

Ets1 and Elk1 transcription factors regulate *Cancerous Inhibitor of Protein phosphatase 2A* expression in cervical and endometrial carcinoma cells

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Abbreviations: CIP2A, cancerous inhibitor of protein phosphatase 2A; PP2A, protein phosphatase 2A; Ets1, E-twenty six; Elk1, Ets like kinase1; RLA, relative luciferase activity; TSS, transcription start site

Cancerous inhibitor of protein phosphatase 2A (CIP2A) has been identified as a proto-oncogene that is overexpressed in various types of human cancers. CIP2A acts by inhibiting protein phosphatase 2A-dependent destabilization of c-Myc, resulting in increased cell proliferation. We have characterized the proximal promoter region of the human *CIP2A* gene in cervical, endometrial and liver carcinoma cells. The 5' flanking minimal proximal promoter of the *CIP2A* gene consists of putative binding sites for Ets1 and Elk1 in forward and reverse orientations. Here, we show that Ets1 and Elk1 binding is essential for *CIP2A* basal expression in several urogenital cancer cell lines. Interestingly, both Ets1 and Elk1 are required together for *CIP2A* expression, as siRNA knockdown of Ets1 and Elk1 together decreased *CIP2A* gene transcription, whereas knockdown of Ets1 or Elk1 alone had no effect. Moreover, ectopic expression of Ets1 and Elk1 together increased *CIP2A* expression. To gain physiological significance of the Ets1 and Elk1 regulation we observed, a panel of matched human cervical carcinoma samples was analyzed for the expression of CIP2A and Ets1 and/or Elk1. We found a direct correlation between the levels of CIP2A and the levels of Ets1 and Elk1. Our results suggest that the binding of Ets1 and Elk1 together to the proximal *CIP2A* promoter is absolutely required for *CIP2A* expression in cervical, endometrial and liver carcinoma cell lines. Thus, different factors regulate CIP2A expression in a cell-type specific manner. As previous work has shown a requirement for only Ets1 in prostate and gastric carcinomas, our results now indicate that *CIP2A* regulation is more complex than previously determined.

Introduction

Cancer is a complex disease that involves genetic alterations, which lead to deregulated growth and development.¹ Oncogenes play an important role in cancer progression.² Mutations due to genetic or epigenetic factors in oncogenes lead to a dominant gain-of-function, such as enhanced cell growth and division, and increased cell survival.³ Cancerous Inhibitor of Protein phosphatase 2A (CIP2A) was discovered as a 90 kDa auto-antigen found to be co-expressed in hepatocellular carcinoma (HCC) patients along with a 62 kDa auto-antigen and was observed to be overexpressed in gastric cancer tissues.⁴ CIP2A was initially characterized as an oncoprotein that inhibits protein phosphatase 2A (PP2A)-dependent dephosphorylation of the oncoprotein c-Myc at Serine 62 (S62), thus stabilizing c-Myc activity.⁵ Several publications have demonstrated that interaction of CIP2A with c-Myc is an important aspect in the progression of different types of cancers, such as human cervical, gastric

and breast cancer cells.^{6–8} Most recently, CIP2A has been shown to regulate death associated protein kinase (DAPK) via inhibition of PP2A.⁹

There is a wealth of data showing that *CIP2A* expression is elevated in many different types of cancers. Shi et al.¹⁰ showed increased expression of *CIP2A* in prostate cancer patients in comparison to patients with benign prostatic hyperplasia (BPH). Similarly, an increase in *CIP2A* expression has been observed in tissue samples obtained from gastric, breast, oral, prostate, cervical, esophageal squamous cell, non-small cell lung, colon, ovarian and colorectal cancer patients.^{11–19} The overexpression of *CIP2A* in breast, non-small cell lung and early stage tongue cancer has been correlated with poor prognosis, suggesting that *CIP2A* levels could serve as a marker for disease progression.^{11,16,20}

Coenen et al.²¹ demonstrated CIP2A to be a novel mixed lineage leukemia translocation partner in acute myeloid leukemia (AML) patients. Increased expression of *CIP2A* has been identified

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Figure 1. Transcription factor binding sites of the *CIP2A* proximal promoter. The 5' flanking region of the *CIP2A* promoter is shown with the transcription start site (TSS) indicated at +1. The sequence has been numbered from the TSS. The following potential transcription factor binding sites were identified: **1**, Glucocorticoid receptor α ; **2**, Retinoic acid receptor α ; **3**, Pax5; **4**, Ets1; **5**, Elk1; **6**, NF- κ B; **7**, Sp1; and **8**, AP-2—using the ALGEN-PROMO or Ali-baba 2.0 computational software. The region between -171 and -95 has Ets1/Elk1 palindromic binding sites, which are important for basal expression of human *CIP2A*.

in patients with AML and chronic myeloid leukemia (CML).^{22,23} High *CIP2A* levels in CML patients correlate with higher risk for blast crisis due to increased phosphorylation of c-Myc.²³ Increased *CIP2A* has been identified in fibroblast-like synoviocytes (FLS), which play an important role in rheumatoid arthritis (RA) progression, suggesting *CIP2A* as an ideal therapeutic target.²⁴

Considering the increased expression of *CIP2A* in different types of cancer and its important role in development of the disease through its inhibition of PP2A tumor suppressor activity, we identified and characterized the transcription factors that control its expression. In this study, we utilized functional deletion constructs and have shown that the region between -123 and -95 of the *CIP2A* gene promoter is essential for the basal transcription in human cervical (HeLa), liver (HepG2) and endometrial carcinoma (ECC-1) cells. Importantly, we have demonstrated that the transcription factors Ets1 and Elk1 are together required for regulating the transcription of the *CIP2A* gene in human cervical and endometrial carcinoma cells. Based on our results and those from others, we suggest that targeting both Ets1 and Elk1 may be a viable therapeutic strategy for treatment of endometrial and cervical cancers.

Results

Nucleotide sequence of *CIP2A* gene. The *CIP2A* nucleotide gene sequence (GenBank accession no AC092693.8) was used

to construct 5' deletion and full length 2.4 kb basal promoter clones. The first exon was found between +1 and +70 nucleotides (Fig. 1). The 5' flanking region upstream of the transcription start site (TSS) +1 region was considered to harbor the promoter region for transcriptional regulation of *CIP2A* gene.

Characterization and Identification of *CIP2A* proximal promoter region. In order to identify functional transcription factor binding sites in the 5' flanking region of *CIP2A* gene promoter, a series of PCR deletion clones were constructed in the pGL4 basic luciferase vector (Fig. 2A). All promoter deletion clones were assayed for activity in human cervical carcinoma (HeLa) and liver hepatoblastoma (HepG2) cell lines. The fold change in relative luciferase activity (RLA) of individual deletion clones was compared with that of the pGL4 basic vector (negative control).

Initially, we found that the full length *CIP2A* promoter (-2379/+70) showed a 50-fold increase in RLA in HeLa (Fig. 2B) and a 5-fold increase in HepG2 (Fig. 2C), compared with the basic vector, whereas the *CIP2A* -95/+70 clone showed no activity above background (Fig. 2; Table 1). Further analyses of promoter function indicated that clone -123/+70 contained the minimal proximal promoter activity of the human *CIP2A* gene. Interestingly, the -941/+70 construct showed the highest increase in RLA (Fig. 2B and C; 253- and 30-fold for HeLa and HepG2, respectively). These data suggest that there may be enhancer and/or co-repressor binding sites upstream of the minimal proximal promoter, which we are currently investigating further. All data are summarized in Table 1 and shown in Figure 2A–C.

Due to our interest in studying female urogenital cancers, we then determined the minimal *CIP2A* promoter sequence housing activity in human endometrial (ECC-1) carcinoma cells. A subset of the deletion constructs (Fig. 2D) were analyzed. A 71- and 283-fold increase in RLA was observed with *CIP2A* -123/+70 and *CIP2A* -171/+70 clones, respectively (Fig. 2E), in comparison with the *CIP2A* -95/+70 clone, which displayed only a 5-fold increase in RLA when compared with pGL4. As was observed for HeLa and HepG2 cells, the *CIP2A* -941/+70 construct showed the highest activity, while the luciferase activity of the full-length construct -2379/+70 was similar to *CIP2A* -171/+70. Thus, the region between -171 and -95 contains the proximal promoter, and the region between -123 to -95 contains the minimal proximal promoter essential for *CIP2A* expression in human cervical, liver and endometrial carcinoma cells. The data are summarized in Table 1.

