p16\textsuperscript{INK4A} Positively Regulates Cyclin D1 and E2F1 through Negative Control of AUF1

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Abstract

**Background:** The cyclin-D/CDK4,6/p16\textsuperscript{INK4a}/pRB/E2F pathway, a key regulator of the critical G1 to S phase transition of the cell cycle, is universally disrupted in human cancer. However, the precise function of the different members of this pathway and their functional interplay are still not well defined.

**Methodology/Principal Findings:** We have shown here that the tumor suppressor p16\textsuperscript{INK4a} protein positively controls the expression of cyclin D1 and E2F1 in both human and mouse cells. p16\textsuperscript{INK4a} stabilizes the mRNAs of the corresponding genes through negative regulation of the mRNA decay-promoting AUF1 protein. Immunoprecipitation of AUF1-associated RNAs followed by RT-PCR indicated that endogenous AUF1 binds to the cyclin D1 and E2F1 mRNAs. Furthermore, AUF1 down-regulation increased the expression levels of these genes, while concurrent silencing of AUF1 and p16\textsuperscript{INK4a}, using specific siRNAs, restored normal expression of both cyclinD1 and E2F1. Besides, we have shown the presence of functional AU-rich elements in the E2F1 3' UTR, which contributed to p16/AUF1-mediated regulation of E2F1 post-transcriptional events in vivo. Importantly, genome-wide gene expression microarray analysis revealed the presence of a large number of genes differentially expressed in a p16\textsuperscript{INK4a} dependent manner, and several of these genes are also members of the AUF1 and E2F1 regulons. We also present evidence that E2F1 mediates p16-dependent regulation of several pro- and anti-apoptotic proteins, and the consequent induction of spontaneous as well as doxorubicin-induced apoptosis.

**Conclusion/Significance:** These findings show that the cyclin-dependent kinase inhibitor p16\textsuperscript{INK4a} is also a modulator of transcription and apoptosis through controlling the expression of two major transcription regulators, AUF1 and E2F1.

Introduction

The cyclin D-CDK4,6/p16/pRB/E2F cascade has been found to be altered in more than 80% of human tumors [1,2]. Indeed, the disruption of this pathway is a mandatory requirement for 100% of lung cancer [3]. During G1 phase, pRB is inactivated by sequential phosphorylation events mediated by various cyclin-dependent kinases (CDKs) leading to the release of the E2F transcription factors, the activation of many genes and progression of the cell cycle [4]. Indeed, activation of the E2F1 transcription factor leads to coordinated induction of multiple downstream effectors implicated in both cell proliferation and death [5,6]. Interestingly, E2F1 may function as an oncogene or as a tumor suppressor gene, and it seems that the threshold level of the E2F1 protein as well as the cell type have a determinant role in the function of the gene [5].

p16\textsuperscript{INK4a} (hereafter referred to as p16) is a cyclin-dependent kinase inhibitor that plays important roles in tumor suppression [7,8,9,10]. The p16 coding-gene has been found homozygously deleted, mutated or transcriptionally inhibited by methylation in a large number of different human tumor types [8,9,11,12]. Mice lacking p16 are tumor prone and develop different types of cancer, particularly after exposure to carcinogens [13,14]. Furthermore, p16 promoter methylation and transcriptional silencing have been detected even in histologically normal mammary tissue of cancer-free women. This suggests that p16 inactivation may represent a cancerous precondition and an early event in promoting genomic instability that leads to tumorigenesis [15].

Recent data have revealed the existence of interactions between the various genes of the pRB pathway. Indeed, it has been reported that the expression of 74 genes is repressed by both p16 and pRB [16]. It has also been shown that the cyclin D1 protein stimulates E2F1 [17,18]. Furthermore, over-expression of cyclin D1 contributed to malignancy by up-regulation of FGFR1 via the pRB/E2F pathway [19]. In the present report we addressed the role of p16 in the regulation of cyclin D1 and E2F1, two major players in the pRB pathway. We have shown that the transcription factor E2F1 is also a target of the RNA binding AUF1 protein, and that the tumor suppressor p16 positively controls cyclin D1 and E2F1 through negative regulation of AUF1. Furthermore, we...
provide clear evidence that p16 is also a master regulator of gene expression and apoptosis in human cells.

Materials and Methods

Cell lines, cell culture and chemicals

U2OS, EH1 and EH2 [20] (The three cell lines are a generous gift from Dr. G. Peters), MEFs p16 (WT) and their p16-specific knockout counterpart [13], Huh7 (hepatocarcinoma cell line) and HFSN1 (primary normal human skin fibroblast) [21]. These cells were routinely cultured in DMEM medium supplemented with 10% FBS. Actinomycin D, IPTG, Flavopiridol and doxorubicin were purchased from Sigma (USA).

Cellular lysate preparation and immunoblotting

This has been performed as previously described [20]. Antibodies directed against p16 (50.1), Cyclin D1 (HD11), E2F1 (KD95), hnRNP DO (T10), HuR (3A2), α-tubulin (TU-02), PCNA (PC-10), Bax (B-9), Bcl-2 (C-2), NF-κB (F-6), Bcl-xL (H-3), GAPDH (FL-335), Cdk4 (C-22), Cdk6 (H-11) and β-actin (C-11) were purchased from Santa Cruz (USA) and Cleaved Caspase-3 (Asp-175) from Cell signaling (UK).

RNA purification, RT-PCR and real time RT-PCR

Total RNA was purified using the TRI reagent (Sigma) according to the manufacturer’s instructions. The concentration of RNA was determined using NanoDrop® ND-1000 Spectrophotometer (Nanodrop Inc., Wilmington, DE, USA). Single stranded complementary DNA (cDNA) was obtained from reverse transcription of 1 μg of RNA using the RT-PCR kit (BD Biosciences) and following the manufacturer protocol. cDNA was then amplified with 1U Taq polymerase, dNTPs (50 mM), and primers (25 pmol each). The mixture was first heated at 94°C for 5 min and then 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, then 72°C for 10 min. PCR products were seen after electrophoresis on ethidium bromide stained 2% agarose gel. For real time RT-PCR Syber green and platinum Taq polymerase (Invitrogen) were used and the amplifications were performed utilizing the Bio-Rad iQ5 multicolor Real time PCR detection system.

