ARTICLE
Journal of Cellular Biochemistry 105:236–244 (2008)

Journal of Cellular Biochemistry

E1A Physically Interacts With RUNX3 and Inhibits Its Transactivation Activity

Eun-Jeong Cha,[1] Byung-Chul Oh,[2] Hee-Jun Wee,[1] Xin-Zi Chi,[1] Yun-Mi Goh,[1] Kyeong-Sook Lee,[1] Yoshiaki Ito,[3] and Suk-Chul Bae[1*]

[1] *Department of Biochemistry, School of Medicine, Institute for Tumor Research, Chungbuk National University, Cheongju 361-763, South Korea*

[2] *Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, 7-45 Songdo-dong, Yeonsu-ku, Inchon 406-840, South Korea*

[3] *Institute of Molecular and Cell Biology, 61 Biopolis Drive, Proteos, Singapore 138673, Singapore*

ABSTRACT

The adenoviral gene, termed *early region 1A* (*E1A*), is crucial for transformation and has been used very effectively as a tool to determine the molecular mechanisms that underlie the basis of cellular transformation. pRb, p107, p130, p300/CBP, p400, TRRAP, and CtBP were identified to be E1A-binding proteins and their roles in cellular transformation have been established. Although the major function of E1A is considered to be the regulation of gene expression that is critical for differentiation and cell cycle exit, one of the most significant questions relating to E1A transformation is how E1A mediates this regulation. RUNX3 is a transcription factor that was first described as a gastric cancer tumor suppressor but is now known to be involved in many different cancers. Exogenous expression of *RUNX3* strongly inhibits the growth of cells. Here, we show that the adenovirus oncoprotein E1A interacts with RUNX3 in vitro and in vivo. RUNX3 interacts with the N-terminus (amino acids 2-29) of E1A, which is known to interact with p300/CBP, p400, and TRRAP. E1A interacts directly with the Runt domain of RUNX3 but does not interfere with CBFβ-RUNX3 interactions. In addition, E1A inhibits the transactivation activity of RUNX3 on the $p21^{WAF1/CIP1}$ promoter. Consistent with these observations, the growth inhibition induced by RUNX3 is reduced by E1A. These results demonstrate that E1A specifically binds to RUNX3 and inactivates its transactivation activity. We propose that one of the mechanisms for the oncogenic activity of E1A is the inhibition of RUNX3, similar to that of RB and p300/CBP. J. Cell. Biochem. 105: 236–244, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: RUNX3; E1A; $p21^{WAF1/CIP1}$; TUMOR SUPPRESSOR

Analyses of the molecular mechanisms regulating the oncogenic activities of DNA tumor viruses have contributed enormously to our current understanding of the biology of cancer. Studies on the adenoviral gene, termed *early region 1A* (*E1A*), are one example of this. *E1A* is crucial for transformation [Avvakumov et al., 2002]. The prototype of the E1A gene (HAdV-5 E1A) encodes two major proteins of 289 (E1A13s) and 243 (E1A12s) residues, which arise from alternative splicing of the same transcript and differ only by a 46 amino acid internal sequence. Sequence alignments of the largest E1A proteins from several adenovirus serotypes highlighted four regions of conservation, designated conserved regions (CR) 1–4 [Avvakumov et al., 2002], which are important for many of E1A's biological activities. A number of E1A-binding proteins including pRb, p107, p130, p300/CBP, p400, TRRAP, and CtBP were identified and their roles in cellular transformation have been established [Turnell and Mymryk, 2006].

The function of E1A can be considered to be largely, if not completely, at the transcriptional level. E1A is responsible for activating viral gene transcription and reprogramming host cell gene expression by forcing quiescent cells to re-enter the cell cycle thereby blocking cell differentiation [Frisch and Mymryk, 2002]. One of the most significant questions relating to E1A transformation is how E1A contributes to S phase entry. Indeed, S-phase entry has been studied intensely with respect to the E1A-binding proteins pRb and p300/CBP because of their implications in oncogenesis.

pRb, the product of the retinoblastoma susceptibility (RB) gene, was the first E1A-binding protein to be identified [Whyte et al., 1988]. pRb negatively regulates the G1-S boundary of the cell cycle

Grant sponsor: Korea Science and Engineering Foundation; Grant number: R16-2003-002-01001-02006.
*Correspondence to: Dr. Suk-Chul Bae, Department of Biochemistry, School of Medicine, Institute for Tumor Research, Chungbuk National University, Cheongju 361-763, South Korea. E-mail: scbae@chungbuk.ac.kr
Received 11 April 2008; Accepted 14 April 2008 • DOI 10.1002/jcb.21818 • 2008 Wiley-Liss, Inc.
Published online 20 June 2008 in Wiley InterScience (www.interscience.wiley.com).

and this property is considered to be the basis of the tumor suppressor activity of pRb. By binding to pRb, E1A dissociates pRb-E2F complexes which stimulate E2F-dependent transcription of a number of S-phase-specific genes. This displacement of the Rb protein from E2Fs provides a partial explanation for how E1A drives G_0 cells into S phase [Frisch and Mymryk, 2002; Berk, 2005].

p300 was first identified as the E1A binding protein [Stein et al., 1990]. It was later found that E1A also binds the highly related CBP (cyclic AMP response element-binding protein) [Arany et al., 1995]. p300 and CBP contain histone acetylatransferase activity [Bannister and Kouzarides, 1996; Ogryzko et al., 1996] and are though to function primarily as transcription coactivators for a number of nuclear proteins. These include known oncoproteins (such as myb, jun, fos) and tumor suppressor proteins (such as p53, Rb, SMADs, RUNXs and BRCA1) [Iyer et al., 2004]. Sequestration of p300/CBP by E1A has the general effect of repressing transcription by any factor that utilizes these coactivators [Gallimore and Turnell, 2001].

TGF-β prevents the E2F-mediated activation of S-phase specific gene expression by inducing the cyclin-dependent cell cycle inhibitors, *p15*^{INK4B} and *p21*$^{WAF1/CIP1}$, and keeping Rb hypophosphorylated. It has been demonstrated that E1A inhibits TGF-β-dependent induction of the cyclin-dependent cell cycle inhibitors and thereby overcomes the growth-inhibitory effect of TGF-β [Datto et al., 1997]. This activity is independent of Rb binding but appears to be associated with p300 binding with since the N-terminal region of E1A (aa 2–36) is required for the effect.

