Abstract
Recent advances in the study of RASSF1A, the candidate tumor suppressor gene, indicate a possible role of RASSF1A in cell cycle regulation; however, very little is known regarding molecular mechanisms underlying this control. Using small interfering RNA to knockdown endogenous RASSF1A in the breast tumor cell line HB2 and in the cervical cancer cell line HeLa, we identify that a key player in cell cycle progression, cyclin A2, is concomitantly increased at both protein and mRNA levels. In A549 clones stably expressing RASSF1A, cyclin A2 levels were diminished compared with vector control. A known transcriptional regulator of cyclin A2, p120E4F (a repressor of cyclin A2), has been shown previously by our group to interact with RASSF1A. We show that levels of p120E4F are not affected by RASSF1A small interfering RNA in HB2 and HeLa cells. However, electrophoretic mobility shift assays indicate that knockdown of endogenous RASSF1A in HB2 and HeLa cells leads to a reduction in the binding capacity of p120E4F to the cyclin A2 promoter, whereas in the A549 clone stably expressing RASSF1A the binding capacity is increased. These data are further corroborated in vitro by the luciferase assay and in vivo by chromatin immunoprecipitation experiments. Together, these data identify the cyclin A2 gene as a cellular target for RASSF1A through p120E4F and for the first time suggest a transcriptional mechanism for RASSF1A-dependent cell cycle regulation. (Cancer Res 2005; 65(7): 2690-7)

Introduction
RASSF1A is a 39-kDa (340-amino acid) protein that is encoded for by the RASSF1 gene that is located within a 120-kb region of chromosome 3p21.3 that frequently undergoes allelic loss in lung and breast cancers (1). It is one of several major transcripts that are produced by alternative promoter selection and alternative messenger (mRNA) splicing. RASSF1A is encoded by the RASSF1 exons 1α, 2αβ, and 3 to 6. The RASSF1A protein contains a NH2-terminal diacylglycerol binding domain (50-101 amino acids) and a Ras-association domain (194-288 amino acids) in the COOH terminus. There is now strong evidence that RASSF1A may function as a tumor suppressor protein in many cells of epithelial origin (1–3) and further evidence is emerging of RASSF1A negatively regulating tumor growth (4). Our previous work (5) and other studies (4) suggest that RASSF1A may indeed have multiple functions, which affect tumorigenesis ranging from inhibiting cell cycle progression to influencing other important variables of tumorigenesis, including cell adhesion, cell migration, angiogenesis, transcription, and apoptosis. Recent reports show that RASSF1A is involved in microtubule stability and is colocalized with microtubules during mitosis (6–8). A report from our laboratory (9) suggests an interaction of RASSF1A with the ubiquitously expressed E1A-regulated transcription factor p120E4F, a transcriptional repressor of cyclin A2, adding further weight to the role RASSF1A in cell cycle progression. Indeed, both RASSF1A and p120E4F proteins have been shown to evoke changes in cell cycle regulatory proteins, including the post-transcriptional elevation of cyclin B1, p21Waf1, and p27Kip1 protein levels, reduced expression of cyclin A2, reduced levels of cyclin-dependent kinase (cdk) 2 and cdc kinase activities in the case of p120E4F, and down-regulation of cyclin D1 and D3 in the case of RASSF1A (4, 5).

E4F is synthesized as a 120-kDa protein (p120E4F) that on proteolytic cleavage gives rise to p50E4F, a 50-kDa NH2-terminal fragment (10). Although p50E4F and p120E4F recognize the same DNA motifs in vitro, they differentially regulate gene expression in vivo. p50E4F transactivates expression of the adenoviral E4 gene in an E1A-dependent fashion (11, 12), p120E4F, on the other hand, is likely to play a key role in mammalian cell cycle control (13). Ectopic expression of p120E4F leads to growth suppression, an effect that is mediated by the interaction with the tumor suppressors pRb (14), p14ARF (15), and p53 (16). Furthermore, overexpression of p120E4F in NIH 3T3 cells inhibits progression from G1 to S phase by a mechanism that involves the repression of cyclin A2 (13). This effect is mediated by p120E4F binding to a cyclic AMP–responsive element (CRE), which is required for full transcriptional activation of cyclin A2 gene (17, 18). A recent report by Le Cam et al. (19) using E4F knockout mice established a crucial role for p120E4F in the mitotic progression during embryonic cell cycle. They showed that p120E4F localized to the mitotic spindle during the M phase of early embryos. This is quite interesting in the light of various reports showing RASSF1A also localizing with the spindles during mitosis (6, 20), further raising the possibility of RASSF1A and p120E4F interaction.