The respective primers were:

- β-actin: 5’-CCCAGCACAATGAAGATCAAGATCAT-3’ (forward) and 5’-ATCTTGCTGGAAAGTGAGCACGGGA-3’ (reverse);
- cyclinD1: 5’-CACAGGGACTACAGGAGAGT-3’ (forward) and 5’-CACAGGGACTACAGGAGAGT-3’ (reverse);
- E2F1: 5’-ATGT-
saved, freezed-thawed five times, and centrifuged (10 min, 14,000 rpm, 4°C) supernatants were saved and centrifuged (10 min, 14,000 rpm, 4°C) and supernatants were saved at −80°C.

Immunoprecipitation and RT-PCR

Cell lysates were prepared from confluent cells, and 3 mg were incubated in the lysis buffer (50 mM Tris (pH 8), 100 mM NaCl, 10% glycerol, 1× protease inhibitors, 5 mM DTT and 2 U/μl RNasin) and 5 μg AUF1 mouse monoclonal antibody (mouse IgG1 was used as control) was added and mixed at 4°C for 4 h. Equal volume of protein A agarose was added per immunoprecipitation and mixed overnight at 4°C. After centrifugation, the pellet was re-suspended in 1 ml TRI reagent used for RNA extraction. RT-PCR reactions were performed as described above.

siRNA transfection

pSILENCER-AUF1/siRNA and control-siRNA plasmids were used to carry out transient transfection using human dermal fibroblast nucleofector kit (Amaxa Biosystems) following the protocol recommended by the manufacturer. pIRE.S2F1 and the scrambled-sequence plasmids were used to perform stable transfection of U2OS cells. Transfection was carried out by mixing 3 μg of the plasmid DNA in 1.5 ml of Opti-MEM I medium without serum. A mixture of 1.5 ml of Opti-MEM I medium with 30 μl Lipofectamine (Invitrogen) was added to the DNA, and then was incubated for 20 min before addition to 50% confluent cells.

3′ UTR PCR, construction and cloning of reporter plasmids

The ARE and ARE-like sequences in the E2F1 3′ UTR (1455–2700 nt, NM_005225.2) were identified by searching for AUUUA patterns. Double-stranded DNA was made by annealing oligonucleotides (80 nt) that correspond to sense and antisense orientation of the ARE regions were synthesized (Metabion, Germany) and contain BamHI and XbaI overhangs, respectively. The DNA was cloned into EGFP expression vector (Genelantis, San Diego, CA), which is under CMV constitutive promoter, and verified by specific PCR and sequencing.

Reporter transfection and activity assessment

Reporter constructs containing GFP-3′UTR/ARE were used in transient transfection using Lipofectamine 2000 (Invitrogen). Transfection efficiency and normalization to control was achieved using GFP reporter fused with stable non-ARE BGH 3′UTR. The intra-well variance of any replicate groups in fluorescence is generally <6%, which does not warrant intra-well normalization of transfection [22]. Data are presented as mean value ± SEM of fluorescence intensity using BD Bioimager and imaging software.

Apoptosis analysis by Annexin V/Flow Cytometry

Cells were either not treated or challenged with Doxorubicin. Detached and adherent cells were then harvested after 72 hrs, centrifuged and resuspended in 1 ml phosphate buffered saline (PBS). Cells were then stained with propidium iodide (PI) and Alexa Flour 488 annexin V and analyzed as previously described [23].

Quantification of protein expression level

The expression levels of the immunoblotted proteins were measured using the densitometer (Bio-RAD GS-800 Calibrated Densitometer) as previously described [20].

Figure 2. Effect of p16 on the turn-over of the cyclin D1 and E2F1 mRNAs. HFSN1 cells were treated with actinomycin D for different periods of time as indicated. Total RNA was extracted and the mRNA levels of cyclin D1 and E2F1 were assessed by real-time RT-PCR. Reaction was carried out by mixing 3 μg of the plasmid DNA in 1.5 ml of Opti-MEM I medium without serum. A mixture of 1.5 ml of Opti-MEM I medium with 30 μl Lipofectamine (Invitrogen) was added to the DNA, and then was incubated for 20 min before addition to 50% confluent cells.

Analysis of mRNA stability

Cells were challenged with 5 μg/ml Actinomycin D for various periods of time (0-6 hrs) and then total RNA was purified and assessed using real time RT-PCR.

Sub-cellular fractionation

Nuclear and cytoplasmic extracts were prepared as previously described [21]. Briefly, cytoplasmic fractions were obtained by incubating cells in 200 μl of hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl2) supplemented with protease inhibitors cocktail on ice, and lysed by addition of 25 μl of buffer A containing 2.5% Nonidet P-40 plus inhibitors. Nuclei were pelleted (3500 rpm, 4 min, 4°C), and supernatants were saved, freezed-thawed five times, and centrifuged (10 min, 3,500 rpm, 4°C). Nuclear pellets were incubated in extraction buffer C (20 mM HEPES [pH 7.9], 0.45 M NaCl, 1 mM MEDTA) plus inhibitors and centrifuged (10 min, 14,000 rpm, 4°C) and supernatants were saved at −80°C.
Affymetrix Genechip Array Experiments

Genome-wide gene expression studies was performed using Affymetrix’s latest GeneChip Human Genome U133 Plus 2.0 Array which have more than 54,000 probe sets used to analyze the expression level of more than 47,000 transcripts and variants, including approximately 38,500 well-characterized human genes. To this end, double-stranded cDNA were made from total RNA using the Superscript cDNAs synthesis kit as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). The product of this reaction was used as a template for in vitro transcription to make biotin-labeled cRNA (target cRNA) using biotinylated UTP and CTP. Target cRNA was fractionated and then hybridized to the gene chips for 16 hrs. The experimental procedures and quality control procedures at each critical step (before hybridization as well as post-hybridization) were strictly followed according to manufacturer’s instructions. Washing, staining, and scanning were performed using Affymetrix’s Fluidics Station 450 and GCS 3000 G7, respectively, according to the manufacturer’s instructions and guidelines. All the necessary steps related to Affymetrix Fluidics Station 450 and GCS 3000 G7 were controlled by Affymetrix’s GeneChip Operating Software (GCOS).

Validation of the transcriptomic data by Real time RT-PCR (qRT-PCR)

To validate the microarray results, the expression levels of differentially expressed transcripts of randomly chosen 11 genes were determined by qRT-PCR experiments using ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). To this end, 50 ng of total RNA procured from the same microarray study samples were transcribed into cDNA using cDNA Archive Kit (Applied Biosystems), and then used as template for PCR reaction using specific primers for the candidate genes, and GAPDH was used as internal control. Quantitech SYBR green (Qiagen) was used for detection and analysis. All reactions were conducted in triplicates and the data was analyzed using the delta delta CT method (Livak KJ, Schmittgen TD).

