p15PAF Is an Rb/E2F-Regulated S-Phase Protein Essential for DNA Synthesis and Cell Cycle Progression

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Abstract

The p15PAF/KIAA0101 protein is a proliferating cell nuclear antigen (PCNA)-associated protein overexpressed in multiple types of cancer. Attenuation of p15PAF expression leads to modifications in the DNA repair process, rendering cells more sensitive to ultraviolet-induced cell death. In this study, we identified that p15PAF expression peaks during the S phase of the cell cycle. We observed that p15PAF knockdown markedly inhibited cell proliferation, S-phase progression, and DNA synthesis. Depletion of p15PAF resulted in p21 upregulation, especially chromatin-bound p21. We further identified that the p15PAF promoter contains 3 E2F-binding motifs. Loss of Rb-mediated transcriptional repression resulted in upregulated p15PAF expression. Binding of E2F4 and E2F6 to the p15PAF promoter caused transcriptional repression. Overall, these results indicate that p15PAF is tightly regulated by the Rb/E2F complex. Loss of Rb/E2F-mediated repression during the G1/S transition phase leads to p15PAF upregulation, which facilitates DNA synthesis and S-phase progression.

Introduction

The p15PAF protein, also known as KIAA0101 or OEATC-1, is a 15-kDa nuclear protein initially identified using a yeast two hybrid screen for proteins that bind to proliferating cell nuclear antigen (PCNA) [1]. p15PAF binds to PCNA through the conserved PCNA-interacting protein motif (PIP box, QxxL/I/ M)x[F/Y][F/Y] at amino acids 62-69 (62-QKGIGEFF-69) [1]. No other functional domains or motifs have been identified using bioinformatic methods. p15PAF is overexpressed in multiple types of human cancer, including hepatocellular carcinoma [2], lung cancer [3], breast cancer [4], and pancreatic cancer [5], and its overexpression is associated with poor patient outcome [2-4]. Overexpression of p15PAF promotes cell growth, whereas attenuation of expression by small interfering RNA (siRNA) leads to reduced cell proliferation [5]. These results indicate that p15PAF has a growth-promoting role; however, the molecular mechanisms underlying its effects have yet to be identified.

p15PAF was reported to play a role in DNA repair. In a study by Simpson et al., p15PAF expression was upregulated in response to ultraviolet (UV) irradiation. Besides, the associations of p15PAF and p33ING1b with PCNA were enhanced after UV irradiation [6]. Similar to p15PAF, p33ING1b is a PCNA-interacting protein, and is an isoform encoded by the ING1 tumor suppressor locus. Overexpression of p33ING1b confers increased efficiency of repair of UV-damaged DNA in melanoma cells, and p53 is required for the repair process [7]. Overexpression of p15PAF also protects cells from UV-induced cell death [6]. p15PAF is a direct transcriptional target of ATF3. ATF3 and p15PAF expression are sufficient to trigger the DNA repair machinery against UV damage [8]. p15PAF was also reported to interact with BRCA1 and regulate the centrosome number [9]; however, it is not yet known if p15PAF is a component of the double-strand break repair pathway.

Several PCNA-interacting proteins are regulators of cell cycle progression or components of DNA synthesis machinery [10]. In this study, we evaluated the role of p15PAF in cell cycle progression and DNA synthesis, and showed that p15PAF is a direct transcriptional target of the Rb/E2F pathway.

Materials and Methods

Immunohistochemical stain

The tissue distribution of p15PAF protein was evaluated using a tissue array containing adult tissues of major organs, obtained from normal regions of surgically resected patient specimens. A 2 mm tissue core was removed for each organ using a manual tissue array device (Beecher Instruments, Silver Spring, MD, USA) and inserted into a recipient paraffin block. The arrayed tissues were cut into 4 μm slices and placed on positively charged slides. Tissue sections were dewaxed and rehydrated. Antigen retrieval was performed by incubating slides in 0.01 M citric acid buffer (pH 6.0) at 100 °C for 10 min. After blocking with 3% hydrogen peroxide and 5% fetal bovine serum (FBS), slides were incubated with a monoclonal antibody against p15PAF (Abnova, Taipei, Taiwan) at 1:500 dilution at 4 °C overnight. Slides were then incubated with polymer-horseradish peroxidase (HRP) reagent (BioGenex, San Ramon, CA, USA). Peroxidase activity was visualized using diaminobenzidine tetrahydrochloride solution.
Sections were counterstained with hematoxylin. Dark brown nuclear staining was defined as positive, and no staining was defined as negative. For negative controls, the primary antibody was replaced with 5% FBS. This study was approved by the Research Ethical Committee of National Taiwan University Hospital. Written informed consent was obtained from all patients.