Here, we report a series of experiments that identify cyclin A2 as a target of RASSF1A through p120E4F. We show diminished levels of p120E4F binding to the cyclin A2 promoter following RASSF1A small interfering RNA (siRNA) treatment in HB2 and HeLa cells both in vitro and in vivo, whereas in the A549 clone stably expressing RASSF1A we show increased binding of p120E4F to the promoter. The identification of cyclin A2, a key factor in cell cycle control, as a functional cellular target for RASSF1A through p120E4F provides a mechanism that might reveal the cell cycle regulatory functions of this factor.
Materials and Methods

Cell culture. HB2 and HeLa cell lines (obtained from American Type Culture Collection, Teddington, United Kingdom) and A549 (non–small cell lung cancer cell line) clones (CL1, CL4, and CL5, stably expressing RASSF1A and V.18 vector control, which do not express RASSF1A; ref. 5) were maintained in DMEM (Invitrogen Life Technologies), Inc., San Diego, CA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 ng/mL streptomycin in a 37°C incubator and 5% CO2.

siRNA treatment of cells. The double-stranded siRNA oligonucleotide targeting RASSF1A was synthesized by MWG Biotech United Kingdom Ltd. (Milton Keynes, United Kingdom) and the sequences used were those published previously (4): sense 5’-CACCGUGGGGACCCUCCATT-3’ and antisense 5’-UGAAGUGCCAGACAGGUGTT-3’. A negative control duplex (control siRNA, Ambion Europe Ltd., Huntingdon, United Kingdom) was used to show that transfection did not induce nonspecific effects on gene expression. The day before transfection, HB2 and HeLa cells were plated onto six-well cell culture plates in 2 mL growth medium without antibiotics and grown to 30% to 50% confluence. On the day of transfection, for each experiment, 1.4 g of either CycA2wt, CycA2Δ, or CycA2pm reporter construct and 0.1 g of pH7-CMV Remilla luciferase expression vector (Promega, Southampton, United Kingdom) was added to the diluted duplex, and the mixture was incubated at room temperature for 20 minutes to allow the siRNA/Oligofectamine complexes to form. This mixture was then added to the transfection well and incubated for 72 hours at 37°C before whole cell lysates were taken for Western blotting/immunoprecipitation or total RNA extracted for reverse transcription-PCR (RT-PCR).

Western blotting. Whole cell lysates were obtained from cultured HB2 and HeLa cells (untreated controls, 72-hour RASSF1A siRNA-treated, and control siRNA-treated) or A549 clones (stable RASSF1A expressor CI.1 and vector control V.18) by harvesting the cells in NP40 lysis buffer. Lysates were incubated on ice for 10 minutes and sonicated for 60 seconds, and insoluble cell debris was removed by centrifugation for 5 minutes at 14,000 rpm at 4°C. Protein samples (20 µg each) were separated by SDS-PAGE (6-15%) and electroblotted to Hybond-P membranes (Amersham Biosciences, Chalfont St. Giles, United Kingdom). Immobilized proteins were detected using appropriate primary and horseradish peroxidase secondary antibodies by enhanced chemiluminescence (Amersham Biosciences). Quantification of the protein bands was carried out using laser densitometry. Complete transfer of proteins was checked by staining with Ponceau S. Bands were quantified using Image J software.
twice with 1 mL of each of the following buffers: chIP lysis buffer, high-
salt chIP lysis buffer [5 mmol/L PIPES (pH 8), 500 mmol/L KCl, 0.5% 
NP40], chIP wash [10 mmol/L Tris (pH 8), 250 mmol/L LiCl, 0.5% NP40], 
and Tris-EDTA. Protein A-Sepharose pellets were resuspended in 300 
µL Tris-EDTA and incubated for 3 hours at 55°C with 10 µg Rnase A 
and 20 µg proteinase K. Cross-linking was reversed by incubation at 65°C 
during 4 hours to overnight. DNA was purified on resin (Wizard protocol, 
Promega) and eluted in 50 µL H₂O. An aliquot of chromatin DNA 
prepared from negative RNA interference (RNAi) HB2 cells, RASSF1A 
RNAi-treated HB2 cells, A549 vector control cells, or RASSF1A-expressing 
A549 cells was taken before immunoprecipitation and further treated 
and purified as the immunoprecipitated DNA. This DNA corresponded to 
the total DNA sample. Immunoprecipitated and total DNA were assayed by 
PCR using the following primers: 5'-GCTTAAAATAATCGGAAGCG-3' 
and 5'-GGCCAAAGAATAGTCGTAGC-3'.