Microarray analysis

The dChip outlier detection algorithm was used to identify outlier arrays [24]. All samples/chips passed the quality controls. The open source R/Bioconductor packages were used for processing and analysis of microarray data [25], which was normalized by the GC Robust Multi-array Average (GC-RMA) algorithm [26,27]. Significantly modulated genes were defined as those with absolute fold change (FC) $\geq 2.0$ and adjusted p-value<0.05. Statistical analyses were performed with the MA-TLAB software packages (Mathworks, Natick, MA, USA), and PARTEK Genomics Suite (Partek Inc., St. Lois, MO, USA).

Results

p16 modulates the expression of cyclin D1 and E2F1 in both human and mouse cells

We analyzed the effect of p16 on the expression levels of E2F1 and cyclin D1 in both human and mouse cells. First, we used the p16-defective osteosarcoma U2OS cell line and its derivatives.
EH1 and EH2 that express p16 under the control of an IPTG inducible promoter [28]. The p16 expression in these cells does not exert any measurable effect on cellular growth [28]. Whole cell extracts from U2OS, EH1 and EH2 cells were prepared and used to analyze the levels of the E2F1 and cyclin D1 proteins using specific antibodies. Figure 1A shows that the protein levels of E2F1 and cyclin D1 are modulated in a p16-dependent manner. Interestingly, IPTG-treated EH2 cells wherein p16 protein level was augmented, the expression levels of the cyclin D1 and E2F1 proteins increased 2 fold as compared to the non-treated cells (Figure 1B). To further elucidate the p16 effect on the expression of these proteins, we evaluated the levels of cyclin D1 and E2F1 in HFSN1 cells stably expressing either control-siRNA or specific p16-siRNA under the control of the U6 promoter in the pRNAT-U6.1 expression vector [20] and also in p16-/- and p16+/+ MEF cells. Figure 1C shows that down-regulation of p16 led to a decrease in cyclin D1 and E2F1 half-lives from several hours to an hour and 15 min and Figure 4. p16 controls the mRNA levels of cyclin D1 and E2F1 through AUF1. (A) HFSN1 cells were transfected with plasmids expressing either AUF1-siRNA (pSILENCER-AUF15) or control-siRNA (pSILENCER). 3 days after transfection total RNA was purified from both cells, and RT-PCR (top panel) as well as real time RT-PCR (histogram) using specific primers for the indicated genes were performed. Error bars indicate standard errors of 3 different experiments (B) RNAs bound to the AUF1 protein were isolated by immunoprecipitation from HFSN1 cells expressing either p16-siRNA or control-siRNA using anti-AUF1 antibody or anti-IgG (as control), and then target transcripts were amplified by RT-PCR visualized on ethidium bromide stained 1% agarose gels. Amplification of the highly abundant GAPDH transcript, which bound IP materials at background levels, was used as loading control. (C) HFSN1 whole cell lysate was immunoprecipitated using the indicated antibodies and then used for immunostaining analysis. (D) Total RNA was purified from p16 proficient (HFSN1 and EH1) and p16-deficient (U2OS and HFSN1 expressing p16-siRNA) cells, expressing either AUF1-siRNA or control-siRNA. Transcripts for the indicated genes were detected by RT-PCR, and the corresponding products were visualized on ethidium bromide stained 1% agarose gels. The numbers below the bands indicate the corresponding expression levels. GAPDH was used as internal control. These experiments were repeated several times and representative ones are shown. doi:10.1371/journal.pone.0021111.g004

p16 controls the turn-over of the cyclin D1 and E2F1 mRNAs

Next, we sought to investigate whether p16 has any role in the stability of the cyclin D1 and E2F1 mRNAs in the human skin fibroblast HFSN1 cells expressing either p16-siRNA or control-siRNA. Cells were treated with the transcription inhibitor actinomycin D and then reincubated for different periods of time (0–6 hrs). Total RNA was purified and the mRNA levels of cyclin D1 and E2F1 were assessed by real time RT-PCR. Figure 2 shows that down-regulation of p16 led to a decrease in cyclin D1 and E2F1 half-lives from several hours to an hour and 15 min and
This result shows that p16 plays a major role in the stability of the cyclin D1 and E2F1 mRNAs in normal human skin fibroblast cells.

There are RNA binding proteins (RBPs) that bind specific mRNAs and promote either their stabilization or destabilization [29]. HuR and AUF1 are two RBPs that control the decay of several mRNAs, including the cyclin D1 mRNA [30]. Thereby, we evaluated the effect of p16 on the levels of the HuR and AUF1 proteins in U2OS and EH1 cells as well as in HFSN1 cells expressing either p16-siRNA or control-siRNA. Nuclear and cytoplasmic cell lysates were prepared from these cells and the expression levels of HuR and AUF1 in addition to p16 were assessed. Figure 3A shows that the level of HuR, whose expression is cell cycle regulated [31,32], was not affected with the status of p16 in both nuclear and cytoplasmic extracts. On the other hand, p16 negatively controls the mRNA decay-promoting AUF1 protein.

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Figure 5. Involvement of ARE in the E2F1 3′UTR and response to AUF1 and p16. (A) Schematic diagram of the E2F1 3′UTR, ARE region sequences, and locations. (B) The E2F1 3′UTR sequences in different species (C) Sequences from the E2F1 3′UTR (ARE regions 1 to 3), IL-8 3′UTR (ARE control), and a control that lacks ARE were inserted in BamHI/XbaI sites in EGFP expression vector as shown. The Huh7 cell line (2.10^4 cells per well) in 96-well black clear-bottom microplates were transfected with the different 3′UTR constructs. The reporter activity was assessed after 24 hr using BD bio-imaging apparatus and software. The non-ARE 3′UTR was used as control and its fluorescence activity was taken as 100%. Data are presented as Mean±SEM (n = 4) of % of the control. *** denote p values of < 0.005 (student t-test) when compared to non-ARE control. (D) Huh7 cells (left panel) or U2OS (right panel) in 96-well microplates were co-transfected with siRNA against AUF1 or scrambled control (50 ng per well) and reporter constructs (25 ng per well) as indicated. Reporter activity was assessed at 48 hr post-transfection. Data (Mean±SEM, n = 4) were presented as % increase in reporter fluorescence due to AUF1 silencing when compared to the fluorescence in control-siRNA-treated cells. * and *** denote p < 0.05 and < 0.005, respectively (student t-test) when compared to non-ARE control. (E) U2OS and EH1 cells (2.10^4 cells per well) were seeded in 96-well black clear-bottom microplates and then transfected with the different 3′UTR constructs. Reporter activity was assessed as described in (D). ANOVA was performed to compare between U2OS and EH1 data groups.