Cell culture and treatment
HeLa cells, MCF7 cells, and viral package 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS in an incubator at 37 °C, with a humidified atmosphere of 95% air and 5% carbon dioxide (CO2). For synchronization of the cell cycle, HeLa cells were treated with 2 mM thymidine (Sigma-Aldrich, St. Louis, MO, USA) for 14 h and then washed 3 times with phosphate buffered saline (PBS). They were then incubated in normal growth medium for 10 h prior to treatment with aphidicolin (5 μg/mL; Sigma-Aldrich) or thymidine (2 mM) for an additional 14 h to arrest cells at the G1/S boundary.

Cell proliferation assay
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay was used to evaluate the rate of cell proliferation. This colorimetric assay measures the activity of cellular enzymes that reduce MTT dye to insoluble MTT formazan, giving a purple color. One-thousand living cells were seeded into 96-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO2. After an appropriate time interval, MTT was added and incubated for 4 h. The resulting color reaction product was extracted using dimethyl sulfoxide and absorbance was measured at 570 nm.

RNA interference
For the knockdown of endogenous p15PAF, the target sequences p15-4: 5'-GCAACCTGTACACAAATGA-3' and p15-5: 5'-GCTTTGTGAGGCAACCTTATTA-3' were constructed in the shRNA vector pLKO.1. An shRNA vector against luciferase (pLKO.1-shLuc) was used as a negative control. For lentiviral production, 293T cells were transfected with 4 μg plKO.1 lentiviral vector, along with 0.4 μg envelope plasmid pMD.G and 3.6 μg packaging plasmid pCMVΔR8.91. Viruses were collected 24 h and 48 h post-transfection. To prepare p15PAF knockdown cells, HeLa cells were infected with the lentivirus for 24 h. Fresh medium containing 2 μg/mL puromycin (Sigma-Aldrich) was added for 2 d for drug-resistant cell selection.

BrdU incorporation assay
Incorporation of BrdU during the S phase was evaluated using the BD BrdU FITC Assay Kit (BD Biosciences) according to the manufacturer’s instructions.

Immunofluorescence
The HeLa cells were seeded onto coverslips at 60%–80% confluency and incubated overnight. After washing twice with PBS, cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% PBS with Tween 20 (PBST) for 10 min, and then blocked in PBS containing 0.5% bovine serum albumin (BSA) for 30 min, and incubated with the anti-p15PAF antibody (1:5000, Abnova) at 4 °C overnight. Cells were washed 3 times with 0.1% PBS and then incubated with goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody coupled to Alexa® 488 or Alexa® 594 dyes (1:1000, Life Technologies) for 1 h. Nuclei were stained using 4',6-diamino-2-phenylindole (DAP) (1 μg/mL). Confocal imaging was performed using a Zeiss LSM 510 META laser-scanning microscope.

For detection of incorporated BrdU, cells were pulsed with BrdU (10 μM, Roche, Basel, Switzerland) for 30 min at 37 °C and harvested at the indicated time points prior to fixation. Cells were fixed with cold methanol for 20 min at −20°C. DNA was denatured by treating with 4 M hydrogen chloride for 15 min and washed 3 times with PBS. Immunofluorescence was performed as described using FITC-anti-BrdU antibody (1:100, AbCam, Cambridge, MA, USA).