Identification of putative transcription factor binding sites. We analyzed the proximal promoter region (-171 to -95) of the human *CIP2A* gene for potential transcription factor binding sites using ALIBABA 2.0 or ALGEN-PROMO software. Binding sites for GR α (glucocorticoid receptor α), RAR α (retinoic acid receptor α), Pax5, Ets1, Elk1, AP-2 and Sp1 were identified within -171 to +1 (Fig. 1). The binding sites for the Ets1 and Elk1 transcription factors were identified in reverse orientations in the region between -110/-118 and -127/-137.

Site-directed mutagenesis of transcription factor binding sites. Using PCR based site-directed mutagenesis, we introduced point mutations (underlined and in bold) or deletions (underlined and in bold italics) within several putative transcription

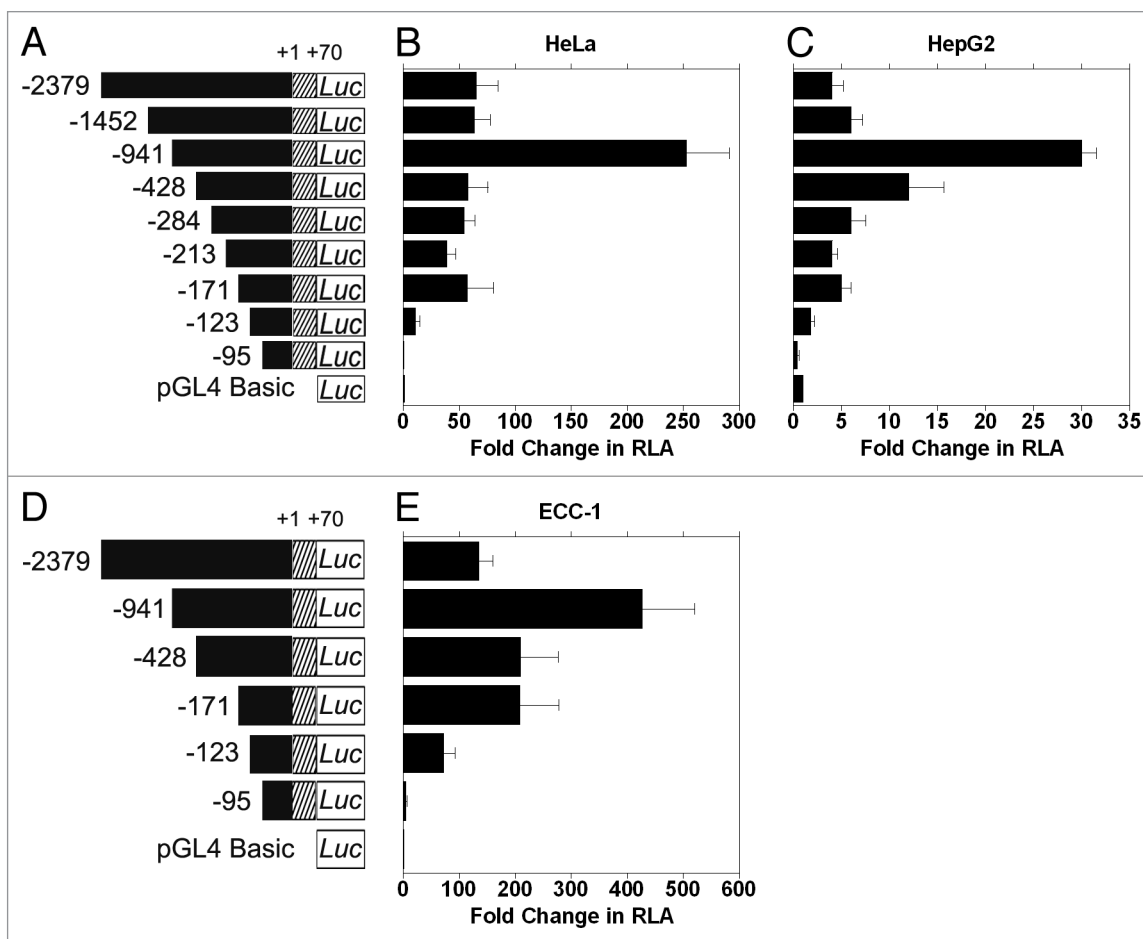


Figure 2. Identification of the *CIP2A* proximal promoter region. (A) and (D) Diagrammatic representation of the full length and sequentially deleted *CIP2A* promoter constructs used in this study. The transcription start site (TSS) is numbered as +1 and the constructs are numbered with reference to the TSS from 5'–3' ends. (B) Human cervical carcinoma cells (HeLa), (C) liver hepatoblastoma cells (HepG2), and (E) endometrial carcinoma cells (ECC-1) were transfected with *CIP2A* promoter constructs shown in Figure 2A or 2D and assayed for luciferase activity after 48 h as described in the Materials and Methods. Fold increase in relative luciferase activity (RLA) was compared with pGL4-basic (set as 1). Normalization in transfection efficiency was performed by co-transfection with pRL-TK (*Renilla* expression vector). The mean \pm SD are from three different experiments, each performed in triplicate.

factor binding sites (Fig. 3A). The *CIP2A* -171/+70 construct was used for all of our mutagenesis studies. Mutant promoter sites were constructed as follows: point mutations for NF- κ B (*CIP2A* Mut1), substitution and deletion mutants for the first Ets1/Elk1 binding site (*CIP2A* Mut2 and Mut3, respectively), point mutations for Pax5 (*CIP2A* Mut4), and deletion and substitution mutations for the second Ets1/Elk1 binding sites (*CIP2A* Mut5 and Mut6, respectively).

Interestingly, the *CIP2A* Mut2 (Ets1/Elk1) and *CIP2A* Mut3 (Ets1/Elk1) constructs displayed a 3 to 4-fold reduced promoter activity in the HeLa cells and a 7 to 16-fold reduction in promoter activity in the ECC-1 cells, when compared with the wild type *CIP2A* -171/+70 construct (Fig. 3B and C). In contrast, mutating the NF- κ B binding site (*CIP2A* Mut1) did not affect the basal luciferase activity in the HeLa and ECC-1 cells. Mutation in the Pax5 binding site (*CIP2A* Mut4) showed cell-type specificity, as its loss in HeLa cells did not effect *CIP2A* transcription (Fig. 3B), but decreased transcription by 2.5-fold in ECC-1 cells (Fig. 3C).

Importantly, when the HeLa and ECC-1 cells were transfected with *CIP2A* Mut5 (Ets1/Elk1, deleted binding site) a 9 to 16-fold loss in *CIP2A* promoter activity was observed. Similarly, the *CIP2A* Mut6 (Ets1/Elk1, nucleotide substitution) displayed a 13 to 39-fold decrease in luciferase activity compared with the wild type construct in HeLa and ECC-1 cells (Fig. 3B and C). These results suggest that the transcription factor binding sites for Ets1 and Elk1 together are critical for basal transcription of the *CIP2A* gene in human cervical and endometrial cancer cells.

In vitro binding of Ets1 and Elk1 to the *CIP2A* minimal proximal promoter region. Since our data suggested the requirement for Ets1 and Elk1 in driving *CIP2A* transcription, we next completed electrophoretic mobility shift assay (EMSA) to confirm our results. We synthesized a wild type (WT) probe harboring the Ets1 and Elk1 binding sites in the forward and reverse orientation from -138 to -107 of the *CIP2A* gene promoter with a consensus oligonucleotide for Ets1 and Elk1. Addition of the WT probe in the presence of nuclear extracts from ECC-1

Table 1. Identification of human *CIP2A* basal proximal promoter

5 deletion construct	Fold increase in relative luciferase activity (RLA)		
	HeLa	HepG2	ECC-1
pGL4 Basic	1	1	1
<i>CIP2A</i> -95/+70	0.4	0.4	5.3
<i>CIP2A</i> -123/+70	11.0	1.8	71.0
<i>CIP2A</i> -171/+70	57.0	5.0	208.0
<i>CIP2A</i> -213/+70	39.0	4.0	-
<i>CIP2A</i> -284/+70	54.0	6.0	-
<i>CIP2A</i> -428/+70	58.0	12.0	210.0
<i>CIP2A</i> -941/+70	253.0	30.0	426.0
<i>CIP2A</i> -1452/+70	63.0	6.0	-
<i>CIP2A</i> -2379/+70	65.0	4.0	134.0

Human cervical carcinoma cells (HeLa), liver hepatoblastoma cells (HepG2), and endometrial carcinoma cells (ECC-1) were transfected with various *CIP2A* promoter constructs shown in **Figure 2A** or **2D** and assayed for luciferase activity after 48 h as described in Materials and methods. Fold increase in relative luciferase activity (RLA) was compared with pGL4 basic (set as 1). Normalization in transfection efficiency was performed by co-transfection with pRL-TK (*Renilla* expression vector). The mean \pm SD are from three different experiments, each performed in triplicate.

cells displayed four protein-DNA complexes (**Fig. 4, lane 2**). Competition with 100-fold molar excess of unlabeled WT probe resulted in a complete inhibition of all four protein-DNA complexes (**Fig. 4, lane 3**). Competition with 100-fold molar excess of unlabeled mutant probe, in which the palindromic binding sites for Ets1 and Elk1 were mutated, did not abolish the fourth protein-DNA complex, though the first three complexes were inhibited (**Fig. 4, lane 4**).