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Figure 6. Flavopiridol does not affect the level of the AUF1 protein. HFSN1 cells were treated with 0.3 and 0.5 μM flavopiridol for 24 hrs. Whole cell extracts were prepared and used for western blot analysis using the indicated antibodies.

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| Affymetrix probesets | Gene Fold Change | Gene Symbol | Gene Name | Biological Function |
|----------------------|------------------|-------------|-----------|---------------------|
| 208051_s_at          | 3.29873          | PAIP1       | Polyadenylate binding protein-interacting protein 1 | Regulation of mRNA and protein stability and metabolism |
| 201019_s_at          | 2.02846          | EIF1A       | Eukaryotic translation initiation factor 1A | Regulation of cellular protein metabolic process and translation |
| 203316_s_at          | -3.2073          | RPE         | Ribose-5-phosphate-3-epimerase | RNA splicing |
| 202017_s_at          | -2.00067         | EPHX1       | Epoxide hydrolase 1, microsomal (xenobiotic) | Response to toxin |
| 208808_s_at          | 2.47779          | HMGB2       | High-mobility group box 2 | DNA packaging, gonad development, negative regulation of macromolecule metabolic process |
| 205394_at            | 2.08141          | CHEK1       | CHEK1 checkpoint homolog (S.pombe) | G2/M transition of mitotic cell cycle, protein phosphorylation, DNA damage response |
| 202870_s_at          | 4.46627          | CDC20       | CDC20 cell division cycle 20 homolog (S.cerevisiae) | Proteasomal ubiquitin-dependent protein catabolic process |
| 228940_at            | -2.1415          | NDUFB5      | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5 | Response to oxidative stress |
| 211713_x_at          | 2.71946          | KIAA0101    | KIAA0101 gene product | Skeletal system development |
| 201344_at            | 2.30847          | UBE2V2      | Ubiquitin-conjugating enzyme E2 variant 2 | Chromatin organization, ubiquitin-dependent protein catabolic process |
| 213661_at            | -3.30605         | DFKZP547E2110 | Anti-silencing function 1A | Cell cycle regulation |
| 204905_s_at          | 2.1388           | EEF1A1      | Eukaryotic translation elongation factor 1alpha 1 | Positive regulation of response to stimulus |
| 201437_s_at          | 2.71295          | EIF4A2      | Eukaryotic translation initiation factor 4A, isoform 2 | Posttranscriptional regulation of gene expression |
| 213004_at            | -3.30579         | ANGPTL2     | Angiopoietin-like 2 | Negative regulation of programmed cell death |
| 1555882_at           | -2.45564         | SPINL       | Spinster-like protein | Spindle organization |
| 40524_at             | -2.20011         | PTPN2       | Protein tyrosine phosphatase, non-receptor type 2 | Enzyme linked receptor protein signaling pathway |
| 1559263_s_at         | 2.2216           | PPIL4       | Peptidylprolyl isomerase (cyclophilin)-like 4 | RNA processing |
| 214500_at            | 4.17384          | H2AFY       | H2A histone family, member Y | Cellular response to stress and DNA damage |
| 204023_at            | 2.53988          | RFC4        | Replication factor c (activato 1) 4, 37kDa | Nucleotide-excision repair, response to DNA damage |
| 202094_at            | 4.22242          | BIRC5       | Baculoviral IAP repeat-containing 5 (survivin) | G2/M transition of mitotic cell cycle, Apoptosis regulation |
| 1555495_s_at         | 2.0944           | SDCAG16     | Serologically defined colon cancer antigen 16 | Response to toxin |
| 211985_s_at          | 2.46326          | CALM2       | Calmodulin 2 (phosphorylase kinase, delta) | Response to calcium ion, regulation of cell division |
| 202274_at            | -2.14338         | ACTG1       | Actin, gamma 1 | Vascular process in circulatory system |
| 208969_at            | 2.1346          | NDUFA5      | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5 | Response to oxidative stress |
| 201433_s_at          | 2.11347          | PTDSS1      | Phosphatidyserine synthase 1 | Regulation of cell proliferation |
| 236561_at            | -2.11136         | TGFBI1      | Transforming growth factor beta 1 induced transcript 1 | Respiratory system development |
| 203396_at            | 2.04501          | PSMA1       | Proteasome subunit, alpha type 1 | Regulation of protein modification and metabolism |
| 209988_s_at          | -2.21421         | ASC1L       | Achaete-scute complex-like 1 (Drosophila) | Cell proliferation, positive regulation of transcription |
| 218350_s_at          | 2.54972          | GMNN        | Geminin, DNA replication inhibitor | Regulation of DNA replication and cell cycle |
| 203180_at            | -2.38611         | ALDH3A1     | Aldehyde dehydrogenase 3 family, member A1 | Regulation of apoptosis |
| 1554161_at           | 2.71614          | SLCSA17     | Solute carrier family 25 member 17 | Response to inorganic substance, nucleotide biosynthesis |
| 214060_at            | -3.90345         | SSBP1       | Single-stranded DNA binding protein | DNA replication, cellular component morphogenesis |
| 242210_at            | -2.99625         | ZNF24       | Zinc finger protein 24 (KDX 17) | Regulation of transcription |
| 201120_s_at          | -2.33754         | PGRMC2      | Progesterone receptor membrane component 2 | Regulation of cell adhesion |
| 1553313_s_at         | -3.15507         | SLC5A6      | Solute carrier family 5 member 6 | Response to calcium ion |
| 203152_at            | 2.02172          | MRPL4       | Mitochondrial ribosomal protein L4 | RNA processing, translation |
| 2028_s_at            | -1.26292         | E2F1*       | E2F1 transcription factor 1 | Regulation of cell cycle, regulation of transcription |
| 208712_s_at          | -1.68361         | CCND1*      | CyclinD1 | Cell cycle checkpoint |
| 211382_x_at          | -3.10006         | CDK11C      | P27.KIP2 | Regulation of kinase activity |
| 227299_s_at          | 2.37098          | CCNI        | Cyclin I | Regulation of cell cycle process |
| 210609_s_at          | 2.3745           | TP53I3      | Tumour protein p53 inducible protein 3 | Positive regulation of programmed cell death |
| 225912_at            | -2.22501         | TP53N1P1    | Tumour protein p53 inducible nuclear protein 1 | Induction of apoptosis |
| 210734_x_at          | -2.47494         | MAX         | MYC associated factor x | RNA biosynthetic process |
| 1567457_at           | 2.40059          | RAC1        | Ras-related C3 botulinum toxin substrate 1 | Regulation of phosphoinositide 3-kinase activity |
AUF1, which is essentially nuclear as previously described [30,33], showed significantly higher levels in the cytoplasmic and the nuclear extracts of p16-defective cells than in their p16-proficient isogenic counterpart cells (Figure 3A). This suggests a role of p16 in the expression of the RNA binding AUF1 protein.