Antibodies. Anti-RASSF1A rabbit polyclonal antibody was kindly 
provided by G.J. Clark (8); anti-β-actin were purchased from Sigma (Poole, 
United Kingdom); anti-cyclin A2 (E43.2) was purchased from the 
monoclonal antibody service, Cancer Research UK (London, United 
Kingdom); anti-p120E4F (88.2) polyclonal was kindly provided by Claude 
Sardet (Institut de Genetique Moleculaire, Centre National de la Recherche 
Scientifique, Montpellier, France; ref. 14).

Statistical analysis. Data are expressed as means ± SD from at least 
three experiments unless otherwise stated. A Student's t test was used to 
compare individual data with control value. A probability of P < 0.05 was 
taken as denoting a significant difference from control data.

Results

Confirmation of RASSF1A siRNA affecting mRNA and 
protein expression of RASSF1A in HB2 and HeLa cells. To 
specifically silence the RASSF1A gene, HB2 and HeLa cells, which 
express RASSF1A endogenously, were transfected with siRNA- 
targeting RASSF1A. The expression of RASSF1A mRNA was 
measured by RT-PCR using total RNA isolated from HB2 cells 
(Fig. 1A). No effect on the RASSF1A gene were visible 24 hours 
after transfection; however, by 72 hours, there is silencing of the 
RASSF1A gene and this lasted for at least a further 72 hours. 
No effects of siRNA were observed on the expression of GAPDH, 
which was used as an internal control for specificity and loading. 
As a control for specificity of siRNA, a negative control siRNA, 
containing a 21-bp scramble sequence with no significant 
homology to human gene sequences, was used (Fig. 1B, top).

Figure 1. Effect of RASSF1A siRNA on RASSF1A 
mRNA and protein expression in HB2 and HeLa cells. A, RT-PCR of total RNA extracted from HB2 cells 
showing levels of RASSF1A mRNA following 
RASSF1A siRNA over a 9-day period. GAPDH 
transcripts served as a loading control. Levels of 
RASSF1A mRNA are diminished after day 3 of RNAi 
treatment (n = 3). B, RT-PCR showing levels of 
RASSF1A mRNA following 3 days (72 hours) of 
RASSF1A siRNA treatment in HB2 cells (top) and 
HeLa cells (bottom) compared with untreated controls 
and control siRNA-treated cells. GAPDH transcripts 
served as a loading control. RASSF1A siRNA 
effectively knocks down RASSF1A mRNA expression 
in both these cell lines (n = 4). C, Western blot analysis 
for RASSF1A and RASSF1C of whole cell lysates 
obtained from HB2 (top) and HeLa (bottom) cell lines 
following RASSF1A siRNA treatment compared with 
untreated controls and control siRNA-treated cells. 
Blots were stripped and reprobed with a 
β-actin antibody to show protein loading (n = 4). D, RT-PCR 
showing levels of RASSF1C mRNA following 3 days 
(72 hours) of RASSF1A siRNA treatment in HB2 and 
HeLa cells compared with untreated controls and 
control siRNA-treated cells.
Similar results to the HB2 cells were also obtained for HeLa cells (Fig. 1B, bottom). In addition, Western blot analysis also showed that RASSF1A siRNA severely suppressed expression of RASSF1A protein in HB2 (Fig. 1C, top) and HeLa (Fig. 1C, bottom) when compared with control cells that were either untreated or transfected with the control siRNA. No effects of siRNA were observed on the expression of RASSF1C (differentially spliced form of RASSF1) or β-actin, which was used as an internal control for specificity and loading. RASSF1A RNAi also did not have any effect on RASSF1C mRNA expression in either HB2 or HeLa cells (Fig. 1D).