The RUNX family of transcription factors plays critical roles in cell specification during development and in neoplastic transformation. In mammals, the RUNX gene family consists of *RUNX1/AML1*, *RUNX2*, and *RUNX3* (see van Wijnen et al., 2004, for details regarding the nomenclature). All RUNX family members form a heterodimer with a shared partner, CBFβ, and that the well-conserved Runt domain in RUNX family members is important for the heterodimerization and sequence-specific DNA binding [Ogawa et al., 1993; Wang et al., 1993; Ito, 2004]. There are three isoforms of CBFβ (CBFβ1, CBFβ2, and CBFβ3) that result from alternative splicing. Of these, CBFβ2 is known to be the major isoform [Ogawa et al., 1993]. *RUNX1* and *RUNX2* are required for hematopoiesis and osteogenesis, respectively, and are genetically altered in leukemia [Look, 1997] and bone disease [Lee et al., 1997; Mundlos et al., 1997]. *RUNX3* is required for the development of CD8-lineage T cells [Taniuchi et al., 2002] and TrkC-dependent dorsal root ganglion neurons [Inoue et al., 2002; Levanon et al., 2002] and function as a tumor suppressor in gastric cancer [Li et al., 2002]. Inactivation of *RUNX3* is also associated with various cancers including lung, colon, pancreas, liver, prostate, bile duct, breast, larynx, esophagus, endometrium, uterine cervix, testicular yolk sac and bladder [Kim et al., 2005]. Recently, *RUNX3* was identified as one of the five most informative genes for the CpG island methylator phenotype of colorectal cancer [Weisenberger et al., 2006].

The tumor suppressor activity of RUNX3 is associated with TGF-β signaling since primary gastric epithelial cells isolated from *Runx3*-deficient ($Runx3^{-/-}$) mice were less sensitive to the growth inhibitory effect and apoptosis-inducing activity of TGF-β [Li et al., 2002]. SMADs, the downstream mediators of TGF-β signaling, have been shown to physically interact with the RUNX proteins [Hanai et al., 1999]. The interaction between RUNX3 and SMADs is required for TGF-β-induced transcriptional up-regulation of *p21* during growth arrest [Chi et al., 2005] and Bim [Yano et al., 2006] during apoptosis.

In this study we show that E1A binds directly to RUNX3 and inactivates its function. This is another example of E1A-mediated inactivation of a tumor suppressor and suggests that inactivation of RUNX3 contributes to the oncogenic activity of E1A by deregulating cell cycle control and perhaps by inhibiting the proapoptotic activity of RUNX3.

MATERIALS AND METHODS

PLASMIDS

The E1A expression plasmid (pLPCX-E1A12s) was a kind gift from S. W. Lowe (Cold Spring Harbor, NY). *E1A12s* (AAW65494) and its deletion mutants were engineered by PCR and cloned into the pCS4-HA vector as *Eco*RI/*Xho*I fragments. *RUNX3* and its deletion mutants were engineered by PCR and cloned into the pCS4-Myc vector as *Eco*RI/*Xho*I fragments. The pQE11-E1A12s, pACYC-RD (amino acids 64–190) and pET28a-CBFβ2 bacterial expression plasmids were constructed by subcloning cDNAs encoding E1A12s, the Runt domain of RUNX3 and the full-length human CBFβ2 into 6xHis tagging vectors, respectively. The reporter plasmids, pGL3-p21-2300 and pGL3-p21-2300-mut abcde were described by Chi et al. [2005].

CELL CULTURE AND TRANSFECTION

All cell lines (293T, BOSC, MCF-7, and HeLa) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO) and 100 units/ml penicillin–streptomysin. Transient transfections were carried out using the Lipofectamine Plus reagent (Invitrogen).

ESTABLISHMENT OF STABLE CELL LINES AND CELL NUMBER COUNTING

Control and E1A12s stable cell lines were generated by transfecting MCF7 cells with the pLPCX empty vector or the pLPCX-E1A12s plasmid using the Lipofectamine Plus reagent (Invitrogen). Stably transfected MCF7 cell lines were obtained by puromycin selection (0.4 μg/ml). E1A expression was confirmed by Western blot. MCF7 stable cell lines expressing adenoviral *E1A12s* (MCF7-E1A-cl1 and cl2) or the empty vector (MCF7-puro) were transiently transfected with the pCS4-Myc-RUNX3 plasmid or empty vector. After 20 h, the cells were plated in triplicate into 24-well plates (2×10^4 cells/well). Viable cells were stained with Trypan blue and counted 72 h after transfection using a Hemacytometer.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Cells were transfected using Lipofectamine, and 12 μg of protein extract from each transfection was subjected to SDS–PAGE, followed by immunoblot analysis using the appropriate antibodies. For immunoprecipitation experiments, BOSC cells were transfected using Lipofectamine and 400 μg of protein extract was incubated with an anti-HA (12CA5) antibody and precipitated with protein G beads as described previously [Chi et al., 2005]. The