Next, we explored the effect of p16 on the expression of the AUF1 mRNA. To this end, total RNA was extracted from HFSN1 cells expressing p16-siRNA or control-siRNA as well as from U2OS and its derivatives EH1, EH2 and also from IPTG-treated EH2 cells. RT-PCR utilizing specific primers was used for the assessment of the AUF1 and CDKN2A mRNAs. Figure 3B shows an inverse correlation between the expression levels of the CDKN2A and AUF1 mRNAs. Indeed, the AUF1 mRNA level is much higher in p16-defective cells (p16-siRNA, U2OS) than in p16-proficient cells (control, EH1, EH2) (Figure 3B). To further elucidate this inverse relationship, EH2 cells were treated with 1 mM of IPTG, which led to 2.5 fold induction in the AUF1 mRNA level (Figure 3B). The increase in the expression level of AUF1 in p16-siRNA expressing cells was confirmed by quantitative real-time RT-PCR (Figure 1D, lower panel). This indicates that p16 negatively regulates the AUF1 mRNA.

To investigate whether p16 controls the stability of the AUF1 mRNA, HFSN1 cells expressing either p16-siRNA or control-siRNA were treated with actinomycin D and then reincubated for different periods of time (0-6 hrs). Total RNA was purified and the mRNA level of AUF1 was assessed by real time RT-PCR. Figure 3C shows that while the AUF1 mRNA half-life in the control cells is 1 hr and 50 min, it reached 4 hrs and 45 min in the AUF1-siRNA-expressing cells, indicating that p16 plays a major role in the AUF1 mRNA decay. Together, these results show that p16 negatively controls the expression of AUF1 by regulating its mRNA turn-over.

The E2F1 3’UTR harbors functional AU-rich elements

In order to search for functional mRNA destability element in the E2F1 3’UTR, we analyzed the 3’UTR sequence, which is 1245 nt long, for the presence of conserved and AU-rich element clusters (Figure 5A). Both regions 2 and 3 contain two overlapping AUUUA pentameric repeats (Figure 5A, right panel). These regions were highly conserved among different species (Figure 5B). In order to evaluate the contribution of each of these regions in the E2F1 mRNA instability, we cloned each of these regions in a vector containing the stable bovine growth hormone (BGH) 3’UTR downstream of the EGFP reporter (Figure 5C, upper panel), and Huh-7 cells were transfected with the three constructs.

Next, we investigated the effect of AUF1 down-regulation on the mRNAs levels of E2F1 and cyclin D1 using both RT-PCR and real time RT-PCR. Figure 4A shows that while the level of AUF1 mRNA was significantly reduced in the AUF1-siRNA-expressing cells, the expression level of the E2F1 and cyclin D1 mRNAs increased significantly in these cells, as compared to the control cells. These results were confirmed by quantitative real-time RT-PCR (Figure 4A, lower panel). This provides the first indication that the E2F1 mRNA is also a potential target of AUF1.

Furthermore, we studied the effect of p16 on the binding of AUF1 to the E2F1 and cyclin D1 mRNAs using HFSN1 cells expressing either p16-siRNA or control-siRNA. AUF1-mRNAs ribonucleoprotein complexes were obtained by immunoprecipitation (IP) using anti-AUF1 antibody and were used for RT-PCR amplification using specific primers. Figure 4B shows amplification of cyclin D1 mRNA and also E2F1 mRNA, indicating the binding of AUF1 protein to E2F1 mRNA. Importantly, the levels of the E2F1 and cyclin D1 mRNAs that were bound to AUF1 were higher in p16-siRNA-expressing cells than in the control cells. This increase correlated with the increase in the immunoprecipitated AUF1 protein in p16-siRNA expressing cells (Figure 4C), confirming what has been shown in figure 3A, and indicating that AUF1 binds specifically to these mRNAs in a p16-dependent manner. This shows that AUF1 plays a major role in mediating the effect of p16 on the expression of E2F1 and cyclin D1.

To further elucidate this role of AUF1, a double knock-down (p16 and AUF1) experiment was performed using specific siRNAs in HFSN1 cells. Total RNA was purified and amplified by RT-PCR using specific primers. Figure 4D shows that while the levels of the cyclin D1 and E2F1 mRNAs decreased in p16-deficient cells as compared to the control cells, their levels became normal when AUF1 was down-regulated in p16-defective cells. Similarly, when AUF1 was knocked-down in the p16-defective U2OS cells, the expression of the two genes increased to a level similar to that observed in p16-proficient EH1 cells (Figure 4D). Together, these data strongly indicate that p16 positively controls the expression of the cyclin D1 and E2F1 mRNAs through negative regulation of AUF1.

AUF1 controls E2F1 expression in an ARE-dependent manner

Since AUF1 binds the E2F1 mRNA and activates its decay (Figure 4), we sought to further confirm that AUF1 controls E2F1 mRNA decay through AREs. To this end, total RNA was extracted from HFSN1 cells expressing AUF1-siRNA and control-siRNA. AUF1-mRNAs were amplified by RT-PCR using specific primers. Figure 4B shows amplification of cyclin D1 mRNA and also E2F1 mRNA, indicating the binding of AUF1 protein to E2F1 mRNA. Importantly, the levels of the E2F1 and cyclin D1 mRNAs that were bound to AUF1 were higher in p16-siRNA-expressing cells than in the control cells. This increase correlated with the increase in the immunoprecipitated AUF1 protein in p16-siRNA expressing cells (Figure 4C), confirming what has been shown in figure 3A, and indicating that AUF1 binds specifically to these mRNAs in a p16-dependent manner. This shows that AUF1 plays a major role in mediating the effect of p16 on the expression of E2F1 and cyclin D1.

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The E2F1 3’UTR harbors functional AU-rich elements

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The reporter activity fused to the 3’UTR with the E2F1 AU-rich regions 2 and 3 was significantly reduced (46% and 64%, respectively) as compared to the control 3’UTR that contains no ARE. On the other hand, the region 1 had no effect (Figure 5C, lower panel). As a positive control, the IL-9 ARE-3’UTR caused 78% reduction of the reporter activity. This EGFP reporter activity system correlated well with ARE- mRNA decay patterns in several cellular models [32,34,35]. These results suggest that the E2F1 3’UTR bears functional AREs.