In order to confirm the loss of the fourth DNA-protein complex as the binding site for Ets1 and Elk1, a 100-fold excess molar competition was performed with the Ets1 and Elk1 consensus sequences, which led to the loss of the fourth protein-DNA complex (**Fig. 4, lanes 5–6**). Moreover, the pattern of protein-DNA complexes observed with the labeled Ets1 and Elk1 consensus probe were similar to those observed with the WT probe of the *CIP2A* gene (**Fig. 4, compare lane 2 with lanes 8 and 9**). These results indicate that transcription factors Ets1 and Elk1 bind to the -138 to -107 region of the *CIP2A* gene and regulate its transcription in endometrial carcinoma cells.

We next performed a gel-supershift analysis to further confirm our results. As a negative control, pre-immune IgG was utilized (**Fig. 5, lane 3**) and no shift was detected. Addition of Ets1 antibody to the nuclear extract from ECC-1 cells in the presence of the WT probe caused a shift in the protein-DNA complex (**Fig. 5, lane 4**). Interestingly, in the presence of Elk1 antibody, the intensity of the protein-DNA complex was greatly enhanced rather than a shift (**Fig. 5, lane 5**). In the presence of both Ets1 and Elk1 antibodies, we detected a shift and an increase in the intensity of the protein-DNA complex (**Fig. 5, lane 6**). These results confirm that Ets1 and Elk1 bind to the palindromic sequence (-138 to -107) in the *CIP2A* promoter.

In vivo association of Ets1 and Elk1 with the *CIP2A* gene promoter. To confirm our in vitro data, we used chromatin immunoprecipitation (ChIP) analyses and quantitative PCR to assess the direct in vivo association of Ets1 and Elk1 with the *CIP2A* gene promoter. Cross-linked protein-DNA was immunoprecipitated with antibodies against Ets1, Elk1 or pre-immune IgG. Two primer sets were used to examine the specificity of Ets1 and Elk1 binding (**Fig. 6A**). Region 1 contains the two binding sites for Ets1 and Elk1, while region 2 has no binding sites for the transcription factors. We found that Ets1 immunoprecipitation (IP) resulted in a significant 3-fold increase in binding to the *CIP2A* promoter at region 1, while IP with the Elk1 antibody resulted in a significant 2-fold increase in binding to the *CIP2A* promoter compared with IgG in HeLa cells (**Fig. 6B**). Similarly Ets1 and Elk1 IP from ECC-1 cells showed a significant 2-fold increase in binding to *CIP2A* promoter occupancy compared with control IgG (**Fig. 6C**). In contrast, the Ets1 and Elk1 immunoprecipitates did not amplify *CIP2A* promoter fragment in the region between -2379 to -2101 in either cell line (**Fig. 6D and E**). These results demonstrate that Ets1 and Elk1 associate with *CIP2A* gene promoter in vivo in cervical and endometrial carcinoma cells.

Functional analysis of Ets1 and Elk1 in *CIP2A* expression. Since our studies indicated that Ets1 and Elk1 transcription factors together are required for regulating basal *CIP2A* expression, we completed siRNA analyses in order to substantiate the specificity of Ets1 and Elk1 for *CIP2A* expression. HeLa cells were transfected with siRNA specific for *ETS1*, *ELK1*, or *ETS1* and *ELK1* together. Both siRNAs displayed specificity for the target mRNA, as *ETS1* mRNA levels were altered only upon *ETS1* siRNA treatment and *ELK1* mRNA was only affected with *ELK1* siRNA treatment, as determined by qRT-PCR analysis (**Fig. 7A and B**). A significant 40% decrease in *CIP2A* mRNA expression level was observed when HeLa cells were transfected with siRNA toward *ETS1* and *ELK1* together (**Fig. 7C**), whereas no effect on *CIP2A* mRNA expression levels were observed when siRNA against *ETS1* or *ELK1* alone was used.

We determined endogenous *CIP2A* protein levels when *ETS1*, *ELK1*, or *ETS1* and *ELK1* were targeted with siRNA in HeLa cells 72 h after siRNA transfection to determine if a decrease in mRNA expression correlated with a decrease in *CIP2A* protein level. As shown in **Figure 7D**, a decrease in *CIP2A* protein level was observed when cells were treated with both *ETS1* and *ELK1* siRNA together, while there was no change in *CIP2A* protein level in the presence of either *ETS1* or *ELK1* siRNA alone. Thus, a loss in Ets1/Elk1 expression (and protein level) causes a decrease in *CIP2A* protein level, suggesting a direct role for Ets1 and Elk1 together in the coordinate regulation of *CIP2A* expression.

To corroborate our observations, we completed an add-back assay to confirm the specificity for Ets1 and Elk1 in basal expression of *CIP2A*. We transfected HeLa cells with either non-targeting siRNA (**Fig. 8A, lane 1**) or siRNA against the 3' UTR regions of *ETS1* and *ELK1*, effectively depleting the cells of endogenous Ets1 and Elk1 protein (**Fig. 8A, lane 2**). We

co-transfected cells with either empty vector (Fig. 8A, lanes 1 and 2) or vectors overexpressing *ETS1* or *ELK1* or *ETS1* and *ELK1* together (Fig. 8A, lane 3, 4, 5), which were resistant to *ETS1* and *ELK1* 3' UTR siRNA, and analyzed cell lysates by western blot. A significant decrease in CIP2A protein levels was observed in HeLa cells upon treatment with both *ETS1* and *ELK1* 3'-UTR siRNA (Fig. 8A, lane 2) when compared with non-targeting siRNA (Fig. 8A, lane 1). Furthermore, ectopic expression of either *ETS1* or *ELK1* alone did not rescue CIP2A expression in HeLa cells (Fig. 8A, lanes 3, 4) treated with *ETS1* and *ELK1* 3' UTR siRNA. Importantly, the loss of CIP2A protein was rescued in HeLa cells when *ETS1* and *ELK1* were overexpressed together (Fig. 8A, lane 5) in the presence of *ETS1* and *ELK1* 3' UTR siRNA. Importantly, *CIP2A* mRNA was significantly decreased when cells were treated with *ETS1* and *ELK1* 3' UTR siRNA (Fig. 8B, compare 1 and 2) when analyzed by qRT-PCR, and *CIP2A* expression was not recovered when *ETS1* or *ELK1* were overexpressed individually (Fig. 8B, compare 3 and 4 to 1). Importantly, *CIP2A* expression was returned to normal levels when both *ETS1* and *ELK1* were overexpressed (Fig. 8B, compare 5 to 1). These results confirm the specificity of Ets1 and Elk1 transcription factors in regulating the basal transcription of *CIP2A*.

Expression levels of CIP2A, Ets1 and Elk1 in human cervical tumor samples. To begin to assess the physiological relevance of our results, we examined CIP2A, Ets1 and Elk1 protein levels in human cervical tumor samples. Six different matched pairs of cervical tumor and normal adjacent tissue (NAT) were utilized for western analyses to determine the protein levels of CIP2A, Ets1, Elk1 and actin. Strikingly, we observed an increase in the protein expression levels of CIP2A in all the six tumor samples compared with the NAT samples (Fig. 9A). Four of the six tumor samples also showed marked increase in Elk1 (Fig. 9B) protein and all five tumors tested displayed increased Ets1 (Fig. 9C) protein when compared with normal tissue samples. Importantly, all six tumor samples showed an increase in at least one transcription factor. Together, these results strongly suggest that Ets1 and Elk1 regulate the basal transcription of *CIP2A* in human cervical tumors.