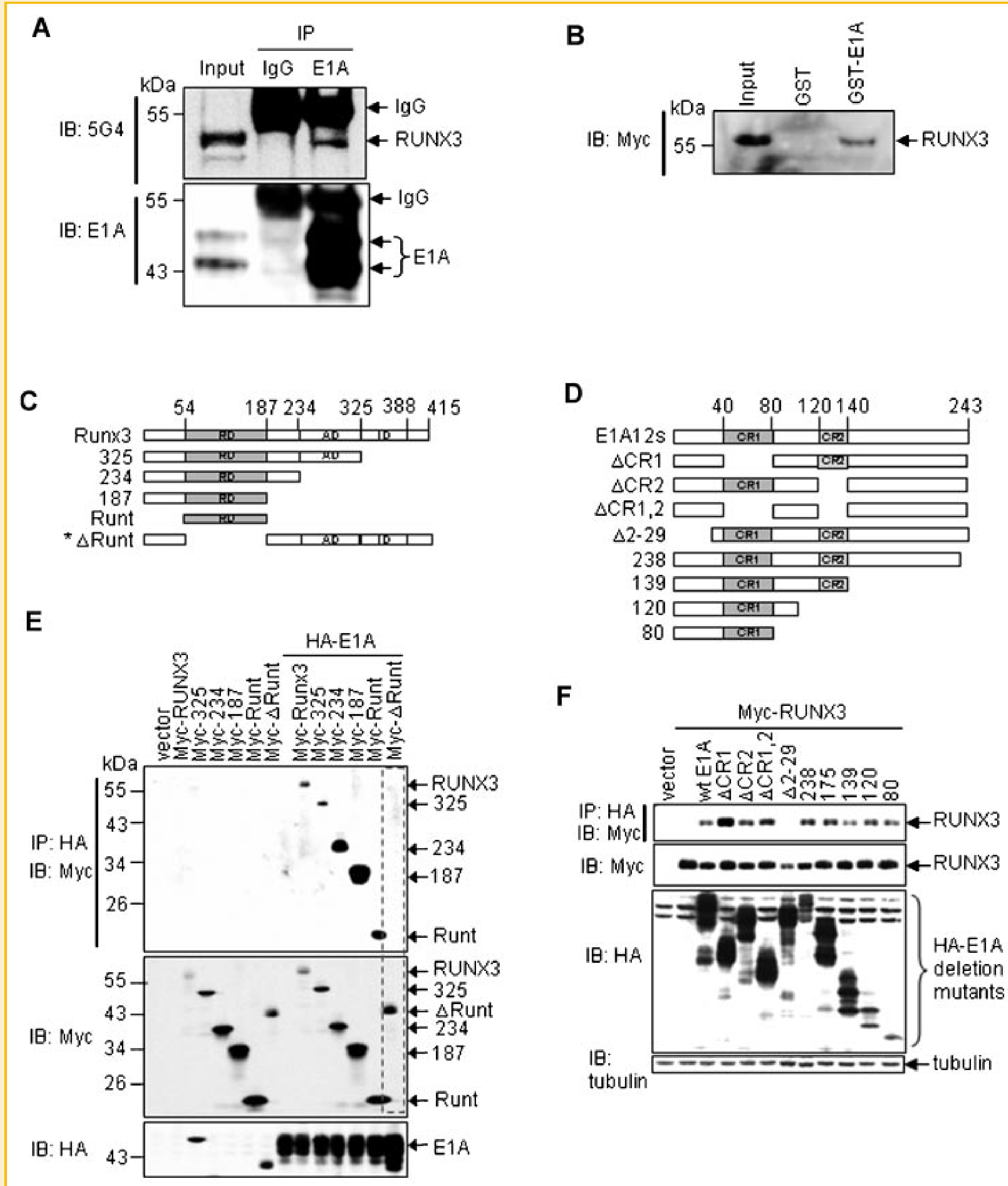

Fig. 1. In vivo interaction between RUNX3 and E1A. A: E1A immunoprecipitation. To examine the physical interaction between endogenous E1A and RUNX3, cell lysates were prepared from HEK293 cells and immunoprecipitated with an anti-E1A antibody or IgG as a control. The immunoprecipitates were analyzed by Western blotting with anti-RUNX3 (5G4) antibody or anti-E1A antibody. B: GST pull down assay. E1A12s fused to glutathione *S*-transferase (GST). Myc-tagged RUNX3 was obtained from transfected BOSC cells. RUNX3 was subjected to in vitro pull-down assays with full length E1A12s fused to GST. The bound complexes were analyzed by Western blotting using a 5G4 antibody. C: Schematic representation of the *Myc-RUNX3* constructs used for the immunoprecipitation studies. Runt indicates the Runt domain. D: Schematic representation of the *E1A* constructs used for immunoprecipitation studies. E: BOSC cells were transfected with a *Myc-RUNX3* construct and *HA-E1A12s* as indicated. Interactions between the RUNX3 deletion mutants and E1A were examined by immunoprecipitation with an anti-HA antibody followed by immunoblotting with anti-Myc and anti-E1A antibodies. E1A-RUNX3 interactions were detected for all RUNX3 constructs except for the one lacking the Runt domain (ΔRunt). F: BOSC cells were transfected with *Myc*-tagged full-length *RUNX3* and the *HA-E1A12s* constructs shown in (D) as indicated. Interactions were examined by immunoprecipitation with an anti-HA antibody followed by immunoblotting with an anti-Myc antibody. E1A-RUNX3 interactions were detected for all E1A constructs except for the Δ2-29 mutant.

immunoprecipitates were then separated by SDS–PAGE, transferred onto a membrane and probed with an appropriate antibody. For endogenous protein immunoprecipitation analysis, 1.5 mg of protein extract from HEK293 cell was used. After precipitation with protein G Sepharose beads, the precipitates were eluted by boiling in SDS-sample buffer and the proteins were resolved by SDS–PAGE. Immunoprecipitates were analyzed by Western blotting using anti-RUNX3 antibody (5G4).