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UTR-linked activity in an ARE-dependent manner, using the two E2F1 ARE-3'UTR constructs that reduced the reporter activity. To this end, we down-regulated the AUF1 expression using specific siRNA in the Huh cell line. Figure 5D shows that AUF1 silencing increased the activity of the reporter expressed from the E2F1 ARE-3'UTR fused constructs. Similarly, as ARE-

Table 2. List of genes under the control of both p16 and E2F1.

| Affymetrix probesets | Gene Fold Change | Gene Symbol | Gene Name | Biological Function |
|----------------------|------------------|-------------|-----------|---------------------|
| 211464_x_at          | -2.108381        | Casp6       | Caspase 6, apoptosis-related cysteine peptidase | Regulation of protein localization, regulation of apoptosis |
| 205681_at            | 6.50843          | Bcl-2       | B-cell CLL/lymphoma 2 | Negative regulation of apoptosis |
| 1861_at              | -2.12347         | BAD         | BCL2-associated agonist of cell death | Positive regulation of apoptosis |
| 207535_s_at          | 3.67377          | NfKb       | Nuclear factor of kappa light polypeptide gene enhancer in B-cells (p65/p100) | Adaptive immune response, extracellular matrix organization |
| 211828_s_at          | -2.29209         | TRAF2       | TNF receptor-associated factor 2 | RNA splicing |
| 208712_at            | -1.66361         | CCND1       | CyclinD1 | Cell cycle checkpoint |
| 205034_at            | 7.27143          | CCNE2       | CyclinE | Regulation of cyclin-dependent protein kinase activity, cell cycle checkpoint |
| 213226_at            | 4.05707          | CCNA        | CyclinA | G2/M transition DNA damage checkpoint |
| 202534_x_at          | 5.08339          | DHFR        | Dihydrofolate reductase | Nucleotide biosynthetic process |
| 214056_at            | 2.07083          | Mc11        | Myeloid cell leukemia sequence 1 (BCL2-related) | Response to cytokine stimulus, cell fate determination, negative regulation of cell death |
| 200893_at            | 2.26377          | SFRS10      | Splicing factor, arginine/serine-rich 10 (transformer 2 homolog, Drosophila) | RNA processing |
| 201129_at            | 2.59299          | SFRS7 (SR protein 9G8) | Splicing factor arginine/serine-rich 7, 35kDa | RNA processing |
| 201586_s_at          | 3.57618          | SFPQ (PSF)  | Splicing factor proline/glutamine-rich (polypurine tract binding protein) | DNA recombination |
| 1552627_a_at         | -2.37653         | ARHGAP4     | Rho GTPase activating protein 4 | Positive regulation of mesenchymal cell proliferation, regulation of cell migration |
| 205024_s_at          | 3.03581          | RAD52       | RAD52 homolog (RecA homolog, E. coli) | Sister chromatid cohesion, cellular response to stress |
| 204127_at            | 4.59846          | RFC3        | Replication factor C (activator 1) 3, 38kDa | DNA strand elongation during DNA replication, nucleotide-excision repair, response to toxin |
| 223746_at            | -2.01097         | STK15       | Serine/threonine kinase 15 | Protein amino acid autophosphorylation, regulation of apoptosis |
| 221085_at            | 7.83536          | TNF3F9      | Tumor necrosis factor (ligand) superfamily, member 9 | Tumor necrosis factor receptor binding, cytokine activity |
| 213575_s_at          | -2.77451         | TRA1        | Transformer-1 | mRNA binding and splicing |
| 210563_x_at          | -2.18188         | CFLAR (FLIP) | CASP8 and FADD-like apoptosis regulator | Cysteine-type peptidase activity |
| 205192_at            | -2.13176         | MAP3K14     | Mitotogen-activated protein kinase kinase kinase 14 | Cell cycle regulation, protein amino acid phosphorylation |
| 221695_s_at          | -2.9927          | MAP3K5      | Mitotogen-activated protein kinase kinase kinase 5 | Cell cycle regulation, protein amino acid phosphorylation |
| 208378_x_at          | -2.17358         | FGF-2       | Fibroblast growth factor 2 | Regulation of cell division |
| 210973_s_at          | -2.04823         | FGF3        | Fibroblast growth factor receptor 2 | Positive regulation of cell proliferation |
| 217279_x_at          | -2.00664         | MMP16       | Matrix metalloproteinase 16 (membrane-inserted) | Regulation of cell adhesion |
| 211527_x_at          | -4.52681         | VEGF-B      | Vascular endothelial growth factor B | Regulation of cell division, transmembrane receptor protein tyrosine kinase signaling pathway, positive regulation of gene expression |
| 201005_s_at          | -2.97704         | CD9         | CD9 antigen (p24) | Wound healing, cellular component morphogenesis, negative regulation of cell proliferation |
| 217294_s_at          | -7.39815         | ENO2        | Enolase 2 | Hydro-lase activity, protein and nucleic acid binding, transcription cofactor activity, metabolism |
| 225669_at            | 2.08248          | IFNA2       | Interferon (alpha, beta and omega) 2 | Posttranscriptional regulation of gene expression, cell cycle arrest, cell death |
| 228325_at            | -3.80088         | KIAA0455    | KIAA0455 | Skeletal system development |
| 224762_at            | 2.14831          | KIAA0767    | KIAA0767 | Skeletal system development |
| 202283_s_at          | -2.59665         | SERPINF2    | Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium) | Regulation of inflammatory response |
| 208905_at            | 2.32975          | CYCS        | Cytochrome c, somatic | Cellular macromolecule catabolic process |

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Cdk4 and Cdk6 were halved in response to 0.5 μM AUF1 by immunoblotting. Figure 6 shows that while the levels of Cdk4, Cdk6 and p16 were prepared and used to assess the level of Cdk4, Cdk6 and p16, and Figure 7 shows that the AUF1 protein is involved in the post-transcriptional control of AUF1 mRNA decay.

