Identification of Aurora-A as a Direct Target of E2F3 during G2/M Cell Cycle Progression*

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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 the Aurora-A protein level during the cell cycle through the ubiquitin-proteasome pathway. However, the mechanism of transcriptional regulation of Aurora-A remains largely unknown. Here, we demonstrated 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.

This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, lane 6 from the E2F3 panel in Fig. 1A was reused in lane 8 of the same panel. Lane 1 from the E2F3 panel from Fig. 4A was reused as lane 3 of the same panel. The authors state that the image reuse does not affect the overall conclusions of the study.
EXPERIMENTAL PROCEDURES

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. Cell transfection was performed with Lipofectamine Plus reagent (Invitrogen).

Synchronization and Cell Cycle Analysis—Cell synchronization at the G1/S phase was performed using a double-thymidine block (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 washes with serum-free medium, the cells were released from the thymidine block by culture in fresh medium containing 24 mM 2-deoxythymidine (Sigma) was added to the culture medium at a final concentration of 2 mM for 12 h. Following two washes with serum-free medium, the cells were released from the thymidine block by culture in fresh medium containing 24 mM 2-deoxythymidine. After 9 h of incubation, the second thymidine block was initiated and completed after 14 h. The cells were released from the block by washing in warm phosphate-buffered saline and replacing with complete culture medium. At different time points, the cells were fixed in 70% ethanol. The fixed cells were rinsed with phosphate-buffered saline and then stained with solution containing 50 μg/ml propidium iodide, 0.05% Triton X-100, and 0.1 mg/ml RNase A (Sigma). Cell synchrony at various cell cycle stages was monitored by flow cytometry. To synchronize cells in the M phase, HeLa cells were incubated in nocodazole for 14 h. Whole cell lysates and total RNA were isolated from parallel experiments for Western and Northern blot analyses.

Plasmids—pGL3-Aurora-A/354, pGL3-Aurora-A/1486, pGL3-Aurora-A/354, and pGL3-Aurora-A/1486/354 were kindly provided (PerkinElmer Life Sciences). To create deletion mutants, DNA fragments corresponding to Aurora-A promoter mutant constructs were generated using QuikChange site-directed mutagenesis kit (Stratagene), including by DNA sequencing. The oligonucleotide primers used were as follows: sense −124/49, sense −124/49, sense −124/49, sense −124/49, and antisense 5′-GATAGAAAAGCAAGAGAGTG-3′; mutation A, sense 5′-GGTCGGCTTGGATCCCTTTCAGCGTGCGC-3′; and antisense 5′-AAGGCCGACACCACCCATGGCCAGGACTTGCTC-3′; and antisense 5′-AAGG-GGCACCACCCATGGCCAGGACTTGCTC-3′.

Luciferase Assay—The cells were cultured in 24-well plates and transfected with pGL3-Aurora-A reporters, a β-galactosidase expressing internal control, and the effector 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 a luciferase kit (Promega). The β-galactosidase activity was measured by using Galacto-Light (Tropix). Transfection efficiency was normalized by β-galactosidase expression. Luciferase activity was expressed as relative activity to β-galactosidase. Each experiment was repeated in triplicate.

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 of total RNA from each sample was separated on 1.0% agarose. After transferring the membrane, the probe in Express hybridization solution (Clontech). For Western blot, equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes. Following blocking in TBS-T containing 5% milk, the membranes were probed with antibodies indicated in the figure legends.

Infection—Knockdown of Aurora-A promoter construct was performed by infection of HeLa cells with lentiviruses expressing different shRNAs of Aurora-A, of which recognize the amino acids coding for E2F3 and the other the 3′-untranslated region. HeLa cells were plated in 60-mm plates. Following culture for 24 h and the addition of polybrene, the cells were infected with pLKO.1-shRNA/E2F3 viruses at multiplicity of infection 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 (5 μg/ml).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed essentially as previously described (25). Solubilized chromatin was prepared from a total of 2 × 107 asynchronously growing HEK293 cells that were transfected with E2F3. The chromatin solution 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 pre-cleared chromatin solution was divided and utilized in immunoprecipitation assays with either an anti-E2F3a antibody (N-20; sc-879, Santa Cruz) or an anti-actin antibody. Following wash, the antibody–protein–DNA complex was eluted from the beads by incubating in 1% SDS, 0.1 mM NaHCO3, at room temperature for 20 min. The protein–DNA cross-linking was reversed, and protein and RNA were removed by incubation at 65 °C for 30 min.