Cyclin A2 levels increase following RASSF1A siRNA in HB2 and HeLa cells but decrease in A549 clones stably expressing RASSF1A. Protein levels of cyclin A2 were shown to be upregulated in HB2 (Fig. 2A) and HeLa (Fig. 2B) cells ~4-fold, as determined by densitometric analysis, following RASSF1A RNAi compared with untreated controls and negative RNAi controls. However, in a panel of A549 clones stably expressing RASSF1A (Cl.1, Cl.4, and Cl.5), cyclin A2 protein levels are decreased by 5- to 6-fold compared with A549 vector control (V.18, which do not express RASSF1A; Fig. 2C).

Cyclin A2 is transcriptionally regulated by endogenously and exogenously expressing RASSF1A. To establish whether RASSF1A regulated cyclin A2 at the level of transcription, we used RT-PCR to measure mRNA levels before and after siRNA (Fig. 3). Cyclin A2 mRNA levels increased significantly in both HB2 (Fig. 3A) and HeLa (Fig. 3B) following RASSF1A siRNA compared with untreated controls and control siRNA control, revealing a good correlation between RT-PCR and Western blot data. In A549 clones stably expressing RASSF1A (Cl.1, Cl.4, and Cl.5), there was a significant decrease in cyclin A2 mRNA levels compared with vector control (Fig. 3C); again, this was entirely consistent with the Western blot data.

RASSF1A inhibits the cyclin A2 promoter through increasing p120E4F-mediated repression. Cyclin A2 is regulated by the transcription factor p120E4F (13) and we have shown previously p120E4F to interact with RASSF1, principally with RASSF1A (9). We confirmed this association in HB2 and HeLa cells as well as in A549
clones stably expressing RASSF1A (data not shown). We therefore assessed the functional consequence of the RASSF1A-p120E4F interaction. p120E4F behaves as a transcriptional repressor, and it has been shown that inhibition of cyclin A2 is an event required for p120E4F-dependent cell cycle arrest in G1, and that the cyclin A2 promoter activity can be repressed by the binding of p120E4F to the CRE site (13). The activity of a reporter construct containing the promoter region of the human cyclin A2 gene, containing the p120E4F binding site, cloned upstream of the luciferase gene (CycA2wt; Fig. 4A) was therefore tested in transient transfection assays. Transfection of HB2 and HeLa cells (control siRNA-treated cells or untreated cells) with the reporter construct resulted in ~4-fold repression of luciferase activity (Fig. 4B). However, this repression was not observed with RASSF1A siRNA-treated cells nor was this repression seen in control siRNA-treated HB2 and HeLa cells or untreated HB2 and HeLa cells transfected with the cyclin A2 promoter deleted (CycA2Δ) for the p120E4F binding site. In the A549 clone stably expressing RASSF1A (Cl.1), the repression of the cyclin A2 promoter was ~6-fold (Fig. 4C) following transfection with the CycA2wt reporter construct. This result correlates very well with the protein levels of cyclin A2 (Fig. 2C), which was shown to be decreased ~5- to 6-fold in the A549 expressing clone. The repression in luciferase activity, however, was not observed with A549 vector control cells (V.18, which do not express RASSF1A) nor was this repression seen with A549 stable clone (Cl.1, stable expression of RASSF1A) transfected with the CycA2wt or CycA2Δ. However, Western blot analysis revealed that protein levels of p120E4F are not affected following RASSF1A siRNA in HB2 and HeLa cells or in the A549 clone stably expressing RASSF1A (Fig. 4D). Taken together, these data show that in the presence of RASSF1A the cyclin A2 promoter is inhibited through p120E4F-mediated repression.