Western blotting
Protein samples (50 μg each) were separated using 10%–15% SDS-PAGE and then electrotransferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK). The membranes were allowed to react with the primary and secondary antibodies at optimum dilution, and the immunoreactive signals were detected using an enhanced chemiluminescence kit (Millipore, Bedford, MA, USA). The antibodies used included p15PAF (1:1000, Abnova), p21 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:5000, Sigma-Aldrich), Rb (1:1000, 11D7, generously provided by Dr. Wen-Hwa Lee, University of California, Irvine, CA), cyclin D1 (1:1000, Neomarkers, Fremont, CA, USA), cyclin B1 (1:1000, Neomarkers), and PCNA (1:1000, Neomarkers).

Reporter assay
The p15PAF promoter fragment −208 to +94 bp was obtained using PCR. The primers used were p15PAF/SacI-208: 5’-AGAGCTCATTCTGTGATGCTTTACAGAAAC-3 and p15PAF/HindIII+94: TAAAGTTCCAGCAGAGGTTTTC. The PCR product was digested with SacI and HindIII.
fragments were cloned into a promoterless pGL3-basic vector. Mutations were introduced by site-directed mutagenesis in the putative E2F-binding sites using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The AERK1 and MCL1 promoters, E2F4 and E2F6 expression plasmids, were gifts from Dr. Ju-Ming Wang (Institute of Bioinformatics and Biosignal Transduction, National Cheng Kung University). The RH expression vector pSLX-CMV-Rh was kindly provided by Dr. Ming-Fu Chang (Institute of Biochemistry and Molecular Biology, National Taiwan University). Transient transfection was performed using the Turbofect reagent (Thermo Fisher, Waltham, MA, USA). Reporter constructs were cotransfected with expression vectors of Rb, E2F4, E2F6 or the control plasmid, and the Renilla luciferase plasmid TK-Renilla, into MCF7 cells, which were grown in 12-well plates at 70% confluence. Twenty-four h after transfection, cell extracts were prepared, and luciferase activities were quantified using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) in an Orion II luminometer (Berthold Detection Systems, Pforzheim, Germany). All experiments were performed in triplicate.

Chromatin immunoprecipitation

After culturing to 60%–70% confluence, formaldehyde (1% final concentration) was added for 10 min crosslinking, and then glycine (125 mM final concentration) was added to arrest crosslinking. DNA was sheared by sonication. The sheared chromatin fragments were immunoprecipitated with an antibody specific for E2F4 (Thermo Scientific), E2F6 (Abcam), or control IgG (Santa Cruz) at 4°C for 16 h. The antibody-chromatin complexes were precipitated using protein-G-sepharose slurry (Sigma-Aldrich) at 4°C for 1 h. After dissociation from the immunoprecipitated chromatin, DNA underwent PCR amplification using primers for GAPDH and the putative E2F-binding sites.

Chromatin-extracted immunofluorescence

Control and p15PAF knockdown HeLa cells were seeded onto coverslips at 60%–70% confluence and incubated overnight. After washing twice with PBS, cells were extracted with CSK buffer (10 mM PIPES-KOH (pH 7), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 0.5% Triton X-100) and fixed with 4% paraformaldehyde. After blocking with PBS containing 0.5% BSA for 30 min, the cells were incubated with the indicated primary antibodies at 4°C overnight. They were then washed 3 times with 0.5% PBST and incubated with goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies, coupled to Alexa 488 or Alexa 594 dyes (1:1000, Life Technologies) for 1 h. Nuclei were stained using DAPI (1 μg/mL). Confocal imaging was performed using a Zeiss LSM 510 META laser-scanning microscope. The primary antibodies used were p15PAF (1:5000, Abnova) and p21 (1:500, Santa Cruz).

GST-tagged protein-protein interaction assay

Full-length open-reading frames of p15PAF and p21 were cloned into pET-15b (Novogen, Madison, WI, USA). His-tagged proteins were expressed in E. coli strain BL21 (DE3) (Stratagene) and purified using nickel ion chromatography. pGEX-4T-1 containing the full-length open-reading frame of PCNA was transformed into BL21, and PCNA-GST fusion protein expression was induced using 0.5 mM isopropyl-D-1-thiogalactopyranoside (IPTG) for 4 h at 18°C. The GST fusion proteins were purified using glutathione-sepharose beads (GE Healthcare, Little Chalfont, UK). His-tagged p21 (25 ng) and different amounts of His-tagged p15PAF were then incubated with PCNA-GST fusion protein in binding buffer (PBS containing 1% NP-40 and 2 mM DTT) for 3 h at 4°C. The glutathione-sepharose beads, along with the PCNA-GST fusion protein and binding proteins, were collected by centrifugation (1 min, 16,000 g), washed extensively in binding buffer, and analyzed employing SDS-PAGE and western blotting using anti-p21 antibody.

