-cyclin B1 to the centrosome. The activated cdc2-cyclin B1 then commits the cell to mitosis (43). While CNTD2 interacts with Aurora-A and activates Aurora-A and cdc2 kinase activity, it does not form complex with cdc2 (data not shown) and its protein level is relative stable through the cell cycle. Since N-terminal region (aa 1-110) of Aurora-A, which is required for interaction with CNTD2, is different from those of Aurora-B and Aurora-C, we found no interaction between CNTD2 and Aurora-B or Aurora-C (data not shown).

The kinase activity of Aurora-A is regulated by autocatalytic phosphorylation of Thr288 in its activatory T-loop (44). This autocatalytic activity of Aurora-A is facilitated by cofactors such as Bora, Ajuba, PAK1, HEF1 and TPX2 (6,8,43,45,46). The mechanism of cofactor-mediated Aurora-A activation is best understood in the case of TPX2. Co-crystallisation revealed that binding of TPX2 to Aurora-A not only induces it to adopt an active conformation but also prevents dephosphorylation of Thr288 by protein phosphatase 1 (PP1) (47). TPX2, Bora and Ajuba are also substrates of the kinase (7,8,43). Unlike these Aurora-A interaction proteins, CNTD2 increases Aurora-A protein stability and kinase activity but is not phosphorylated by Aurora-A.
Several oncogenes have been identified from chromosome 19q13 amplicon, including AKT2, PAK4, pancreatic differentiation 2 (PD2), SERTAD3 and transcriptional regulator intersex-like (IXL) (15,48-51). We have previously shown amplification/overexpression of AKT2 in human ovarian, pancreatic and breast cancers. Inhibition of AKT2 inhibits tumor growth and sensitizes cells to chemotherapeutic agent-induced cell death (15,52,53). In this report, we demonstrated that CNTD2 resides between AKT2 and PAK4 and is amplified/overexpressed in human ovarian, pancreatic and lung cancers. Overexpression of CNTD2 is associated with late stage/high grade ovarian tumors and accompanied with poor clinic outcome. Therefore, CNTD2 could be a potential therapeutic target for these malignancies. Future investigations are required for characterization of the molecular mechanism of CNTD2 stabilization and activation of Aurora-A as well as the role of CNTD2 in oncogenesis in vivo.
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CHAPTER V

DISCUSSION AND CONCLUSION

Aurora kinases represent a novel family of serine/threonine kinases crucial for cell cycle control. The first Aurora kinase was discovered in *Drosophila* (1). Because its mutations resulted in a failure of centrosome separation, leading to the formation of monopolar spindles, it was given the name "Aurora," reminiscent of the North Pole. The mammalian Aurora family comprises three related kinases that share the highest degree of sequence homology in their catalytic domains (2, 3). The Aurora-A and -B kinases have emerged as essential regulators of cell division. While Aurora-A is implicated in regulating mitotic entry, centrosome maturation, and spindle assembly; Aurora-B is required for correct chromosome segregation and cytokinesis. For Aurora-C, expression seems to be restricted to normal testicular tissue, the normal function of which is not well documented until recent reports show that Aurora-C is also a chromosomal passenger protein, and that it binds directly to INCENP and survivin *in vitro* (4-6). Experimental data suggest that inappropriately high or low levels of Aurora kinase activity are linked to genetic instability. Despite their sequence homology and common association with cycling cells, the subcellular distribution, partners, and substrates and therefore functions of Aurora-A and -B are essentially nonoverlapping (7, 8).

In this work, we focused on Aurora-A kinase. Through study of upstream regulator, downstream target and functional associating partner (Fig. 5.1), we provided new insights into better understanding of the molecular mechanism of Aurora-A kinase in human oncogenesis.
Aurora-A alteration is found in various human malignancies, at mRNA and/or protein levels more than DNA copy number change (9-11). The discrepancy suggests that Aurora-A overexpression is likely to be regulated not only by gene amplification, but also by other mechanisms such as transcriptional or translational activation and suppression of protein degradation. However, transcriptional regulation of Aurora-A during the cell cycle is largely unknown. We demonstrated, for the first time, that transcription factor E2F3 directly binds to Aurora-A promoter and tightly regulates Aurora-A expression during G2/M phase, which provides a mechanism for regulation of Aurora-A at transcription level during the cell cycle.