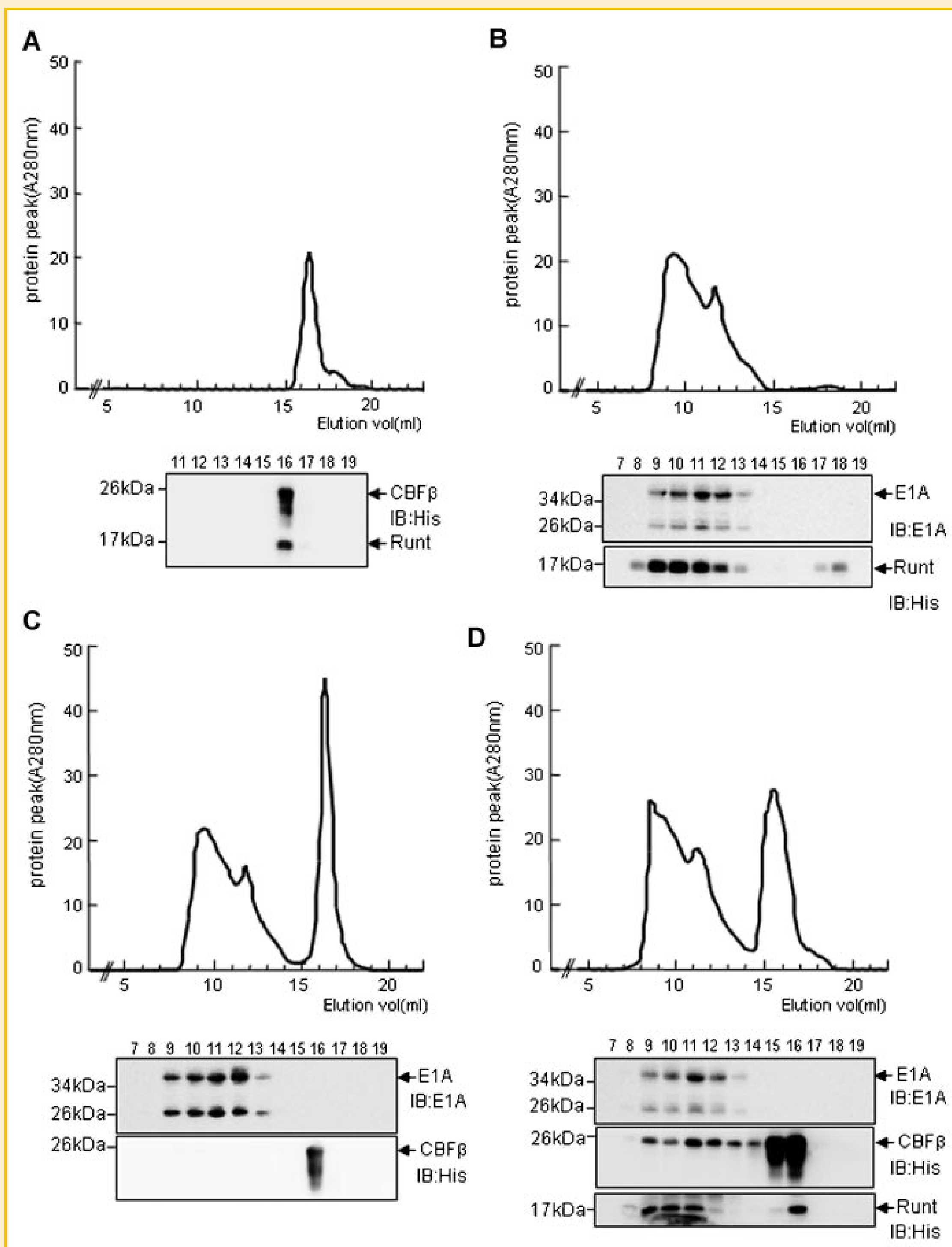

Fig. 2. Protein interactions between the Runt domain, CBFβ and E1A in vitro. The elution profiles of mixtures containing (A) the Runt domain and CBFβ2 (B) the Runt domain and E1A12s (C) CBFβ2 and E1A12s and (D) CBFβ2, E1A12s, and Runt. Below each elution profile, the immunoblot results of the relevant fractions are shown. Equal amounts of protein from each fraction were separated by SDS–PAGE, followed by immunoblotting with an anti-His or anti-E1A antibody. The numbers above the lanes indicate the relevant fraction number.

GST PULL DOWN ASSAY

GST fusion protein was expressed in Escherichia coli strain BL21 (DE3) and purified by the standard method. Glutathione-Sepharose beads loaded with the GST-E1A protein were incubated with lysates of Myc-Runx3 transfected BOCS cells for 4 h at 4°C. The cell lysate (1.5 mg) was used for the pull down assay. The beads were washed with lysis buffer and then eluted and analyzed by Western blotting using the anti-Myc antibody (9E10).

REPORTER ASSAYS

HCT116 or 293 cells were transiently transfected with the pGL3-p21-promoter-luciferase or 6×OSE-luciferase reporter plasmid [Chi et al., 2005] and plasmids that expressed *RUNX3*, *E1A12s*, or *E1A* deletion mutants. The pCMV-β-gal plasmid (Promega) was used as an internal control. Luciferase activity was measured after 42 hours using the Luciferase Reporter Assay Kit (Promega) with a Centro microplate luminometer LB 960 (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was normalized to the activity of β-galactosidase from pCMV-β-gal. Results were obtained from at least two independent experiments, each run was in triplicate.

GEL FILTRATION CHROMATOGRAPHY

The Runt domains of RUNX3, E1A-243R (E1A12s), and CBFβ2 were all purified from IPTG induced BL21 (DE3) strains that were transformed with the pACYC-Runt domain (amino acids 64–190) (Q13761), pQE11-E1A (AAW65494), and pET28a-CBFβ2 (Q13951), respectively. Proteins were further purified by gel filtration chromatography on a Superdex 200 column (FPLC system, Amersharm Pharmacia Biotech). After the purification of each protein, equal molar concentrations of the Runt domain, CBFβ2, and E1A were mixed in various combinations to test whether stable complexes were generated. After incubation for 1 h at 4°C, the protein mixtures were loaded onto a HiLoad Superdex 200 column pre-equilibrated with equilibration buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) at a flow rate of 1.0 ml/min. Proteins in the peak fractions were pooled and analyzed by SDS–PAGE and immunoblotting.

RESULTS

PHYSICAL INTERACTION BETWEEN RUNX PROTEINS AND E1A IN VIVO AND IN VITRO

To examine the physical interaction between RUNX3 and E1A in vivo, whole cell lysates were prepared from HEK293 cells and E1A protein was immunoprecipitated with anti-E1A monoclonal antibody. The HEK293 cell line is an immortalized cell line of primary human embryonic kidney cells transformed by human Adenovirus type 5 (Ad5) DNA and endogenously expresses *E1A* [Louis et al., 1997]. As shown in Figure 1A, the RUNX3 protein was detected in anti-E1A antibody precipitates. This result indicates that endogenous RUNX3 physically interacts with endogenous E1A in vivo.

To determine whether RUNX3 and E1A interact in vitro, we generated a glutathione *S*-transferase (GST)-E1A12s fusion protein. The GST-pull down analysis showed that RUNX3 directly associated with E1A12s in vitro (Fig. 1B).

To define the region of RUNX3 that is required for its interaction with E1A, coimmunoprecipitation experiments were performed with BOSC cells co-expressing E1A12s and full-length or deletion derivatives of RUNX3. Schematic representations of the RUNX3 deletion constructs and E1A deletion constructions are shown in Figure 1C,D. We found that the Runt domain of RUNX3 was sufficient for interaction with E1A12s (Fig. 1E, lane Myc-Runt). Additionally, deletion of this domain abolished the interaction (Fig. 1E, lane Myc-ΔRunt). The region of E1A responsible for its interaction with RUNX3 was also mapped in similar experiments by co-transfecting the cells with RUNX3 and E1A deletion constructs. We determined that the N-terminal domain of E1A (amino acids 2-29) is required for its interaction with RUNX3 (Fig. 1F, lane Δ2-29).