Results

p15PAF is expressed in proliferative tissues

To evaluate p15PAF tissue distribution, we constructed a tissue array containing nontumorous adult tissues of major organs. Immunohistochemical staining revealed p15PAF nuclear staining in the epidermis, lymph node, endometrium, and small and large intestines, but not in the brain, liver, heart, pancreas, lung, and myometrium (Fig. 1). In the lymph node, p15PAF was specifically expressed in germinal centers. In the small and large intestines, p15PAF was predominantly expressed in the lower half of the epithelial crypts, where proliferative cells reside. In the epidermis, p15PAF was expressed in the suprabasal cells only, which are the major proliferative epidermal cells. This pattern of tissue distribution indicated that p15PAF expression is restricted to proliferative cells.

Figure 1. p15PAF expression in normal adult tissues. Nuclear expression of p15PAF was detected in the lymphocytes in the germinal center (arrow) of lymph node (B), the epithelial cells in the lower half of crypt (arrow) in the colonic mucosa (D), and the suprabasal keratinocytes (arrow) of epidermis (F), but not in the neurons and glial cells of brain (D), hepatocytes of liver (E), and (F) pneumocytes of lung. doi:10.1371/journal.pone.0061196.g001
after release, indicating the predominant expression of p15PAF during the S phase. Western blotting also showed high p15PAF expression 3 h and 6 h after release from aphidicolin, and downregulated p15PAF expression 9 h after release (Fig. 2C). To verify this result, we pulse-labeled HeLa cells using 5-bromo-2'deoxy-uridine (BrdU). Immunofluorescence showed that nearly all cells with BrdU staining were also positive for p15PAF and vice versa (90% concordant rate; Figs. 2D and 2E). These results indicated that p15PAF is predominantly expressed during the S phase.

**p15PAF is essential for cell cycle progression and DNA synthesis**

We obtained 5 different lentiviral constructs carrying p15PAF shRNA from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan), and used them to transduce HeLa cells. Cells transduced with shRNAs #4 and #5 displayed marked reductions

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**Figure 2. p15PAF is expressed in S phase.**

(A) HeLa cells were synchronized at G1/S boundary by thymidine/aphidicholin double block. Immunofluorescence staining of p15PAF was performed at the indicated time point after release. Flow cytometry was also performed to determine the cell cycle distribution. Expression of p15PAF peaked at 6 h (mid-to-late S phase) after release. (Scale bar = 30 µm). (B) Quantification of the percentage of p15PAF-positive cells at the indicated time point. (C) HeLa cells were synchronized at the G1/S boundary thymidine/aphidicholin double block and were collected for Western blotting following release into the cell cycle. Cyclin D1 and B1 were used as markers of S and G2 phase, respectively. (D) After pulse labeling with BrdU, HeLa cells were stained with anti-p15PAF and anti-BrdU antibodies. The cells with positive BrdU staining were also positive for p15PAF. (Scale bar = 30 µm). (E) Quantification of the result of immunofluorescence staining. 90% of the cells showed concordant staining of p15PAF and BrdU.

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in p15\textsuperscript{PAF} mRNA and protein expression (Fig. 3A). We therefore used them in subsequent analyses. Using the MTT assay, we showed that stable knockdown of p15\textsuperscript{PAF} by shRNAs \#4 and \#5 led to the inhibition of HeLa cell proliferation (Fig. 3B). To evaluate the effects of p15\textsuperscript{PAF} knockdown on cell cycle progression, we synchronized HeLa cells at the G1/S boundary using a double thymidine block, followed by release at the G1/S boundary (Fig. 3C). Knockdown of p15\textsuperscript{PAF} suppressed cell cycle progression, with the cells arrested at the G1 phase. (D) Knockdown of p15\textsuperscript{PAF} inhibited the incorporation of BrdU into DNA in the S phase.