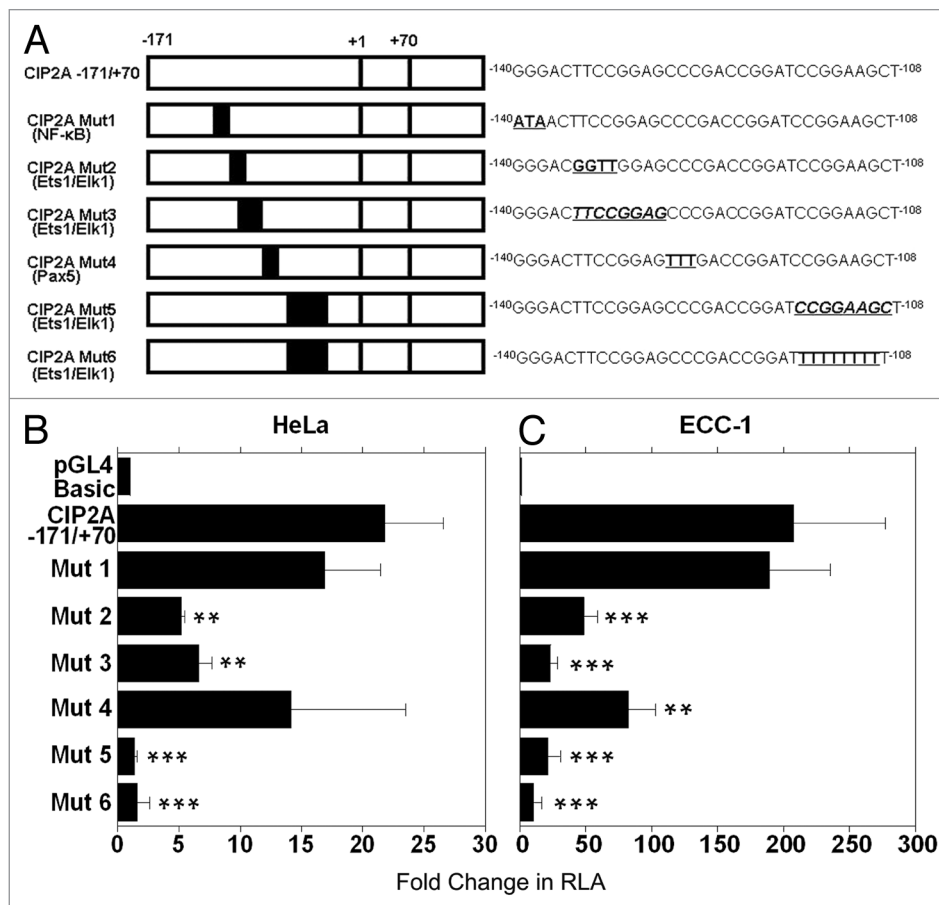


Figure 3. The Ets1 and Elk1 binding sites control transcription of *CIP2A*. (A) Six different mutants were constructed as explained in Materials and Methods. Mutated binding sites in the *CIP2A* promoter region are indicated in bold and are underlined. Deletions are indicated in bold, italics, and are underlined. *CIP2A* Mut1 targeted the binding sites for transcription factor NF-κB, *CIP2A* Mut2 targeted the binding site for transcription factor Ets1, *CIP2A* Mut3 was targeted toward the Elk1 binding site, and *CIP2A* Mut4 targeted the binding site for transcription factor Pax5, while *CIP2A* Mut5 and *CIP2A* Mut6 targeted the binding sites for Ets1 and Elk1. (B) HeLa and (C) ECC-1 cells were transfected with a mutant clone or the wild-type promoter (*CIP2A* -171/+70) and assayed for luciferase activity after 48 h as described in Figure 2. Transfection efficiency was normalized by co-transfection with pRL-TK (*Renilla* expression vector). The mean ± SD are from three different experiments, each experiment performed in triplicate (**p < 0.001, *p < 0.01, **p < 0.05 with *CIP2A* -171/+70 compared with the control pGL4 basic vector and the mutant compared with *CIP2A* -171/+70 wild-type construct).

Discussion

Studies from Junttila et al.⁶ first described the role of CIP2A as an oncogene interacting with protein phosphatase 2A (PP2A) and c-Myc. Recently, multiple publications have also described increased expression of CIP2A in various types of cancer cells and tumor samples, including gastric, breast, prostate, cervical, ovarian and colorectal cancer and a variety of other cancers.^{7,8,11-24} These observations suggest a direct correlation between CIP2A overexpression, cancer progression, aggressivity and poor prognosis in cancer patients.^{11,16}

We are the first to elucidate the transcriptional regulation of *CIP2A* in female urogenital cancers. In the present study, we have functionally characterized the human *CIP2A* gene basal proximal and minimal proximal promoter in ECC-1 and HeLa

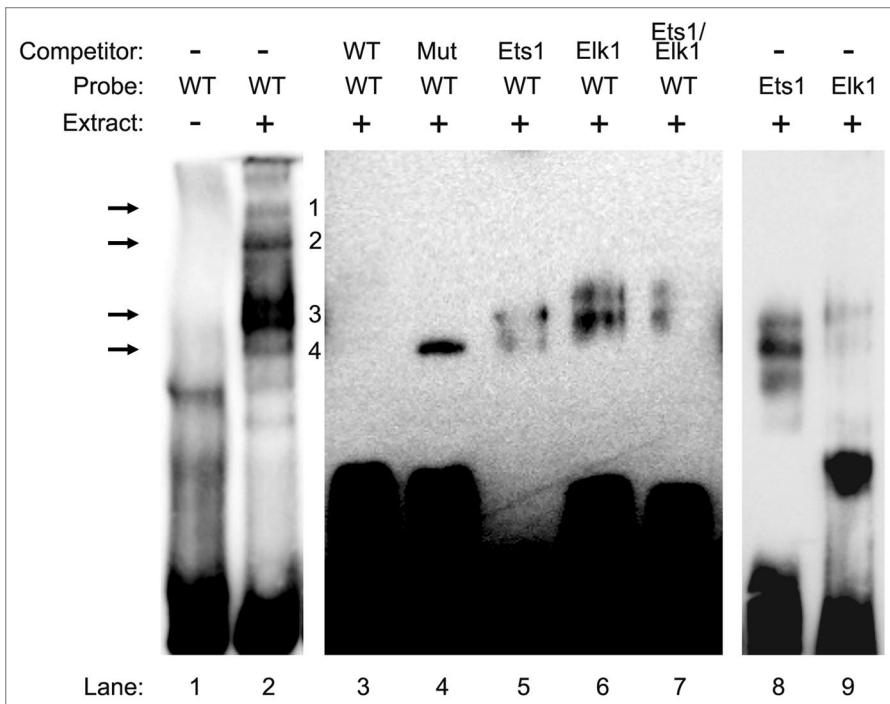


Figure 4. In vitro binding of Ets1 and Elk1 to the proximal promoter region of the *CIP2A* gene. Electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts (9 μ g) from ECC-1 cells that were incubated with the wild type (WT) probe (-138 to -107) from the human *CIP2A* gene as described in Materials and Methods. In competition experiments, a 100-fold molar excess of the designated probes were utilized to demonstrate the specificity of each binding reaction. Arrows indicate the formation of specific protein-DNA complexes. The experiment was repeated twice with similar results.

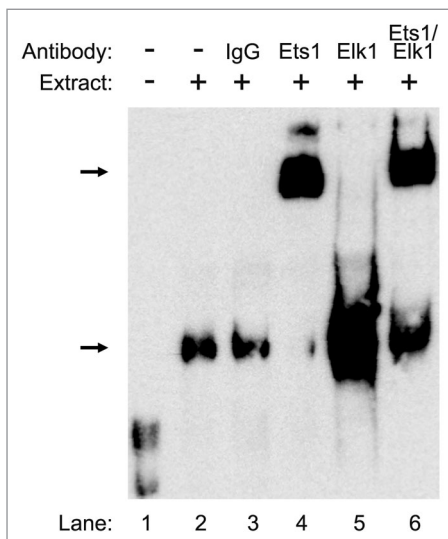


Figure 5. Confirmation of Ets1 and Elk1 binding to the proximal promoter of the *CIP2A* gene. Nuclear extracts (9 μ g) from ECC-1 were incubated with WT probe (-138 to -107) from the *CIP2A* gene along with 2 μ g of antibody directed toward Ets1, Elk1, or Ets1 and Elk1 together. The negative control consisted of rabbit-pre-immune control IgG. The formation of DNA-protein complexes is designated by solid arrows. The experiment was repeated twice with similar results.

cell lines. We provide evidence that the GGAA binding transcription factors, Ets1 and Elk1, together are involved in the basal regulation of *CIP2A* expression in human cervical and endometrial carcinoma cells.