THE RUNT DOMAIN, CBFβ AND E1A INTERACT IN VITRO

In order to examine the interactions between the Runt domain, CBFβ2 and E1A12s in vitro, equal molar concentrations of each of the bacterially expressed proteins were mixed in various combinations and analyzed by gel filtration. The Runt domain (RD) alone eluted primarily in fractions 17–18 and CBFβ2 eluted in fraction 16 (data not shown). When a mixture containing the Runt domain and CBFβ2 was applied to the column, the two proteins co-eluted in fraction 16 (Fig. 2A). This result confirms previous work which showed that the Runt domain complexes with CBFβ2. Next, we mixed the Runt-domain (RD) with E1A12s and injected it into the column. E1A12s eluted in fractions 9–13 and the majority of the Runt domain co-eluted in the same fractions (Fig. 2B). This result indicates that the Runt domain directly interacts with E1A in vitro. When a mixture of E1A12s and CBFβ2 were applied to the column, the elution profile of each protein was completely separate,

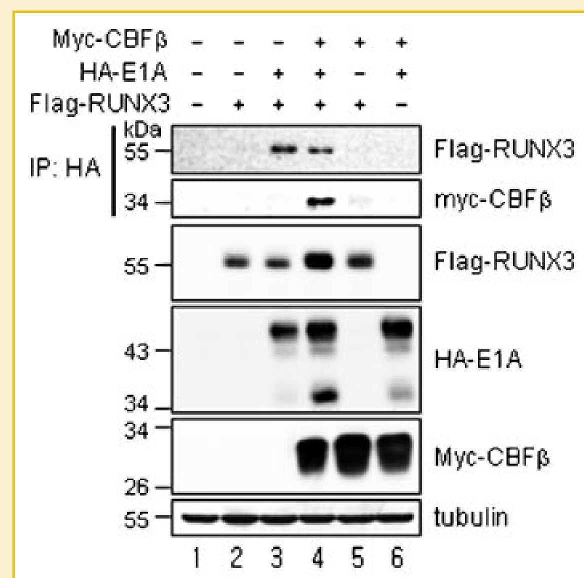

Fig. 3. In vivo interaction of RUNX3, CBFβ and E1A. BOSC cells were transfected with *Flag-RUNX3*, *HA-E1A12s* and *Myc-CBFβ2* as indicated and analyzed by immunoprecipitation with an anti-HA antibody. Cell extracts were also immunoblotted with an anti-Flag or anti-Myc antibody. The level of tubulin is shown as a loading control.

suggesting that E1A12s does not complex directly with CBFβ2 (Fig. 2C).

When the Runt domain, E1A12s and CBFβ2 were combined and applied to the column, all three proteins eluted in fractions 9–12, indicating that these proteins form a ternary complex. Although a large portion of CBFβ2 did not elute with E1A12s, it is noteworthy that under these conditions the elution profile of CBFβ2 shifted to the left (from 16 to 15–16) and that free CBFβ2 protein now eluted in fraction 15. From these results, we interpret that these three proteins make a ternary complex but this complex is not stable in vitro.