The mRNA levels of 11 genes coregulated by both p16 and AUF1 were analyzed by quantitative RT-PCR in HFSN1 cells expressing either p16-siRNA or control-siRNA, and are expressed as fold difference. The error bars represent standard deviation of three different values. *: The fold difference in the expression of these genes was less than 2 in the array data. doi:10.1371/journal.pone.0021111.g007

p16 controls E2F1 expression via ARE-mediated events

To further elucidate the link between p16, AUF1 and E2F1, we assessed the reporter activities described above in both p16-proficient (EH1) and p16-deficient (U2OS) cell lines. Importantly, the reporter activities, for both regions 2 and 3, were more than 12 fold higher in EH1 than in U2OS cells (Figure 5E). This shows the effect of p16 on these activities, and further proofs the role of p16 in the control of E2F1 mRNA decay.

Flavopiridol-dependent inhibition of Cdk4/Cdk6 protein kinases does not affect the expression level of AUF1

The two major targets of p16 are the protein kinases Cdk4 and Cdk6. Thereby, we sought to investigate the possible role of these proteins in mediating p16 effect on AUF1. Previous studies have indicated that flavopiridol inhibits the kinase activities of both Cdk4 and Cdk6 [36,37]. Therefore, HFSN1 cells were treated with flavopiridol (0.5 and 0.5 μM) for 24 hrs. Whole cell extracts were prepared and used to assess the level of Cdk4, Cdk6 and AUF1 by immunoblotting. Figure 6 shows that while the levels of Cdk4 and Cdk6 were halved in response to 0.5 μM flavopiridol, the level of AUF1 remained unchanged. This suggests that Cdk4 and Cdk6 may not be involved in the negative regulation of AUF1 by p16.

p16 regulates the expression of several genes under the control of E2F1 or AUF1

The fact that p16 negatively controls the ubiquitous RNA-binding protein AUF1 and the transcription factor E2F1 suggests that p16 might control the expression of several genes. To test this hypothesis, we used oligonucleotide gene expression microarrays to identify genes whose expression is modulated in a p16-dependent manner. Therefore, total RNA was isolated from both p16-siRNA-expressing cells and their control counterparts and was hybridized to microarrays. The difference in gene expression was considered significant only when the variation was ≥2.00. Interestingly, the knocking-down of p16 modulated the expression of 2170 genes, with 1010 (46.5%) were up-regulated and 1160 (53.4%) were down-regulated. Most of these genes (1501) are involved in cell cycle regulation, and the others are implicated in various cancer-related mechanisms such as apoptosis, DNA repair, senescence, angiogenesis and aging. Importantly, the array data showed 4 fold increase in the expression of AUF1 in p16-deficient cells and 46 genes known to be AUF1 targets [38] are also under the control of p16 (Table 1). In addition, 33 genes known to be E2F1 targets [39] are also under the control of p16 (Table 2). To validate the microarray results, the expression level of 11 genes was assessed by quantitative RT-PCR. Figure 7 shows that the expression of all the analyzed genes is modulated in a p16-dependent manner, confirming the microarray data. This shows that the expression of a large number of genes is indeed under the control of the tumor suppressor p16 protein.

p16-dependent modulation of E2F1 affects doxorubicin-induced apoptosis and apoptotic proteins

Next, we studied the effect of p16-dependent modulation of E2F1 level on the expression of apoptotic-regulatory proteins, known to be under the control of E2F1 [40]. Figure 8A shows that the levels of the pro-apoptotic proteins Bax and cleaved Caspase-3 are respectively 2.4 and 6.5 fold higher in EH1 as compared to U2OS. On the other hand, the expression levels of the anti-apoptotic proteins Bcl-2, Bcl-XL and NF-xB were respectively 1.6, 2.6 and 18 fold lower in EH1 as compared to U2OS. This indicates that the expression level of various apoptosis proteins is modulated in a p16-dependent manner. To show that these variations are E2F1-dependent, E2F1 was ectopically introduced into the p16-defective U2OS cells and whole cell extracts were prepared from U2OS and U2OS expressing either the control plasmid or E2F1-expressing plasmid. Figure 8B shows that the expression level of E2F1 increased 3.4 fold in E2F1-expressing U2OS cells as compared to the control. Consequently, there was a clear correlation between the E2F1 expression level and the expression of the pro- and anti-apoptotic proteins. In E2F1-expressing U2OS cells, the expression levels of Bax and cleaved Caspase-3 were 2.2 and 2.9 fold higher than in the control cells, while the expression levels of Bcl-2, Bcl-XL and NF-xB were 2.5, 2.8 and 2.6 fold lower than in the control, respectively (Figure 8B). This suggests that p16-dependent modulation of the expression of the apoptotic genes could be mediated through E2F1.

To further investigate this, U2OS cells expressing control plasmid or E2F1-expressing plasmid as well as the p16-proficient isogenic EH1 cells were either sham-treated or challenged with different doses of doxorubicin for 3 days, and the level of apoptosis was assessed by Annexin V/flow cytometry. Figure 8C and Figure 8D show that the proportion of spontaneous apoptosis in U2OS cells (2.14%) was enhanced by the over-expression of E2F1 (26.29%). Similarly, doxorubicin-induced apoptosis was higher in U2OS cells expressing E2F1 than in the control cells (Figure 8C and Figure 8D). Indeed, while apoptosis in response to doxorubicin was only 20% in U2OS cells, it reached about 45% in EH1 cells and 66% in U2OS cells wherein E2F1 was over-expressed (Figure 8D). This indicates that the expression of E2F1 in p16-defective cells restores p16-related apoptosis, which
suggests that the apoptotic effect of p16 is mediated through the E2F1 transcription factor. To confirm this, we investigated the effect of p16 and doxorubicin on the level of pro- and anti-apoptotic proteins in U2OS, EH1 and U2OS expressing E2F1. Figure 8E shows that doxorubicin treatment (2 μM for 3 days) up-regulated the E2F1 protein in the 3 different cell lines. However, the level of E2F1 is more than 2 fold higher in EH1 and U2OS expressing E2F1 than in U2OS. Importantly, doxorubicin effect on the cleaved caspase-3 and Bax levels was only marginal in U2OS, but it led to 2 fold increase in the p16-proficient EH1 cells and U2OS expressing E2F1 as compared to their respective non-treated cells (Figure 8E). Concomitantly, the levels of NF-κB, Bcl-2 and Bcl-xL were reduced in EH1 and U2OS-expressing E2F1 cells as compared to their levels in U2OS, and doxorubicin treatment decreased their levels in the 3 cell lines, but its effect was more pronounced in p16-proficient and E2F1-expressing U2OS cells (Figure 8E). Furthermore, the level of the Bax/Bcl-2 ratio significantly increased in p16-proficient EH1 cells and also in p16-deficient cells expressing E2F1 as compared to p16-deficient U2OS cells (Figure 8F). This further shows the role of p16 in inducing doxorubicin-dependent apoptosis, and that E2F1 over-expression overcomes p16-deficiency in this process.