Our work shows that the minimal proximal promoter of *CIP2A* lies between -123/+70 (Fig. 2). Both Ets1 and Elk1 bind to this palindromic region in order to regulate *CIP2A* expression (Fig. 3B and C). Previously, a head-to-head Ets1 binding site separated by 4 bp in the *stromelysin-1* gene was identified to be essential in basal transcription of the promoter.²⁵ Moreover, the *p53* promoter is regulated by Ets1 binding to palindromic Ets-binding sites.²⁶ The presence of palindromic Ets1 binding sites enhances an association between Ets1/Ets1 homodimers by creating a stable interaction in the DNA binding domain, which overcomes the auto-inhibitory domain in the Ets-DNA binding region.²⁷ The Ets-DNA binding domain is flanked by N- and C-terminal inhibitory regions that cause auto-inhibition and impair DNA binding.²⁸

We observed a decrease in *CIP2A* luciferase activity in ECC-1 cells when the Mut4 construct was expressed (Fig. 3C); this construct harbors a mutation in the Pax5 binding site. However, no significant decrease in *CIP2A* promoter activity was seen when compared with the minimal proximal promoter region (-123/+70, data not shown), which suggests that Pax5 is not the major transcription factor regulating *CIP2A* in ECC-1 cells. The decreased luciferase activity due to expression of *CIP2A* Mut4 might be due to cooperative interactions between Pax5 and Elk1 as characterized in earlier studies.²⁹ Previous work has demonstrated that Pax5 recruits Elk1 and Net transcription factors to form a functional ternary complex in the B-cell specific promoter. Structural studies with Pax5 and Ets1 have shown a change in the Ets1 nucleotide binding motif from GGAA to GGAG upon interaction with Pax5.^{30,31} Additionally, a recent observation by Khanna et al.³² has described mutations in the overlapping Ets1 binding sites within the *CIP2A* gene promoter, which result in decreased promoter activity in human gastric adenocarcinoma (AG1478) cells. Thus, it is possible that different regions of the *CIP2A* gene regulate basal transcription in different cell types.

The importance of Ets1 and Elk1 in controlling the basal transcription of *CIP2A* gene was obtained using EMSA (Fig. 4) and gel super-shift (Fig. 5) assays. Interestingly, we did not detect a shift when incubating nuclear extracts with the anti-Elk1 antibody; rather, we detected an increase in the intensity of the protein-DNA complex (Fig. 5, lane 5). The increase in intensity also indicates a positive interaction of the antibody with protein-DNA complex, as previous observations by Kurachi et al.³³ have

shown a similar increase in protein-DNA complex in the presence of anti-Ets1 antibody. It could be possible that a small shift in the presence of anti-Elk1, as observed by Yang et al.,⁵⁴ was masked by an increased protein-DNA interaction. These results confirm the binding of Ets1 and Elk1 transcription factor(s) in the region between -138 and -107 of the *CIP2A* gene promoter in endometrial carcinoma cells.

Using ChIP analysis, we showed that Ets1 and Elk1 bind to the *CIP2A* promoter in HeLa and ECC-1 cells (Fig. 6B and C). We are the first to demonstrate that Elk1 regulates *CIP2A* expression, and to suggest that Elk1 and Ets1 coordinately regulate expression in human cervical and endometrial cancer cells. Based on these results, we believe that any attempt to therapeutically decrease *CIP2A* expression through inhibition of specific transcription factors will be guided by what type of cancer is being treated. In the case of cervical and endometrial cancers, two inhibitors may be required to decrease *CIP2A* expression efficiently.

We have shown that Ets1 and Elk1 together control the basal transcription of *CIP2A* (Fig. 7C and D) in female urogenital cancers. It has been observed that knockdown of the Ets1 transcription factor alone decreases *CIP2A* protein levels in human gastric (AG1478) and prostate (PC-3 and LNCaP) carcinoma cells.³² We confirmed these results in PC-3 cells (data not shown), suggesting that there is cell-type specific regulation of *CIP2A*. The siRNA rescue experiment (Fig. 8) further demonstrates the necessity for Ets1 and Elk1 together in cell-type specific regulation of the *CIP2A* gene. Thus, Ets1 and Elk1 are essential for basal transcription of *CIP2A* in endometrial and cervical carcinoma cell lines. The differences in our results from those of others can be explained by cell-type specific regulation of gene expression, such as previously reported for *AKR1C1* transcription. Sp1 was found to regulate *AKR1C1* transcription in human colon cancer cells resistant to methotrexate,³³ while the same gene was regulated by nuclear factor- κ B in human ovarian, liver and lung carcinoma cells.³⁴

The E-26-specific (Ets) family is comprised of 29 transcription factors involved in regulating and inhibiting the expression of various genes involved in several biological processes, such as apoptosis, development, differentiation, proliferation and transformation.³⁵⁻³⁷ The Ets DNA binding domain binds to the GGA(A/T) core motif referred to as the Ets binding site (EBS).³⁸ In the present study, the GGAA core binding motif was identified in the *CIP2A* promoter region in a palindromic orientation and found to be fully functional. Ets like kinase 1 (Elk1) is a ternary complex factor (TCF) initially identified to regulate the *c-fos* promoter in cooperation with serum response elements (SRE).^{39,40} The oligonucleotide consensus sequence CCG GAA GTR shows more affinity for Elk1 binding in association with a second transcription factor SRE, which binds to the consensus sequence CC(A/T)₆GG.^{41,42}

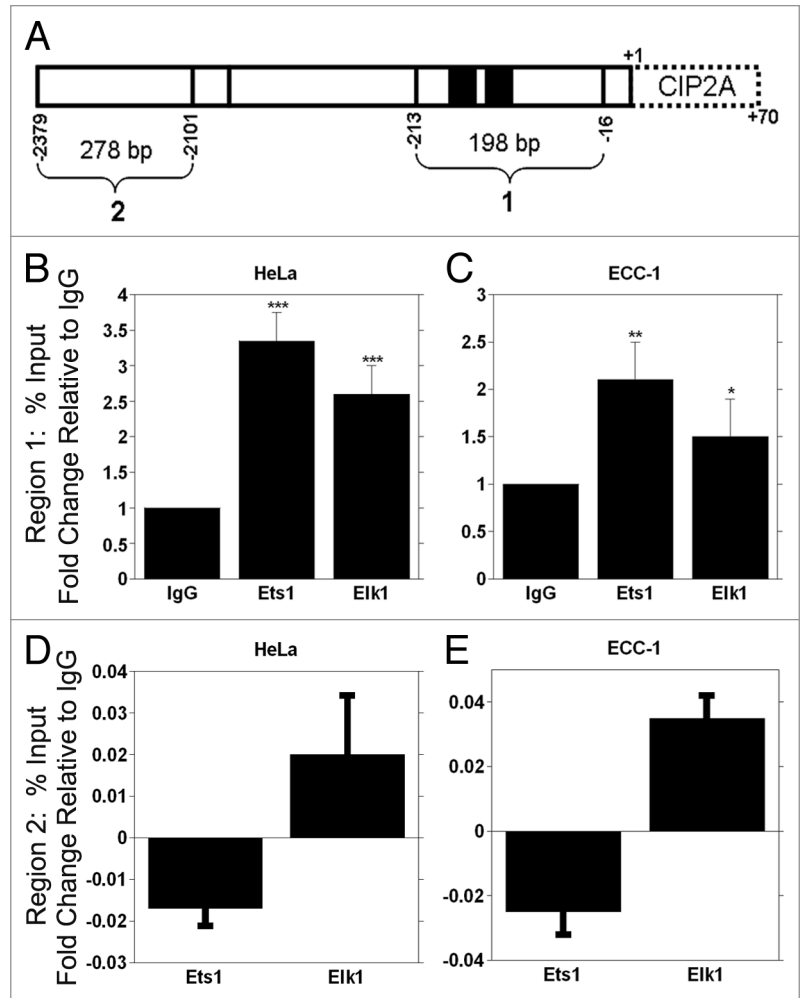


Figure 6. ChIP analysis of Ets1 and Elk1 binding to the *CIP2A* promoter. (A) Schematic representation of the Ets1 and Elk1 binding sites within the proximal promoter region. The primers utilized for amplification of DNA region are noted in Table 3. ChIP qPCR analysis of Ets1 and Elk1 association with the *CIP2A* promoter for region 1 in (B) HeLa and (C) ECC-1 and region 2 for (D) HeLa and (E) ECC-1 are shown. Mouse IgG serves as a negative control. Region 1 is specific for the region containing the Ets1 and Elk1 binding sites in the *CIP2A* promoter, while region 2 is a distal part of the *CIP2A* gene that is devoid of Ets1 and Elk1 binding sites. The fold enrichment from IgG, Ets1 and Elk1 immunoprecipitation are shown and were calculated relative to input as % input. The fold change in occupancy was calculated by setting the fold enrichment of IgG to 1. The results are from two different experiments, each experiment performed in duplicate (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ with Ets1, Elk1 compared with the control IgG).