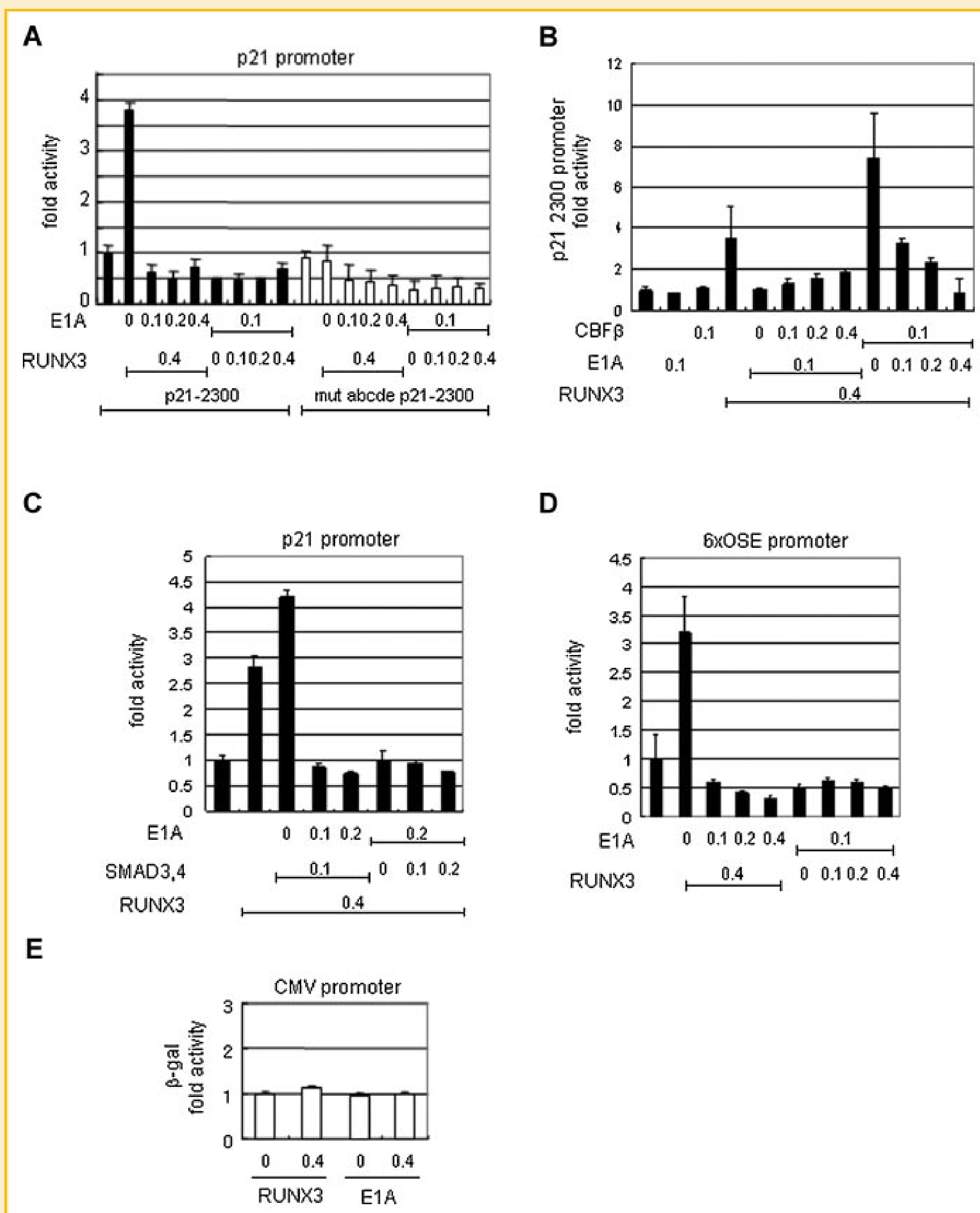

Fig. 4. E1A represses the transactivation activity of RUNX3. HCT116 cells were transfected with a *p21*-promoter reporter (pGL3-p21-2300-luciferase) or its Runt domain binding site mutant (pGL3-p21-2300-mut abcde) construct and various expression plasmids. Luciferase reporter activity was measured and normalized to that of the pCMV-β-gal control. Variable amounts of (A) *RUNX3* and *E1A12s*, (B) *RUNX3*, *E1A12s* and *CBFβ2*, and (C) *RUNX3*, *E1A12s*, *SMAD3* and *SMAD4* were expressed as indicated. D: The reporter was replaced by a 6xOSE-promoter reporter plasmid (pGL3-6xOSE-luciferase). E: pCMV-β-gal was cotransfected with *RUNX3* or *E1A12s* expression plasmids (0.4 μg) and β-gal activity was measured by ELISA. The data are representative of at least two independent series of triplicate experiments.

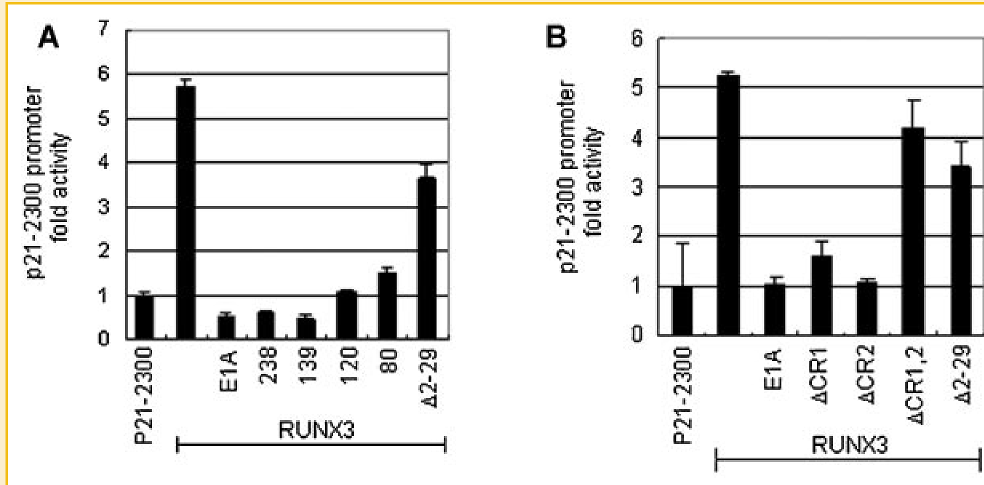

Fig. 5. Mapping of the inhibitory regions of E1A. A,B: HCT116 cells were transfected with pGL3-p21-2300-luciferase, pCS4-Myc-RUNX3 and deletion constructs of *E1A*. The luciferase reporter activities were measured and were normalized to a pCMV-β-gal transfection efficiency control.

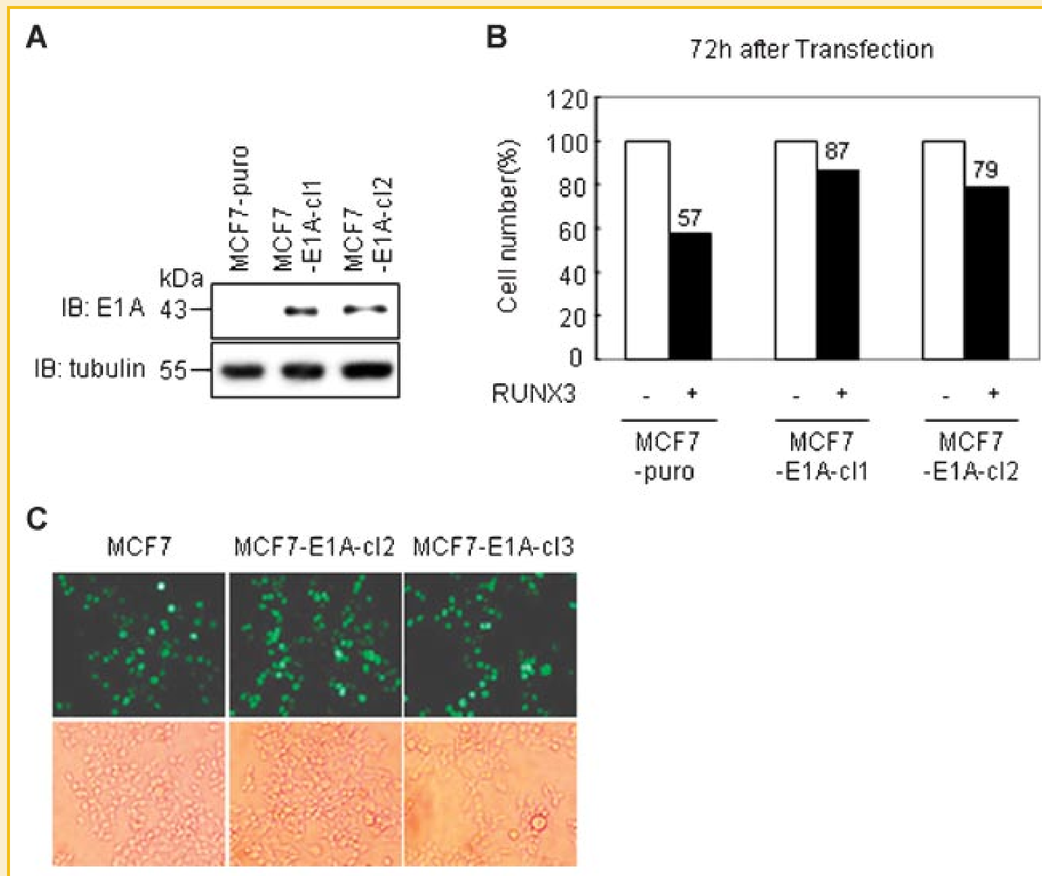

Fig. 6. *E1A* inhibits *RUNX3* activity. A: MCF7 cells were transfected with an *E1A* expression plasmid or a control empty vector. An MCF7-puro control cell line and *E1A* expressing MCF7-E1A-cl1 and MCF7-E1A-cl2 cell lines were generated by puromycin selection. Expression of *E1A* was detected by Western blotting using an anti-E1A antibody. B: MCF7-puro, MCF7-E1A-cl1 and MCF7-E1A-cl2 cells were transiently transfected with a *RUNX3* expressing plasmid and viable cells were counted. The ratios of transfected viable cells to untransfected cells are shown. C: The transfection efficiency was measured after the transient transfection of an *EGFP* expression plasmid. Upper panels: Fluorescence microscopy images of a representative area of the plate. Lower panels: phase contrast microscopy image.