RASSF1A regulates the binding of p120E4F to the cyclin A2 promoter. Electrophoretic mobility shift assay (EMSA) was used to determine the effect of RASSF1A on the binding affinity of the p120E4F transcription factor to the cyclin A2 CRE promoter region. Fig. 5A is a representative mobility shift assay gel showing that RASSF1A RNAi in HB2 cells results in ~4- to 5-fold decrease in p120E4F binding affinity to the cyclin A2 CRE promoter region (lane 4) compared with untreated control (lane 3) and negative RNAi control (lane 2). Specificity of binding was confirmed by cold competition studies using 100-fold molar excess of the unlabeled cyclin A2 CRE oligonucleotide probe (lane 6). Additional confirmation of binding specificity was detected using a polyclonal antibody to p120E4F, which resulted in a supershift of the band (lane 7) and using 100-fold molar excess unlabeled activator protein-1 (AP-1) oligonucleotide probe as a nonspecific competitor (lane 5), which did not compete with p120E4F binding. A similar EMSA profile is also seen in HeLa cells (lanes 8-11). EMSA results (Fig. 5B) for the A549 clone stably expressing RASSF1A (Cl.1) show a significant increase in the binding capacity of p120E4F for the cyclin A2 promoter region (lane 3) compared with the A549 vector control (V.18; lane 2). Specificity of binding was confirmed by competition studies using 100-fold molar excess of the unlabeled cyclin A2 CRE oligonucleotide probe (lane 5) as well as using a polyclonal antibody to p120E4F, which resulted in a supershift of the band (lane 4). These results show RASSF1A enhancing the binding capacity of p120E4F to the cyclin A2 promoter in vitro.

RASSF1A affects the binding of p120E4F to the cyclin A2 promoter in vivo. To further show that RASSF1A could interfere with the binding of p120E4F in vivo, we did chIP experiments with an anti-p120E4F polyclonal antibody. PCR amplification at the CRE-p120E4F locus of the cyclin A2 promoter obtained with chromatin purified from control siRNA-treated HB2 (Fig. 6A, top) and HeLa cells (Fig. 6A, bottom) gave a strong signal, whereas the control immunoprecipitation with normal rabbit serum (IgG) gave only a background amplification signal, although p120E4F-bound DNA was present in that chromatin extract as shown by the signal obtained with the secondary E4F immunoprecipitation. However, in RASSF1A siRNA-treated HB2 and HeLa cells, there was no amplification signal. The chromatin purified from the A549 clone stably expressing RASSF1A (Cl.1; Fig. 6B) was in accordance with the control siRNA-treated HB2 and HeLa cells. Vector control A549 cells (V.18, which do not express RASSF1A), however, produced no amplification signals. These data reinforce the observation that RASSF1A modulates the binding of p120E4F to the cyclin A2 promoter.

Discussion

RASSF1 transcriptional silencing has been observed in many cancers and the tumor-suppressing function of this gene has been implicated in reducing the proliferation of cancer cells (1, 2). Although several specific pathways and functions have been suggested for RASSF1A (4, 5), the protein product of the RASSF1 gene, the underlying mechanisms involved have yet to be
delineated. Recent studies have pointed toward a cell cycle regulatory role of RASSF1A (4, 9) and this study adds to the growing weight of evidence that this may be an important role of RASSF1A. The data presented in this study use RNAi technology, which mimics the naturally occurring gene silencing observed in tumors, to identify cyclin A2 as a target of RASSF1A in cells of breast tumor origin (HB2) and in the cervical cancer cell line (HeLa), both of which express RASSF1A endogenously; in addition, our results show that this targeting is of a transcriptional nature. We show that the differentially spliced and non-tumor-suppressing form of RASSF1 (RASSF1C) is not affected by RASSF1A siRNA at both protein and mRNA levels. We also show that RASSF1A interacts with a known transcriptional regulator of the cyclin A2 promoter (p120E4F) not only in the HB2 or HeLa tumor cell lines but also in an A549 clone stably expressing RASSF1A (Cl.1). Furthermore, using the luciferase reporter assay, we show RASSF1A to inhibit the cyclin A2 promoter through increasing p120E4F-mediated repression; moreover, using EMSA and chIP experiments, we confirm that RASSF1A affects the binding of p120E4F to the cyclin A2 promoter both in vitro and in vivo.

The process of growth, one of the fundamental aspects of the development of an organism, is tightly controlled by the coordination of proliferation, differentiation, and apoptosis, with disruption of these processes commonly resulting in tumor formation. Cell cycle progression is controlled by protein complexes, such as cyclins, cdks, and cdk inhibitors. The sequential activation and subsequent inactivation of cyclin-cdk complexes govern the progression of eukaryotic cells throughout cell cycle (22). In cell cycle, the period from the late G1 to S phase is the most important restriction point for cell proliferation. Whether cells pass the G1-S restriction point determines the continuity of cell proliferation (23). We show cyclin A2, a key factor controlling cell cycle progression at both S-phase entry and G2-M transition, to be increased at both mRNA and protein levels following RASSF1A