Microarray analysis by Boros et al.^{43,44} identified a large group of promoters that function through Elk1 independently of the SRE binding region. We show, however, that the regulation of *CIP2A* by Elk1 is SRE-independent but does require Ets1 promoter binding.

Since the Ets families of proteins share the EBS binding motif, it is challenging to identify the specific transcription factor involved in gene regulation based solely on binding to this element. Moreover, the Ets family of proteins have a varied tissue distribution: Ets1, Erg, Fli-1, ETV4, Elf1, ESE-1 and PU.1 are found in lymphoid organs, brain, vascular endothelial cells,

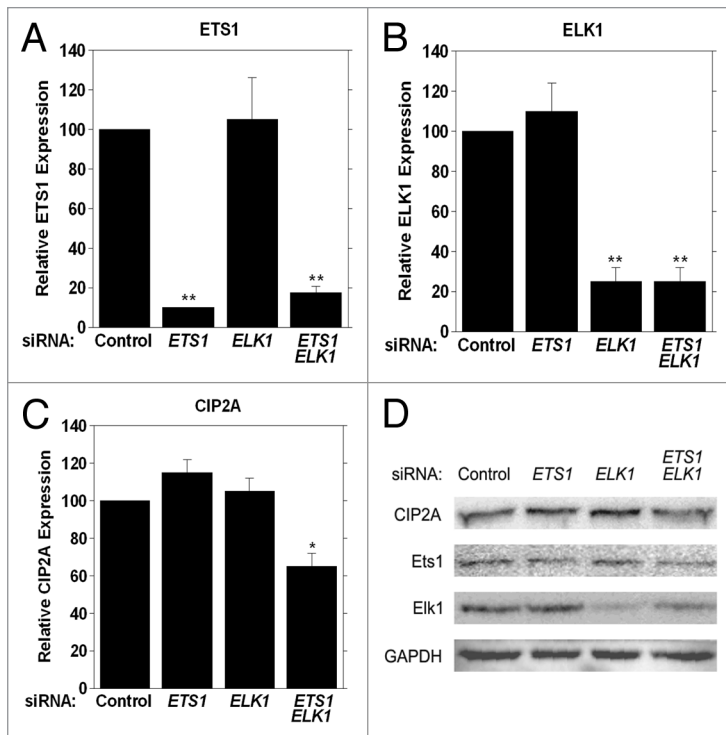


Figure 7. Functional analysis of Ets1 and Elk1 in regulating *CIP2A* transcription in HeLa cells. Cells were transiently transfected with 100 nM of *ETS1*, *ELK1*, or *ETS1* and *ELK1* siRNA together or non-targeting siRNA (Control) as the negative control and (A) *ETS1*, (B) *ELK1* and (C) *CIP2A* mRNA expression analyzed by qRT-PCR 24 h after transfection. (D) Western blot analysis of CIP2A, Ets1, Elk1 and GAPDH protein expression levels in HeLa cells transfected with scrambled siRNA or siRNA toward *ETS1*, *ELK1* or *ETS1* and *ELK1* together after 72 h.

hematopoietic cells, kidney, liver, epithelial cells, B cells, macrophages and neutrophils, while Ets2, GABP α , TEL and TCFs are found to be ubiquitous.³⁶ Based on our results and the data of others, it is evident that the complex distribution of Ets family members may play a role in regulating the tissue-specific expression of *CIP2A*. Microarray analysis demonstrated the possibility of overlapping promoter binding patterns for the Ets transcription factors Ets1, Elf1 and GABP α .⁴⁵ Similarly, our data demonstrate that both Ets family members, Ets1 and Elk1, cooperatively bind and are required for *CIP2A* expression in human cervical and endometrial carcinoma cells. Interestingly, Ets1 and Elk1 together have recently been identified to regulate the expression of *dipeptidyl-peptidase III (DPP-III)* gene, which is involved in ovarian cancer aggressivity.⁴⁶

CIP2A aids in the development of drug resistance in cancer cells. Recent reports have correlated the overexpression of *CIP2A* with development of doxorubicin resistance in breast cancer cells (MDA-MB-231)⁴⁷ and overexpression of *CIP2A* in hepatocellular carcinoma cells (HCC) PLC5 leads to resistance to bortezomib.⁴⁸ The development of drug resistance and increased *CIP2A* expression can be correlated with Ets1 expression levels, as previous reports have observed a direct relationship between Ets1 expression and cisplatin and gemcitabine resistance in human ovarian and pancreatic carcinoma cells.^{49,50} Our results show a correlation in the protein expression levels of *CIP2A*, Ets1 and

Elk1 in six matched pairs of human cervical tumor samples. Increased *CIP2A* protein correlated with an increase in the Elk1 and/or Ets1 expression levels (Fig. 9). Importantly, each tumor sample showed increased expression of either Ets1 or Elk1 alone or together. The combined identification of *CIP2A* expression and expression of transcription factors regulating *CIP2A* expression may prove to be a prognostic marker in human cervical carcinoma.

Materials and Methods

Cell culture. The human cervical carcinoma cell line HeLa was grown in DMEM supplemented with 10% fetal calf serum (Invitrogen, 16140071) and penicillin-streptomycin (Sigma, P433) at a final concentration of 100 μ g/ml. The hepatocellular carcinoma cells HepG2 were maintained in DMEM (Invitrogen, 11995065) with 10% FCS and gentamicin (Invitrogen, 15710064) at a final concentration of 10 μ g/ml. The endometrial carcinoma cells ECC-1 were grown in RPMI-1640 (Invitrogen, 11875093) supplemented with 5% FCS. All cells were grown at 37°C in a 5% CO₂ incubator.

Human cervical carcinoma tissue samples. Six matched pair of human cervical carcinoma tissue samples were purchased from ILS Bio, LLC (Chestertown, MD) and were analyzed for protein levels of *CIP2A*, Ets1, and Elk1. Sample descriptions are provided in Table 2.

Construction of *CIP2A* luciferase reporter vector and 5' deletion analysis. The BAC clone RP11-161J9 (Rosewell Parker Cancer Institute Human BAC library) that harbors the 5' flanking region of *CIP2A* gene (GenBank accession no AC092693.8) was utilized to design the primers for construction of *CIP2A* promoter-luciferase plasmids. DNA isolated from HeLa cells was used as templates to generate PCR fragments using Taq polymerase (Takara, RR350A), which were cloned into the reporter vector at the NheI-XhoI (New England Biolabs, R0131, R0146) poly-cloning sites by incorporating the corresponding restriction sites in the forward and reverse primers. The full length construct -2379/+70 luciferase promoter consisting of an approximately 2.4 kb region upstream and 70 bp downstream of the transcription start site (TSS) was cloned into the pGL4.10 [*luc2*] vector (Promega, E6651). PCR amplified promoter regions -1452/+70, -941/+70, -428/+70, -284/+70, -213/+70, -171/+70, -123/+70 and -95/+70 were cloned in NheI-XhoI sites of pGL4-basic vector. The nucleotide sequence of the clones was verified by sequencing.

Transient transfection and luciferase assay. Cells were seeded in 6-well plates at a density of 5x10⁵ cells/well for HeLa, HepG2 and 8x10⁵ for ECC-1 cells, 24 h before transfection. Transfection was performed utilizing Lipofectamine 2000 reagent (Invitrogen, 11668019) following the manufacturers' recommendations. In each experiment, 2 μ g of control vector (pGL4-basic without *CIP2A* promoter insert, empty vector) or the reporter vector (*CIP2A* full length promoter fragment or sequentially deleted *CIP2A* PCR fragments in pGL4-basic vector) was co-transfected along with 250 ng of pRL-TK (*Renilla* luciferase, Promega,

E2241) as an internal control. Following incubation with DNA for 4 h cells were feed with 2 mL of fresh growth medium for an additional 44 h. Luciferase assay was performed as described by Pallai et al.³⁴ utilizing a 384 well robotic plate reader (Envision, Perkin Elmer).

Identification of potential putative transcription factor binding sites in *CIP2A* gene promoter. Potential transcription factor binding sites within the *CIP2A* gene promoter were screened using ALGEN-PROMO^{51,52} or ALIBABA 2.0 programs (www.gene-regulation.com).