THE RUNT DOMAIN, CBFβ2 AND E1A12S INTERACT IN VIVO

In order to examine whether the Runt domain, CBFβ and E1A, form a ternary complex in vivo, constructs encoding *Flag-RUNX3*, *HA-E1A12s*, and *Myc-CBFβ2* were expressed in BOSC cells. Protein interactions were determined by immunoprecipitation with an anti-HA antibody followed by Western blotting with an anti-Flag or anti-Myc antibody. Our results revealed that CBFβ2 did not coimmunoprecipitate with E1A12s in the absence of RUNX3 (Fig. 3, lane 6); however, it did coimmunoprecipitate with E1A in the presence of RUNX3 (Fig. 3, lane 4). These results demonstrate that E1A interacts with CBFβ2 through RUNX3 and suggests that RUNX3, CBFβ2 and E1A12s form a ternary complex in vivo.

E1A REPRESSES THE TRANSACTIVATION ACTIVITY OF RUNX3

E1A has been shown to abrogate many TGF-β-mediated gene responses and TGF-β-induced growth inhibition [Pietenpol et al., 1990]. We have shown previously that *RUNX3* is required for TGFβ-dependent *p21* induction in gastric epithelium [Chi et al., 2005]. We therefore investigated whether E1A influenced the transactivation activity of RUNX3 on the *p21* promoter in HCT116 cells which are deficient of p300. Interestingly, we found that RUNX3-dependent *p21* promoter transactivation was strongly inhibited by E1A12s (Fig. 4A). A previous study has reported that CBFβ and Smads enhance the transactivation activity of RUNX3 [Chi et al., 2005]. Our results show that E1A12s effectively inhibits RUNX3-mediated transactivation of the *p21* promoter even in the presence of an excess amount of CBFβ2 (Fig. 4B) or SMAD3/4 (Fig. 4C). To rule out the possibility that E1A inhibits p21-reporter activity by inactivating unknown factors, we performed similar reporter assays using the pGL3-6xOSE-luciferase reporter plasmid which contains 6 tandem repeats of the RUNX binding sequence. The result revealed that RUNX3-mediated 6×OSE-luciferase reporter activity was also inhibited by E1A12s (Fig. 4D). However, CMV promoter-driven gene expression was not affected by RUNX3 or E1A (Figs. 1E and 4E). Therefore, E1A specifically inhibits RUNX3-mediated transactivation activity and the inhibition is p300 independent.

THE CR1 AND CR2 DOMAINS OF E1A ARE REQUIRED TO INHIBIT THE TRANSACTIVATION ACTIVITY OF RUNX3

A schematic representation of the *E1A* constructs is shown in Figure 1D. Constructs containing the N-terminus and CR1 domain still effectively inhibited the transactivation activity of RUNX3 on the *p21* reporter. The N-terminal deletion mutant (Δ2-29), which does not interact with RUNX3, inhibited RUNX3 activity, but only weakly (Fig. 5A). Although E1A constructs lacking either CR1 or CR2 could still inhibit RUNX3 activity, the deletion of both CR1 and CR2 abolished the inhibitory effect of E1A (Fig. 5B).

E1A INHIBITS RUNX3 ACTIVITY

Overexpression of *RUNX3* results in cell growth inhibition [Chi et al., 2005] and apoptosis [Yano et al., 2006]; therefore, we examined whether *E1A* expression could overcome these effect of *RUNX3*. For this purpose, we first tried to establish a stable MCF7 cell line that overexpressed *RUNX3*. Unfortunately, we did not obtain any positive clones which expressed detectable levels of exogenous *RUNX3*. This technical difficulty may due to the growth inhibitory and apoptosis inducing effect of *RUNX3*. We therefore established MCF7 stable cell lines that expressed *E1A* (MCF7-E1A-cl1 and MCF7-E1A-cl2) by transfecting an *E1A12s* expression plasmid (Fig. 6A). These cells were transiently transfected with a *RUNX3* expression plasmid or empty vector and cell viability was measured. The growth of MCF7-puro cells was inhibited by 43% after the transfection of *RUNX3*. In contrast, the growth of *E1A* expressing cells was only inhibited by 13–21% (depending on the cell line) following *RUNX3* transfection (Fig. 6B). The transfection efficiency of these cell lines was about 40–50% (Fig. 6C). These results suggest that the overexpression of *RUNX3* in MCF7 cells can strongly inhibit cell proliferation; however, *E1A* expression reduced the activity of *RUNX3*.

DISCUSSION

E1A has been shown to interact with various cellular regulators that play important roles in controlling cell growth in both normal and cancerous cells. In this study, we found that the N-terminal domain of E1A interacts with the Runt domain of RUNX3. Interestingly, the interaction of E1A with RUNX3 strongly repressed RUNX3-mediated transcription of the *p21* promoter construct. The addition of CBFβ or SMADs, which are known inducers of RUNX3 activity, could not restore its activity. E1A constructs which lack the N-terminus failed to inhibit RUNX3-mediated transcription.

E1A has been shown to inhibit p53-mediated transcription of *p21* through its interaction with p300/CBP, a coactivator for p53 signaling [Steegenga et al., 1996]. The N-terminal region of E1A binds to p300/CBP which inhibits p53-mediated transcription [Lill et al., 1997]. RUNX3 as well as p53 are upstream regulators of *p21* and they both require p300 as a co-activator; therefore, the inactivation of p300 by E1A might inhibit the transactivation activity of RUNX3 indirectly. It has been demonstrated that E1A also inhibits TGF-β-dependent induction of *p21* [Datto et al., 1997]. This inhibition is mediated by the N-terminal region of E1A which is required for the interaction with p300. It is worth noting that TGF-β can induce *p21* through a p53-independent pathway [Datto et al., 1995]. These results suggest the presence of an unidentified mechanism for E1A-mediated *p21* down-regulation.