Figure 4. RASSF1A enhances the inhibitory effect of p120E4F on cyclin A2 transcription. A, diagrams of luciferase reporter genes under the transcriptional regulation of the wild-type human cyclin A2 promoter (CycA2wt), the cyclin A2 promoter with the p120E4F deleted from the CRE site (CycA2zm), and the promoter point mutated at the CRE (CycA2zm). CRE, CAAT box, and CCRE/CHR elements. B, luciferase activities of the various cyclin A2 promoter constructs were measured in untreated HB2 or HeLa cells, control siRNA-treated HB2 or HeLa cells, or RASSF1A siRNA-treated HB2 or HeLa cells. Fold repression of cyclin A2 promoter activity in the presence or absence of RASSF1A. Experiments repeated four times. Bars, SD. **, P < 0.01, compared with empty PGL3 vector control; ##, P < 0.01, compared with control siRNA-treated cells transfected with CycA2zm. C, luciferase activities of the various cyclin A2 promoter constructs were measured in untreated A549 vector control cells (V.18) or the A549 clone with stably expressing RASSF1A (Cl.1). Fold repression of cyclin A2 promoter activity in the absence or presence of RASSF1A. Experiments repeated four times. Bars, SD. **, P < 0.01, compared with A549 empty PGL3 vector control; ##, P < 0.01, compared with Cl.1 transfected with CycA2zm. D, knockdown of endogenous RASSF1A or stable expression of RASSF1A does not affect p120E4F protein levels. Impact on p120E4F protein levels as determined by Western blot analysis following RASSF1A siRNA in HB2 or HeLa cells or stably expressing RASSF1A in A549 cells (Cl.1). Antibody against β-actin was used to control for protein loading (n = 3).
RNAi in HB2 and HeLa cells, whereas in A549 clones stably expressing RASSF1A the levels of cyclin A2 were significantly decreased. Cyclin A2 exerts its control at the G1-S phase transition by binding to a cdk (cdk2); however, levels of this protein remained unchanged following RASSF1A RNAi in HB2 cells, in HeLa cells, or in the A549 expressing clone (Cl.1; data not shown). A novel observation made in one of our recent studies (9) was the association of the transcription factor p120E4F with RASSF1A in mammalian cells. It has been shown that p120E4F acts as an inhibitory transcription factor and represses cyclin A2 promoter activity and that this repression correlates with p120E4F binding to the CRE site of the cyclin A2 promoter (13). Our RT-PCR data in HB2 cells, in HeLa cells, and in the A549 clone stably expressing RASSF1A (Cl.1) suggest that RASSF1A is regulating cyclin A2 expression at the transcription level. This, together with immunoprecipitation data showing p120E4F associating with RASSF1A in both cell lines (data not shown), points toward the possibility that RASSF1A regulates cyclin A2 expression through p120E4F. Indeed, to date, cyclin A2 is the only cellular target shown to be regulated by p120E4F (13). There are probably other genes regulated by p120E4F that are involved in cell cycle progression. Cell cycle arrest by p120E4F is also enhanced by interacting with retinoblastoma, the p53 transcription factor (14, 16), and the p14ARF tumor suppressor (15). Thus, although the precise cellular signals that regulate endogenous p120E4F have yet to be clearly defined, the evidence to date suggests that p120E4F regulates cell cycle progression in response to several different signals.

Here, we show through luciferase reporter assays, EMSA, and chIP experiments that endogenous and exogenous expression of RASSF1A is able to enhance p120E4F binding to the cyclin A2 promoter; thus, RASSF1A is shown to act as a repressor of cyclin A2. It would appear that RASSF1A is possibly stabilizing p120E4F binding to the CRE element of the cyclin A2 promoter. Regulation of the cyclin A2 promoter during G0 and G1 is governed by contiguous acting elements, the CDE-CHR bipartite element (24–26). Various transcription factors, including the pocket proteins (pRb and p107), are involved in the binding to these elements, but the mechanism remains to be elucidated and is still a matter of controversy (24, 26).