Site-directed mutagenesis. The -171/+70 *CIP2A* proximal promoter fragment was used to generate mutant clones of the *CIP2A* promoter. The Quickchange lightning site-directed mutagenesis kit (Stratagene, 210518) was utilized to generate mutants *CIP2A* Mut1-Mut6. Primers for introduction of point mutations or deletions were designed as instructed by the manufacturer. The nucleotide sequence of the mutated clones was verified by sequencing. The promoter activity of the mutated clones was assayed by transient transfection and luciferase assay as described above.

Electrophoretic mobility shift assay (EMSA) and gel supershift. Nuclear extracts were prepared from ECC-1 cells. 1x10⁷ cells were seeded in 75 cm² flasks 24 h before nuclear proteins were extracted utilizing the nuclear complex CO-IP kit (Active Motif, 54001) as instructed by the manufacturer. The wild type and mutant probes were synthesized as double stranded oligonucleotides (Integrated DNA technology) from the -138 to -107 region of the *CIP2A* gene promoter. Consensus oligonucleotides for Ets1 and Elk1 were synthesized based on the sequence data from Santa Cruz Biotechnology (Santa Cruz, CA) and Panomics (Affymetrix, CA). The sequences of the probes utilized were: WT -5'-GAC TTC CGG AGC CCG ACC GGA TCC GGA AGC TT-3'; Mutant -5'-GAA **AAT TTA** AGC CCG ACC GGA **TAA ATT TAC** TT-3' (mutated bases shown in bold text); Ets1 -5'-GAT CTC GAG CAG GAA GTT CGA-3'; Elk1 -5'-TTT GCA AAA TGC AGG AAT TGT TTT CAC AGT-3'. All the probes were labeled with biotin using the Biotin 3'-end DNA labeling kit (Thermo Scientific, 89818) according to the manual. Nine micrograms of the nuclear extract was utilized for the binding reactions. The EMSA binding reactions were performed at room temperature for 30 min and consisted of the nuclear extract in 1X binding buffer [50% glycerol, 100 mM MgCl₂, 1 µg/µl Poly (dI-dC), 1% NP-40, 1 M KCl, 200 mM EDTA] and 5 µM DNA probe. The mixture was run on 8% non-denaturing polyacrylamide gels in 0.5 × Tris Borate-EDTA buffer at 170 V. The protein-DNA complexes were then transferred to Hybond-N+ nylon membrane using the Trans-Blot semi-dry method (Bio-Rad, CA) and cross-linked using the Spectrolinker XL-1000 UV cross-linker (Spectronics Corporation, NY). Detection of biotin-labeled DNA was performed using the LightShift chemiluminescent EMSA kit (Thermo Scientific, 20148) and visualized by exposure to a charge-couple device camera (GE ImageQuant LAS 4000).

For competition EMSA, 100-fold molar excess of the cold, mutant or consensus oligonucleotides were added to the EMSA binding reaction. For the gel-Supershift assay, following the

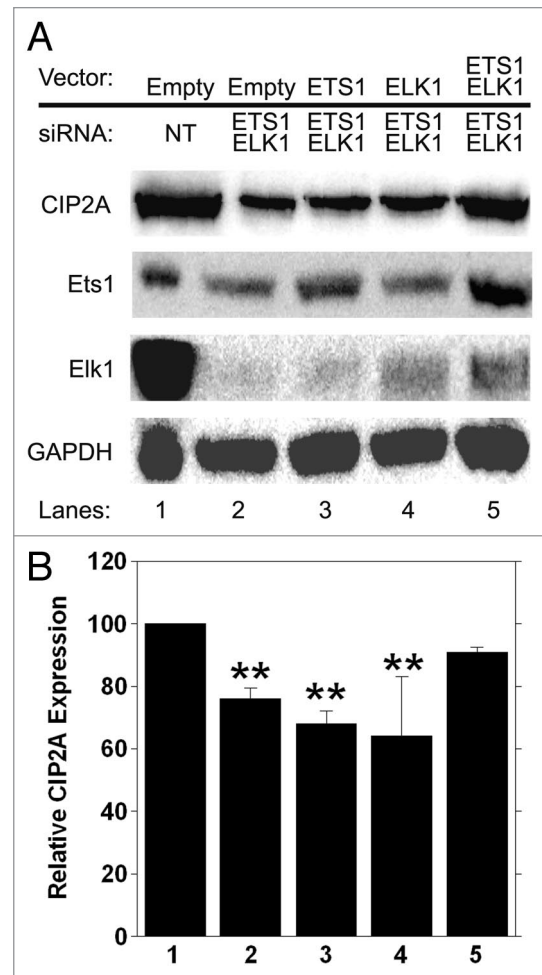


Figure 8. Ectopic expression of *ETS1* and *ELK1* together rescues *CIP2A* expression from 3' UTR siRNA treatment. HeLa cells were transiently transfected with 100 nM of *ETS1* and *ELK1* 3' UTR siRNA together or non-targeting (NT) siRNA as the negative control. *ETS1* and *ELK1* cDNA were cloned into pCDN4-His Max Topo expression vector (Invitrogen, K864-20) utilizing the primers mentioned in Table 4, generating Ets1-Topo and Elk1-Topo. Sequences of the clones were verified and 1 µg was utilized for ectopic expression in HeLa cells following 3' UTR siRNA treatment. Cells transfected with the empty vector served as a negative control. (A) Western blot analysis of CIP2A, Ets1, Elk1 and GAPDH protein expression levels was performed 72 h after transfection and (B) qRT-PCR was conducted at 48 h after transfection to confirm rescue of *CIP2A* mRNA. **p < 0.01.

incubation of the nuclear extracts with the 32 bp WT *CIP2A* promoter fragment for 30 min, 5 µg of Ets1 antibody (Abcam, ab124282), 5 µg of Elk1 antibody (Epitomics, 1277-1) and/or the two antibodies (anti-Ets1 and Elk1) were added to the binding reaction and the mixture incubated at room temperature for an additional 30 min. The pre-immune IgG (Millipore, 12-371) was utilized as negative control in the Supershift assay. The mixture was fractionated on 5% non-denaturing polyacrylamide gel. Transfer and detection was performed as described above.

Ets1/Elk1 siRNA knockdown, siRNA rescue and *CIP2A* expression analysis. Human siRNA specific for Ets1 (Dharmacon, J-003887-06, J-003887-08), Elk1 (Dharmacon,

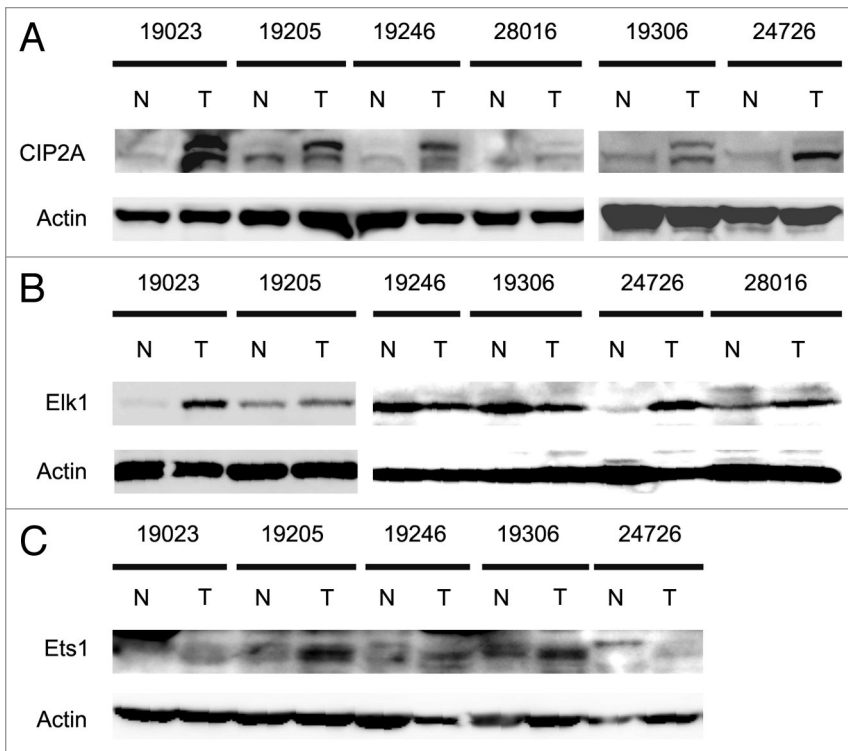


Figure 9. Analysis of CIP2A, Ets1 and Elk1 protein expression levels in human cervical tumor samples. Six different matched pairs of human cervical tumor samples with adjacent normal tissue were obtained and protein extracted as mentioned in Materials and Methods. Patient samples are described in **Table 2**. The expression levels of **(A)** CIP2A, **(B)** Elk1, **(C)** Ets1 and Actin (as loading control) protein were observed by western blotting.