We tested whether the interaction between E1A and RUNX3 directly inhibited RUNX3-mediated transcription. Reporter assays using a *p300* deficient cell line showed that E1A could directly inhibit RUNX3-mediated *p21* transcription. This result revealed a new mechanism for the E1A-mediated down-regulation of *p21* whereby E1A inhibits *p21* transcription by direct interacting with RUNX3, an upstream activator of TGF-β-dependent *p21* expression.

Given that the E1A protein directly binds to cellular regulators that play important roles in controlling cell growth, E1A has been instrumental in the identification of key cellular regulatory proteins that are involved in transformation. In this study, we show that E1A interacts directly with RUNX3 and reduces RUNX3-mediated activation of *p21*. Our findings demonstrate that RUNX3 is a target of E1A and suggest that RUNX3 is an important regulator of cell growth which must be inhibited for transformation by E1A.

ACKNOWLEDGMENTS

We appreciate Dr. K. Shigesada for the kind help interpreting the results. This work was supported by the Creative Research Grant from the Korea Science and Engineering Foundation R16-2003-002-01001-02006.

REFERENCES

Arany Z, Newsome D, Oldread E, Livingston DM, Eckner R. 1995. A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. Nature 374:81–84.

Avvakumov N, Wheeler R, D'Halluin JC, Mymryk JS. 2002. Comparative sequence analysis of the largest E1A proteins of human and simian adenoviruses. J Virol 76:7968–7975.

Bannister AJ, Kouzarides T. 1996. The CBP co-activator is a histone acetyltransferase. Nature 384:641–643.

Berk AJ. 2005. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. Oncogene 24:7673–7685.

Chi XZ, Yang JO, Lee KY, Ito K, Sakakura C, Li QL, Kim HR, Cha EJ, Lee YH, Kaneda A, Ushijima T, Kim WJ, Ito Y, Bae SC. 2005. RUNX3 suppresses gastric epithelial cell growth by inducing p21(WAF1/Cip1) expression in cooperation with transforming growth factor β-activated SMAD. Mol Cell Biol 25:8097–8107.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. 1995. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci USA 17: 5545–5549.

Datto MB, Hu PP, Kowalik TF, Yingling J, Wang XF. 1997. The viral oncoprotein E1A blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B. Mol Cell Biol 17:2030–2037.

Frisch SM, Mymryk JS. 2002. Adenovirus-5 E1A: Paradox and paradigm. Nat Rev Mol Cell Biol 3:441–452.

Gallimore PH, Turnell AS. 2001. Adenovirus E1A: Remodelling the host cell, a life or death experience. Oncogene 20:7824–7835.

Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, Imamura T, Ishidou Y, Fukuchi M, Shi MJ, Stavnezer J, Kawabata M, Miyazono K, Ito Y. 1999. Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. J Biol Chem 274:31577–31582.

Inoue K, Ozaki S, Shiga T, Ito K, Masuda T, Okado N, Iseda T, Kawaguchi S, Ogawa M, Bae SC, Yamashita N, Itohara S, Kudo N, Ito Y. 2002. Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat Neurosci 5:946–954.

Ito Y. 2004. Oncogenic potential of the RUNX gene family: 'overview'. Oncogene 23:4198–4208.

Iyer NG, Ozdag H, Caldas C. 2004. p300/CBP and cancer. Oncogene 23:4225–4231.

Kim WJ, Kim EJ, Jeong P, Quan C, Kim J, Li QL, Yang JO, Ito Y, Bae SC. 2005. RUNX3 inactivation by point mutations and aberrant DNA methylation in bladder tumors. Cancer Res 65:9347–9354.

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G. 1997. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nat Genet 16:307–310.

Levanon D, Bettoun D, Harris-Cerruti C, Woolf E, Negreanu V, Eilam R, Bernstein Y, Goldenberg D, Xiao C, Fliegauf M, Kremer E, Otto F, Brenner O, Lev-Tov A, Groner Y. 2002. The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. EMBO J 21:3454–3463.

Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y. 2002. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 109:113–124.

Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. 1997. Binding and modulation of p53 by p300/CBP coactivators. Nature 387:823–827.

Look AT. 1997. Oncogenic transcription factors in the human acute leukemias. Science 278:1059–1064.

Louis N, Evelegh C, Graham FL. 1997. Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. Virology 233:423–429.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89:773–779.

Ogawa E, Inuzuka M, Maruyama M, Satake M, Naito-Fujimoto M, Ito Y, Shigesada K. 1993. Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. Virology 194:314–331.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferasees. Cell 87:953–959.

Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Munger K, Howley PM, Moses HL. 1990. TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell 61:777–785.

Steegenga WT, van Laar T, Riteco N, Mandarino A, Shvarts A, van der Eb AJ, Jochemsen AG. 1996. Adenovirus E1A proteins inhibit activation of transcription by p53. Mol Cell Biol 16:2101–2109.

Stein RW, Corrigan M, Yaciuk P, Whelan J, Moran E. 1990. Analysis of E1A-mediated growth regulation functions: Binding of the 300-kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity. J Virol 64:4421–4427.

Taniuchi I, Osato M, Egawa T, Sunshine MJ, Bae SC, Komori T, Ito Y, Littman DR. 2002. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell 111:621–633.

Turnell AS, Mymryk JS. 2006. Roles for the coactivators CBP and p300 and the APC/C E3 ubiquitin ligase in E1A-dependent cell transformation. Br J Cancer 95:555–560.

van Wijnen AJ, Stein GS, Gergen JP, Groner Y, Hiebert SW, Ito Y, Liu P, Neil JC, Ohki M, Speck N. 2004. Nomenclature for Runt-related RUNX proteins. Oncogene 23:4209–4210.

Wang S, Wang Q, Crute BE, Melnikova IN, Keller SR, Speck NA. 1993. Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol Cell Biol 13:3324–3339.

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. 2006. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38:787–793.

Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E. 1988. Association between an oncogene and an anti-oncogene: The adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334:124–129.

Yano T, Ito K, Fukamachi H, Chi XZ, Wee HJ, Inoue K, Ida H, Bouillet P, Strasser A, Bae SC, Ito Y. 2006. The RUNX3 tumor suppressor upregulates Bim in gastric epithelial cells undergoing transforming growth factor beta-induced apoptosis. Mol Cell Biol 26:4474–4488.