However, several reports also show that the CRE site plays an important role in transcriptional activation of the cyclin A2 gene (17, 18, 27). The CRE is recognized by CRE binding protein and activating

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**Figure 5.** RASSF1A affects binding of p120E4F to the cyclin A2 promoter. EMSA of the binding of p120E4F protein from nuclear extracts of HB2 cells (lanes 1–7; A) and HeLa cells (lanes 8–11; A) and the A549 RASSF1A-expressing clone (Cl.1; B) to the oligonucleotide probe (for sequence, see Materials and Methods) containing the cyclin A2 CRE promoter region. A, lane 1, labeled probe, no protein; lanes 2 and 3, nuclear extracts from HB2 control siRNA and untreated control, respectively; lane 4, nuclear extracts from RASSF1A siRNA-treated HB2 cells; lane 5, nonspecific competition with 100 times unlabeled oligonucleotide containing the AP-1 consensus sequence (5’–CGCTTGATGACTCAGCCGGAA–3’); lane 6, cold competition with 100 times unlabeled oligonucleotide containing the cyclin A CRE promoter region; lane 7, supershift using p120E4F (88.2) polyclonal antibody; lane 8, labeled probe, no protein; lane 9, nuclear extracts from HB2 control siRNA and untreated control, respectively; lane 10, nuclear extracts from RASSF1A siRNA-treated HeLa cells; lane 11, nonspecific competition with 100 times unlabeled oligonucleotide containing the AP-1 consensus sequence. B, lane 1, labeled probe, no protein; lane 2, nuclear extracts from A549 vector control (V.18); lane 3, nuclear extracts from A549 cells stably expressing RASSF1A (Cl.1); lane 4, supershift using p120E4F (88.2) polyclonal antibody; lane 5, cold competition with 100 times unlabeled oligonucleotide containing the cyclin A CRE promoter region.

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**Figure 6.** RASSF1A enhances the binding of p120E4F to the cyclin A2 promoter in vitro. PCR amplification products (see Materials and Methods for oligonucleotides used) of p120E4F chIP obtained from control siRNA-treated and RASSF1A siRNA-treated HB2 cells (A), HeLa cells (A, bottom), or the A549 clone (Cl.1) expressing RASSF1A and vector control (V.18) cells (B). Antibodies used for the immunoprecipitations (IP). Total DNA was done with an aliquot of the DNA obtained from the various cells as indicated taken before immunoprecipitation.
transcription factor family members that belong to the family of basic leucine zipper proteins, which are able to form homodimers and heterodimers and cross-family heterodimers with members of the AP-1 family. As a result, different factors binding to CRE have been identified in different cell types. p120E4F also recognizes the CRE site and regulates cyclin A2 transcription as a repressor. Our study shows this repression to be under the influence of RASSF1A, which may be acting in a similar capacity to auxiliary proteins, such as HMGA, that have been postulated as regulators of p120E4F (13, 28).

Although, in the case of RASSF1A, association with p120E4F results in increased binding capacity of p120E4F to the cyclin A2 promoter and a greater transcriptional repression. Interestingly, repression of the cyclin A2 promoter in quiescent cells was found to be associated with recruitment of the E2F–4 transcriptional repressor (ref. 29 and references therein). Our finding that another repressor, p120E4F, under the influence of a putative tumor suppressor, RASSF1A, associates with the cyclin A2 promoter defines a second level of repressive regulation for this gene and underlies its critical role in tumorigenesis.

While this work was in progress, a report by Song et al. (30) was published, also suggesting that RASSF1A regulates cyclin A2. However, Song et al. suggest that RASSF1A may be stabilizing mitotic cyclins. These observations are seen in HeLa and depletion of RASSF1A by RNAi in their system resulted in an earlier depletion of cyclin A2 than in control cells. Although at odds with our HB2 and HeLa data, it is possible that these dissimilarities are due to experimental design differences—we use a transient siRNA system and HeLa data, it is possible that these dissimilarities are due to the reasons for these observed differences, it certainly raises more intriguing questions regarding the role of RASSF1A in cyclin A2 regulation and warrants further investigation.

In conclusion, this study establishes that RASSF1A regulates the cyclin A2 gene, a key player in cell cycle progression, through association with the transcriptional repressor p120E4F and increasing its binding capacity to the cyclin A2 promoter. To delineate precisely how this is achieved would require characterization and a better understanding of the role of the RASSF1A/p120E4F complex.

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References

Transcriptional Regulation of Cyclin A2 by RASSF1A through the Enhanced Binding of p120E4F to the Cyclin A2 Promoter