Among the 8 E2F family members identified so far, the transcriptional activators E2F1–3 have been shown to be cell cycle regulated; E2F4 and E2F5 negatively regulate cell cycle with decisions of cell differentiation and quiescence; and E2F6-8 are Rb-independent transcriptional repressors. Gene expression microarray analyses have revealed that E2F3 regulates not only DNA replication for S phase entry, but also mitotic regulatory factors such as cyclin B1, cyclin A2 and cdc2 during G2/M progression (12-14). Our data show that ectopic expression of E2F3 induces mRNA and protein levels of Aurora-A whereas knockdown of E2F3 decreases Aurora-A expression and mitotic cell cycle delay. Notably, chromatin immunoprecipitation reveals that E2F3 binds to Aurora-A promoter in vivo and the interaction primarily occurs during G2/M phase.

Moreover, we demonstrated frequent coexistence of elevated levels of E2F3 and Aurora-A in human primary ovarian carcinoma. Importance of Aurora-A and E2F3 in oncogenesis has been well established by their alterations in human neoplasms and their capacity to induce cell transformation (15-18). We observed strong association of elevated E2F3 expression and Aurora-A in ovarian tumors underscoring the clinical significance of the E2F3-Aurora-A signaling axis. E2F3 could also be one of the major transcriptional factors that contribute to upregulation of Aurora-A in other human primary tumors. Aurora-A, as a mitotic E2F3 target gene, could mediate E2F3 oncogenic function. Thus, E2F3-Aurora-A axis represents an attractive target for cancer therapy.
CHFR (checkpoint with forkhead and ring finger) has been discovered as an early mitosis checkpoint. Unlike the spindle checkpoint, the CHFR checkpoint is not essential for cell proliferation, which can be explained by the high frequency of CHFR inactivation, either by promoter methylation or in a few cases by missense mutations. The overall CHFR inactivation rate is ranging from 15 to 50% in various tumor types (19-26), which is much higher than the frequency of inactivation of all spindle checkpoint genes combined.

It has been shown that CHFR mediates the degradation of polo-like kinase 1(Plk1), Aurora-A and CHFR itself through its ring finger domain which possesses ubiquitin ligase activity. CHFR also contains a FHA domain, which could bind phosphorylated peptides (19, 27), suggesting that protein kinases might act upstream of CHFR and regulate its activity. A previous report shows that Akt/PKB phosphorylates CHFR at Thr-39 in FHA domain after DNA damage, which led to inhibition of CHFR E3 activity and shortening G2 arrest induced by DNA damage (28). We demonstrated in this study that CHFR is a substrate of Aurora-A kinase. Aurora-A phosphorylates CHFR at Ser218 and Ser337 and inhibits its E3 activity.

We also showed that frequent downregulation of CHFR and inverse correlation of expression of Aurora-A and CHFR in human ovarian cancer, which supports the notion that CHFR is an E3 ligase of Aurora-A (29). However, co-expression of CHFR and elevated Aurora-A was observed in a subset of ovarian cancers, which is consistent with the previous findings that only a fraction of tumors with loss of CHFR expression are due to promoter hypermethylation, suggesting that Aurora-A might phosphorylate CHFR and inhibit its tumor suppressor function in these tumors. Taken together, these findings indicate a feedback regulation loop between Aurora-A and CHFR.