Table 2. Pathological characteristics of human cervical carcinoma tissue samples

Identification no.	Cervical cancer phenotype	Donors race
19203 N		Georgian
19203 D	Undifferentiated Carcinoma Grade III	Georgian
19306 N		Georgian
19306 D	Adenosquamous Cell Carcinoma Grade II	Georgian
19205 N		Georgian
19205 D	Squamous Cell Carcinoma (SSC) Grade II	Georgian
19246 N		Georgian
19246 D	Squamous Cell Carcinoma (SSC) Grade II	Georgian
24726 N		Vietnamese
24726 D	Adenocarcinoma Grade III	Vietnamese
28016 N		Vietnamese
28016 D	Adenocarcinoma Grade III	Vietnamese

J-003885-06, J-003885-08) or Ets1/Elk1 together were utilized. siGENOME non-targeting siRNA pool 1 (Dharmacon,

D-001206-13) was utilized as negative control. The human cervical carcinoma cell line HeLa were seeded at a density of 5×10^5 cells/well and were transiently transfected with 100 nM of each of the targeted siRNA or in combination utilizing Lipofectamine 2000 (Invitrogen). RNA and protein were isolated 24 h and 72 h after transfection for CIP2A expression levels.

One microgram of the RNA was used in the reverse transcription reaction along with 4 U of Omniscript reverse transcriptase (Qiagen, 205110), 1 μ M oligo-dT primer (Qiagen, 79237), 0.5 mM dNTP (Qiagen, 205110), 10 U of RNase inhibitor (Qiagen, 129916) and 1X RT buffer (Qiagen, 205110). Reverse transcription was performed at 37°C for 1 h with a final incubation at 93°C for 5 min for inactivation of reverse transcriptase. Two microliters of the product was used in the real-time PCR reaction. The Quantitect SYBR green PCR kit (Qiagen, 204163) was utilized and PCR was performed according to the manufacturer's instructions using the Stratagene MxPro 3000 real time RT cyler (Agilent Technologies). Each PCR reaction consisted of 50% (v/v) of 2 X SYBR green master mixes and 0.2 μ M gene-specific forward and reverse primers. Quantification of glyceraldehyde-6-phosphate dehydrogenase was used to normalize the relative expression levels of *CIP2A*, *ETS1* and *ELK1* mRNA. Each experiment was performed

in duplicates and repeated at least twice.

For the siRNA rescue experiment, 5×10^5 HeLa cells seeded in six well plates were transiently transfected with *ETS1* 3'-UTR siRNA and *ELK1* 3'-UTR siRNA (Dharmacon). siGENOME non-targeting siRNA pool 1 (Dharmacon) was utilized as the negative control. Transfections were performed with 100 nM of the targeted 3'-UTR siRNA in combination or the non-targeting pool with Lipofectamine RNAiMax (Invitrogen) as described by the manufacturer. Forty-eight hours after 3'-UTR siRNA treatment, cells were transiently transfected with 1 μ g of Ets1-Topo and Elk1-Topo mammalian expression vector encoding respective cDNAs utilizing Lipofectamine RNAiMax (Invitrogen). HeLa cells transfected with empty vector served as a negative control. Protein was extracted 72 h after transfection for CIP2A analysis. Rabbit anti-CIP2A (Novus, NB100-68264), rabbit anti-Ets1 (Abcam, ab124282), rabbit-anti-Elk1 (Epitomics, 1277-1) and rabbit anti-GAPDH (Abcam, ab9385) were utilized for western blotting.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation was performed using $1-2 \times 10^7$ HeLa or ECC-1 cells, which were treated with 37% formaldehyde (Sigma, F1635) at 1% final concentration, v/v for 10 min at room temperature to cross-link proteins to DNA. After cross-linking the cells were washed twice with 1X ice-cold PBS containing protease inhibitor cocktail (Sigma, P8340). The cells were collected and centrifuged at

Table 3. Primers utilized for chromatin immunoprecipitation

Sequence no.	Region spanning <i>CIP2A</i> promoter	Sequence
1	-16 to -213 (within the <i>CIP2A</i> proximal promoter, which has the Ets1, Elk1 palindromic binding sites)	Forward: TCC TGG ACC CAC AAA TCA CCT Reverse: CCG GCT TAG GGA CCA CCA C
2	-2101 to -2379 (distal region in the <i>CIP2A</i> promoter, which is void of the Ets1, Elk1 binding sites)	Forward: AAA CTG GAA ATT AAA AGC GTG AGC Reverse: TGC CAT CTT TGT TGG ATT TTG ACT TA

Table 4. Primers utilized for construction of Ets1, Elk1 mammalian expression vectors

Sequence no.	Clone	Primer sequence
1	Ets1-Topo	Forward: ATG AGC TAC TTT GTG GAT TCT GCT G Reverse: TCA CTC GTC GGC ATC TGG CTT GAC GTC CAG
2	Elk1-Topo	Forward: ATG GAC CCA TCT GTG Reverse: TCA TGG CTT CTG GGG CCC TGG GGA GAG CAC

700 × g for 2 min, resuspended in 1 ml of SDS-lysis buffer with protease inhibitor cocktail. The cells were then sonicated twice with a bioruptor (Diagenode, UCD200) at high power, with 30 sec on/off pulse for 15 min. The cell lysate was centrifuged at 10,000 × g for 15 min at 4°C and the supernatant was further subjected to enzymatic digestion utilizing micrococcal nuclease (New England Biolabs, M0247) for 15 min at 37°C. The enzyme activity was inactivated by adding 0.5 M EDTA and incubated on ice for 10 min. Twenty-five microliters of the DNA fraction was kept aside as input for PCR. The remaining DNA fraction was pre-cleared using a mixture of 35 μl of protein G and protein A agarose beads each (50% slurry, Millipore, 16-201, 16-157) for 2 h at 4°C. Immunoprecipitation was performed by adding antibodies toward Ets1 (Abcam, ab124282), Elk1 (Epitomics, 1277-1) or mouse IgG (Millipore, 12-371) as the negative control. The immunocomplex was precipitated by incubation with 70 μl of protein A/G agarose beads for 2 h at 4°C. The protein/DNA complex was eluted using 200 μl of elution buffer (1% SDS, 0.1 M NaHCO₃) from the beads. Cross-linking of protein-DNA was reversed by adding 10 μl of 5 M NaCl at 65°C for 2–3h. The DNA was purified using spin columns (Promega, A9281) and 5 μl of the DNA was used in the qPCR reaction for amplification of 198 bp or 298 bp of the *CIP2A* promoter region (Fig. 5A). qPCR reactions were performed utilizing the primers in Table 3.

Western Blot analysis. Protein was isolated from cells by washing the cells in 1X ice-cold PBS and resuspending in 250 μl of RIPA buffer supplemented with protease inhibitor cocktail (Sigma, P8340). The cells were then sonicated with the bioruptor (Diagenode, UCD200) at high power, with 30 sec on/off pulse for 10 min. The cell lysate was centrifuged at 10,000 × g

for 30 min at 4°C and 50 μg of protein was fractionated on 10% or 12% SDS-PAGE and transferred to a 0.45 μm nitrocellulose membrane, blocked at 4°C overnight in 5% non-fat milk in TBS and incubated with rabbit anti-CIP2A (Novus, NB100-68264), rabbit anti-Elk1 (Novus, NB110-56953), rabbit anti-Ets1 (Novus, NBL1-10355) and rabbit anti-GAPDH (Abcam, ab9485) at 4°C overnight. Blots were then washed with 1X TBST and incubated with anti-rabbit secondary HRP at room temperature for 1 h. Detection of signal was performed by adding chemiluminescent substrate (Millipore, WBKLS0500) and visualized by exposure to a charge-couple device camera (GE ImageQuant LAS 4000).

Statistical analysis. Statistical analysis was performed for calculating the significant differences in luciferase activity between constructs, effect of siRNA in knock-down of *CIP2A* mRNA expression and effect of Ets1, Elk1 knock-down in *CIP2A* mRNA by one way randomized analysis of variance (ANOVA) and Newman-Keuls test with significance level of $p < 0.05$.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest.

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