2 The abbreviations used are: shRNA, small hairpin RNA; ChIP, chromatin immunoprecipitation.

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ATGCGGTGTGCACCTT-3′ and antisense 5′-AAGGGGCACCACCCATGGCCAGGACTTGCTC-3′.
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RESULTS

Expression of the Members of E2F Family and Aurora-A during the Cell Cycle—Although mRNA and protein levels of Aurora-A are elevated at G2/M phase, the underlying molecular mechanism remains elusive (2). 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 whether Aurora-A is regulated by E2F3, we knocked down E2F3 by infection of HeLa cells with lentiviruses (pLKO.1-E2F3/shRNA) expressing five different E2F3 shRNAs, four of which are corresponding to the coding region and the other of which matches the 3′-untranslated region. Fig. 2C showed that E2F3 was considerably reduced in 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 (Figs. 2D and 3B).

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 enter G2/M phase later as compared with the pLKO.1 vector-infected cells (Fig. 3A). Consistent with cell cycle change, the elevation of Aurora-A mRNA and the protein levels were decreased at G2/M phase (Fig. 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 E2 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 Increases and Knockdown of E2F3 Decreases Aurora-A Protein and mRNA Levels—To determine whether 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. 2A). Immunoblotting analysis showed that E2F3 up-regulated Aurora-A expression in a dose-dependent manner (Fig. 2B). To further demonstrate that Aurora-A expression is controlled by E2F3, we knocked down E2F3 by infection of HeLa cells with lentiviruses (pLKO.1-E2F3/shRNA) expressing five different E2F3 shRNAs, four of which are corresponding to the coding region and the other of which matches the 3′-untranslated region. Fig. 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 (Figs. 2D and 3B).

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E2F3 binds to and transactivates the 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. 4A). Moreover, basal level of Aurora-A promoter activity, especially at mitosis, was reduced in E2F3 knockdown cells as compared with pLKO.1 vector-infected HeLa cells (Fig. 4B).

Sequence analysis showed four putative E2F-binding elements (TT(C/G)GCGC(C/G)) within the promoter (Fig. 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 decreased E2F3-induced promoter activity, whereas it still contains all four putative E2F-binding elements, implying that this region is of transactivation function. Moreover, the deletion of the two 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 −189 to −124 or −189 to −96, both of which retain the 2 E2F response elements (C and D) proximal to the transcriptional start site, was significantly induced by E2F3. The promoter activity was completely abrogated by further deletion of the proximal E2F response elements (Fig. 4C). These results suggest that the region from −189 to −96 contains a repression element(s) and that all four E2F-binding sites are responsive to E2F3. The two binding elements proximal to the transcriptional start site are sufficient for E2F3 transactivation of the Aurora-A promoter.

To determine whether E2F3 could directly bind to the E2F-binding sites of the Aurora-A promoter...
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FIGURE 4. Aurora-A promoter is regulated by E2F3. A, ectopic expression of E2F3 activates luciferase activity. HeLa cells were transiently transfected with indicated plasmids. E2F3 expression and luciferase activity were measured and normalized to β-galactosidase. The independent experiments performed in triplicate (top). The bottom graph shows E2F3 activity of cell lysates treated with nocodazole (Noc). B, knockdown of E2F3 reduces basal level of Aurora-A promoter activity. NIH3T3 cells were transfected with indicated Aurora-A deletion mutants and different amounts of E2F3 plasmid. The E2F3-binding sites are labeled as A–D (in squares). Luciferase assay was performed at different phases of the cell cycle. C, E2F3 regulates the Aurora-A promoter. By mutation of individual or combinational E2F3-binding sites (CG → AT) in Aurora-A promoter, we further demonstrated that the two E2F response elements proximal to the transcriptional start site are required for E2F3 transactivation of the Aurora-A promoter (Fig. 5, A and B).

Because mRNA and protein levels of Aurora-A are low at G1 and gradually increase during the G1/M phase, we examined whether 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. 5C shows that E2F3 barely interacts with Aurora-A promoter during the G1/S phase. The binding and transactivation activities of E2F3 toward the Aurora-A promoter were significantly increased upon the cell entering the G2/M phase. These data further support the notion that E2F3 plays a pivotal role in Aurora-A expression during the G2/M phase.

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, the cells were immunostained with anti-Aurora-A antibody. As shown in Fig. 6, unsynchronized cells expressing GFP-E2F3 exhibited higher density and clearer centrobody formation in the cells that did not, further indicating E2F3 up-regulation and Aurora-A.

We and others previously demonstrated 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. Although 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 up-regulation of Aurora-A. To this end, we examined protein levels of E2F3 and Aurora-A in human ovarian cancers. Immunoblotting analyses were performed in a total of eight ovarian cancer cell lines and 72 microdissected ovarian tumor specimens (Fig. 7, A and B). 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 the majority of ovarian cancer cell lines examined. Notably, 78% (32/41) of tumors with high levels of Aurora-A overexpress E2F3 (Fig. 7C). Co-up-regulation of E2F3 and Aurora-A seems to be predominantly observed in serous ovarian carcinomas and clear cell tumors, whereas the number of cases is relatively small (Fig. 7C). These data indicate that a large subset of ovarian tumors with 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.