Discussion

In the present report we have shown that p16 is not only a cell cycle checkpoint protein, but it is also a master regulator of gene expression in absence of cellular stresses. Indeed, p16 negatively regulates the ubiquitous RNA-binding protein AUF1 through controlling the turn-over of its mRNA. Furthermore, p16 positively regulates the expression of cyclin D1 and the transcription factor E2F1 at the post-transcriptional level. We have also shown the presence of 3 AU-rich element clusters in the E2F1 mRNA (Figure 5), which provides the first indication that the oncogene/tumor suppressor gene E2F1 codes for an ARE-mRNA that is under the control of AUF1. It is noteworthy that the
status of p16 had no effect on the level of the cell cycle regulated HuR protein [Figure 3A] and also on the cell proliferation protein PCNA [Figure 1A], which ruled out the possible implication of the cell cycle, and indicates that the modulation in the expression of cyclin D1 and E2F1 is rather p16-dependent. The fact that AUF1 negatively controls p16 [41], points to the presence of a negative feedback loop between the tumor suppressor p16 and the RNA binding AUF1 protein [Figure 9]. While AUF1 is known to regulate p16 through binding and promoting the destabilization of its mRNA [41,42], it still unclear how p16 regulates the expression of AUF1. We have shown here that p16 controls the stability of the AUF1 mRNA. However, p16 is not known to be an RNA binding protein. Therefore, we hypothesized that p16 could affect the AUF1 mRNA decay through one of its major targets Cdk4 and Cdk6 [7]. However, the inhibition of both kinases by Flavopiridol did not affect the level of the AUF1 protein [Figure 6], suggesting that p16-dependent regulation of AUF1 may not be mediated through Cdk4 or Cdk6. Consequently, it is possible that p16 regulates AUF1 expression through another RNA-binding protein or through specific miRNA(s). We are currently investigating these two possibilities to elucidate the exact mechanism(s) whereby p16 controls AUF1 mRNA turn-over.

Concerning the link between p16 and cyclin D1, Wang et al. have previously shown that down-regulation of AUF1 by specific siRNA up-regulated p16 protein and concomitantly increased the level of cyclin D1 [41]. Furthermore, it has been shown that in senescent cells there is a concomitant increase in the levels of both p16 and cyclin D1, while cyclin A and cyclin B1 levels decrease [41,43]. These data are in keeping with the results presented here, showing that cyclin D1 expression is modulated in a p16-dependent manner at least in human skin fibroblasts. In cancer cells, in addition to the osteosarcoma U2OS cells, DOAY and other p16-defective medulloblastoma cells also showed undetectable or very low levels of cyclin D1 (data not shown), which further supports this link between p16 and cyclin D1.

The fact that p16 controls the expression of both AUF1 and E2F1, two important transcription regulators, suggested that this CDK1 can also modulate the expression of a plethora of genes. Using microarray analysis we have shown that p16 controls the expression of more than 2000 genes involved in various cellular metabolisms, and most of them are implicated in cell proliferation and carcinogenesis. Importantly, 46 of these genes are members of the AUF1 regulon, and 35 genes are co-regulated by p16 and E2F1 (Table 1 and Table 2). Among these genes there is another master transcription regulator, NF-κB. The mRNA and protein levels of this gene increased 3.6 fold and 18 fold in p16-deficient cells as compared to their respective controls, respectively (Figure 8A and Table 2). This effect seems to be E2F1-dependent since the overexpression of E2F1 decreased the level of NF-κB in p16-deficient U2OS cells (Figure 8B). Together, these results suggest that p16 exerts a negative control on the expression of NF-κB, through the transcription factor E2F1. This relationship between p16 and NF-κB is of great importance since NF-κB regulates the expression of a large number of genes important for apoptosis, tumorigenesis, viral replication, inflammation and various immune responses [44], which could suggest a key role of p16 in all these processes.

Annexin A2 (ANXA2) is another important cancer gene under the control of p16 and AUF1 (Table 1). The level of the ANXA2 gene increased 2 fold in p16-deficient cells, and it is known that its expression is elevated in a wide range of cancers [45], including melanomas wherein p16 is usually inactive [14,46]. This suggests an important role of p16 in the increase in the ANXA2 expression level in cancer cells.

What are the consequences of p16-dependent modulation of E2F1 expression level on cell proliferation and/or cell death? In fact, the modulation in p16/E2F1 expression did not affect cell proliferation nor the expression of PCNA (Figure 1A), which is under direct control of E2F1 [40]. The role of E2F1 in apoptosis is attributed mainly to E2F1-dependent up-regulation of various pro-apoptotic genes, like caspases and Bax and/or inhibiting survival signals, in particular those mediated by the transcription factor NF-κB or Bcl-2 [47]. Importantly, we have found that p16 is also a positive regulator of the pro-apoptotic protein Bax and an inhibitor of the anti-apoptotic proteins Bcl-2, Bcl-xL and NF-κB. This effect is E2F1-mediated, since the over-expression of this protein restored the normal expression of these apoptosis-related proteins in p16-defective cells. This suggests that p16 is also a modulator of apoptosis through the E2F1 protein (Figure 9). To further elucidate this, we have shown that the over-expression of

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**Figure 9. Schematic representation of the p16-related regulation of AUF1, E2F1 and CyclinD1.** See text for details. doi:10.1371/journal.pone.0021111.g009
the E2F1 protein enhances spontaneous as well as doxorubicin-induced apoptosis in the osteosarcoma p16-defective U2OS cells. Similar results were obtained by the ectopic expression of p16 in the U2OS cells (EH1), indicating that p16 sensitizes the osteosarcoma cells to doxorubicin through E2F1. Furthermore, we have previously shown that p16 modulates apoptosis in response to both UV light and cisplatin [23]. Together, these results show that, like E2F1, p16 plays also a major role not only in cell growth but also in cell death [5].

In summary, these results provide the first clear indication that the cyclin-dependent kinase inhibitor p16 protein also modulates the expression of several key transcription regulators such as AUFI, E2F1 and NF-kB, and other important genes directly or indirectly involved in cancer development and spread. This sheds more light on the molecular basis of the tumor suppressor function of this protein.

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Author Contributions

Conceived and designed the experiments: AA KSK HHA DC NK. Performed the experiments: HHA AAB SFH NAY. Analyzed the data: AA KSK HHA DC NK. Contributed reagents/materials/analysis tools: HHA SFH NKDC. Wrote the paper: AA KSK HHA DC NK.

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