Aurora-A exerts its mitotic function through regulation of a number of proteins (30-34), which include histone H3, TACC3, Eg5, CPEB and TPX2. We and others have previously shown Aurora-A phosphorylation of p53 which leads to inactivation of p53 and G2/M cell cycle progression (35). CHFR has been shown to mediate a delay in cell cycle progression early in mitosis in response to microtubule stress. Identification of
Aurora-A inhibition of CHFR provides additional molecular mechanism for Aurora-A function in control of G2/M cell cycle progression.

Aurora-A has well-established but perhaps not yet fully understood roles in centrosome maturation and duplication, mitotic entry and bipolar spindle assembly. By the G2 phase of the cell cycle through anaphase, it can be detected in the centrosomes, spindle poles and spindle microtubules. Following initial activation by the LIM protein Ajuba in G2, Aurora-A phosphorylates and recruits several microtubule-associated proteins to the centrosome to promote maturation. Cyclin-dependent kinases (CDKs) require not only phosphorylation of the equivalent threonine (Thr160CDK) but also the binding by a partner protein, cyclin A, to be fully activated (36). Aurora-A might also rely on a similar mechanism. After nuclear envelope breakdown, Aurora-A is localized to spindle microtubules and fully activated by its interacting non-enzymatic protein TPX2, which plays an as yet not fully defined role in the Ran-spindle assembly process (37, 38). The activation of Aurora-A is also facilitated by other cofactors/substrates, with different regulatory mechanism and cellular functions.

We identified CNTD2, a novel mammalian B-type cyclin, as a cofactor of Aurora-A. CNTD2 colocalizes and immunoprecipitates with Aurora-A at centrosome. The interaction results in stabilization Aurora-A protein and activation of Aurora-A kinase. Unlike other Aurora-A interaction proteins TPX2, Bora and Ajuba, which are also substrates of the kinase (34, 39, 40), CNTD2 increases Aurora-A protein stability and kinase activity but is not phosphorylated by Aurora-A.

Aurora-A is required for the activation and recruitment of cdc2-cyclin B1 to the centrosome. The activated cdc2-cyclin B1 then commits the cell to mitosis (40). While CNTD2 interacts with Aurora-A and activates Aurora-A and cdc2 kinase activity, it did not form complex with cdc2. Other than the role in Aurora-A regulation, CNTD2 is an oncogene, which is concluded from the facts that ectopic expression of CNTD2 induces malignant transformation, centrosome amplification and genomic instability.

CNTD2 locates to chromosome 19q13 amplicon, which contains several other oncogenes AKT2, PAK4, pancreatic differentiation 2 (PD2), SERTAD3 and
transcriptional regulator intersex-like (IXL) (41-45). We have previously shown amplification/overexpression of AKT2 in human ovarian, pancreatic and breast cancers. Inhibition of AKT2 reduces tumor growth and sensitizes cells to chemotherapeutic agent-induced cell death (41, 46, 47). In this report, we demonstrated that CNTD2 is amplified/overexpressed in human ovarian, pancreatic and lung cancers. Overexpression of CNTD2 is associated with late stage/high grade ovarian tumors and accompanied with poor clinic outcome. Therefore, CNTD2 could be a potential therapeutic target or cancer progression/prognosis marker for these malignancies. The molecular mechanism of CNTD2 regulation of Aurora-A protein stability and kinase activity remains to be investigated.

Fig. 5.1. Working scheme of molecular mechanism of Aurora-A in human oncogenesis. This dissertation has demonstrated that transcriptional upregulation by E2F3, interaction and activation by CNTD2 of Aurora-A; and Aurora-A feedback regulation of CHFR may contribute to Aurora-A-induced tumorigenesis.
References


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APPENDICES
Appendix I

List of Publications

Chapter II of this dissertation has been submitted to Journal of Biological Chemistry, 2008.
Chapter III and IV of this dissertation are in manuscript preparation.

Other publications:


ABOUT THE AUTHOR

Lili He received her Bachelor’s Degree at Beijing Institute of Technology, China in 2003. Ms He entered the Ph.D. program at University of South Florida College of Medicine in April 2004, and received her Master Degree of Science at USF in 2006.

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