E2F3 Regulates G2/M Cell Cycle Progression through Aurora-A in Ovarian Cancer Cells—Having shown close correlation between expression of E2F3 and Aurora-A in ovarian cancer,
FIGURE 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 under "Experimental Procedures" 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. The cell cycle was monitored with flow cytometry (top), and ChIP assay was performed for each time point (second panel). Aurora-A RNA levels (third panel) and promoter activity (bottom panel) are correlated with E2F3 binding to Aurora-A promoter. IP, immunoprecipitation; WT, wild type.
we further examined whether E2F3 regulates cell cycle through Aurora-A in ovarian cancer cells. Because expression levels of E2F3 and Aurora-A are high in OVCAR3 and low in A2780S cells (Fig. 6A), we stably knocked down and expressed E2F3 in OVCAR3 and A2780S cells, respectively (Figs. 8A and 9A). Cells treated with vector alone were used as controls. Aurora-A expression levels were decreased and elevated upon knockdown and expression of E2F3, respectively (Figs. 8A and 9A).

After double-thymidine block, flow cytometry analysis revealed that knockdown of E2F3 delays G2/M phase in OVCAR3 (Fig. 8, C and D), whereas ectopic overexpression of E2F3 in A2780S cells accelerates G2/M progression (Fig. 9, B and C), further supporting the findings in HeLa cells (Fig. 3A).

We next determined whether Aurora-A is required for the E2F3 action in cell cycle of ovarian cancer cells. E2F3 knockdown OVCAR3 cells were transfected with Aurora-A (Fig. 8B). As shown in Fig. 8 (C and D), expression of Aurora-A largely rescues G2/M delay induced by knockdown of E2F3. Moreover, knockdown of Aurora-A in E2F3-transfected A2780S cells largely abrogated E2F3-promoted G2/M progression (Fig. 9C). These results provide additional evidence that Aurora-A is a direct target of E2F3 and mediates E2F3 function in G2/M progression.

**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. it 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 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, the 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

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**FIGURE 6. Expression of E2F3 increases Aurora-A density in centrosome.** HeLa cells were transfected with GFP-E2F3. Unsynchronized cells were immunostained with anti-Aurora-A antibody (top panel). The bottom panel shows the quantification of cells with clear Aurora-A centrosome staining from 300 GFP-E2F3-transfected and nontransfected cells. The experiment was repeated three times.

**FIGURE 7. Overexpression of E2F3 correlates with Aurora-A level in human ovarian tumors.** A and B, Western blot analysis. Representative ovarian cancer cell lines tumor (A) and normal ovarian tissue (B) lysates were analyzed by Western blot with the indicated antibodies. Intensity of E2F3 and Aurora-A (AurA) were quantified via ImageJ software (National Institutes of Health). 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. C, summary of expression of E2F3 and Aurora-A in ovarian cancers. The percentage of co-expression of E2F3 and Aurora-A in the different stages of tumor is listed in the right column. Approximately 44% of the total patients exhibited overexpression of both E2F3 and Aurora-A. Of the samples that presented with elevated E2F3, ~78% had increased Aurora-A levels. The bottom panel is expression of E2F3 and Aurora-A in different histological types of ovarian carcinoma.

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withdrawn
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-terminally 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 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. (37) cloned human Aurora-A promoter and identified two E2F-binding elements that correspond to binding sites A and B in Fig. 4C. The findings presented here show that although E2F3 binds to these two
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sites, their mutations still respond to E2F3 (Fig. 5A). This led us to identify two more E2F-binding motifs proximate to transcriptional start site (Fig. 4C). ChIP and reporter assays show that they are required for the transcriptional activation of Aurora-A gene by E2F3 (Fig. 5, B and C).

Previous reports have demonstrated that E2F3 is frequently overexpressed in a variety of types of human malignancy, and alteration of E2F3 is associated with late stage and high grade tumors. However, alterations of E2F3 in human ovarian cancer have not been well documented, whereas a gene expression array study shows up-regulation of E2F3 in ovarian tumor (39). Based on our current observations, we believe that Aurora-A, as a mitotic E2F3 target gene, could mediate E2F3 function. Pharmacological agents that inhibit Aurora-A axis as an attractive target for cancer therapy. The 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 function. Pharmacological agents that inhibit Aurora-A axis as an attractive target for cancer therapy.

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