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Figure 3. p15\textsuperscript{PAF} is essential for cell cycle progression and DNA synthesis. (A) p15\textsuperscript{PAF} mRNA (upper panel) and protein (lower panel) levels were significantly reduced in HeLa cells stably transduced with lentiviruses sh-p15\textsuperscript{-4} and -5. Sh-Luciferase (sh-Luc) served as the negative control. (B) The MTT proliferation assay revealed a growth inhibitory effect of knockdown of p15\textsuperscript{PAF}. (C) Knockdown of p15\textsuperscript{PAF} suppressed cell cycle progression. After release from double thymidine block, the control cells progressed normally into S and G2 phases but the cells with knockdown of p15\textsuperscript{PAF} were arrested at G1 phase. (D) Knockdown of p15\textsuperscript{PAF} inhibited the incorporation of BrdU into DNA in the S phase.
thymidine block. As shown in Fig. 3C, the control cells progressed normally through the S phase to the G2 phase, and approximately one-third of cells completed the cell cycle and returned to the G1 phase 9 h after release from thymidine. In contrast, cells with attenuated p15PAF expression remained at the G1/S boundary for up to 12 h after release. The major event that occurs during the S phase is DNA synthesis; therefore, we investigated the effects of p15PAF knockdown on DNA synthesis. Our results from the BrdU incorporation assay showed that p15 PAF knockdown markedly inhibited DNA synthesis in asynchronous HeLa cells (Fig. 3D).

p15PAF knockdown upregulates p21 expression and increases its chromatin binding. The cyclin-dependent kinase inhibitor p21WAF1/CIP1 (p21) plays important roles in the regulation of cell cycle progression. To elucidate the mechanism by which p15PAF knockdown arrests the cell cycle, we evaluated p21 expression using western blotting. In unsynchronized HeLa cells, p15PAF knockdown induced marked upregulation of the p21 protein (Fig. 4A) and mRNA expression (Fig. 4B). These results indicated that regulation of p21 expression is mainly at the transcriptional level. p21 is also a PCNA-interacting protein [11] and inhibits the ability of PCNA to activate DNA polymerase δ, the principal replicative DNA polymerase, which inhibits DNA replication [11]. We speculated that p15PAF might compete with p21 for binding to PCNA, and that p15PAF attenuation leads to excess interaction between p21 and PCNA. Therefore, we assayed the chromatin-bound fraction of p21 and identified that p15PAF attenuation resulted in p21 accumulation in chromatin (Fig. 4C). An in vitro GST-tagged protein-protein interaction assay further showed that p15PAF inhibited the binding of p21 to GST-PCNA fusion protein in a dose-dependent manner (Fig. 4D).
The Rb/E2F pathway is the major regulatory mechanism for genes that are required for S-phase entry, such as DNA polymerase subunits, cyclin A, and cyclin E [12]. Therefore, we hypothesized that p15PAF is a direct transcriptional target of the Rb/E2F pathway. We analyzed the p15PAF promoter sequence using the TFSEARCH website, identifying 3 putative E2F binding motifs located at -94 to -87, -29 to -22, and 17 to 25 bp of the transcriptional start site (Fig. 5A). HeLa cells have a defective Rb function caused by the binding of human papilloma virus protein E7 to Rb [13]; therefore, we used the breast cancer cell line MCF7 to evaluate Rb/E2F pathway-mediated p15PAF regulation.

Figure 5. p15PAF is a target of Rb/E2F pathway. (A) Three putative E2F-binding motifs are present in proximal promoter region of the p15PAF gene. The promoter sequence containing these three putative E2F-binding motifs was cloned upstream to the luciferase (Luc) gene in the pGL3-Basic vector. (B) Knockdown of Rb by siRNA enhanced expression of p15PAF in MCF7 cells. (C) Overexpression of Rb repressed the activity of p15PAF, AURKA, and MCL1 promoters. AURKA and MCL1 are known targets of the Rb/E2F pathway and served as positive controls. (D) Mutations of all three putative E2F-binding motifs derepressed the promoter activity of p15PAF. (E) E2F4 and E2F6 suppressed the promoter activity of p15PAF. (F) ChIP assay showed binding of E2F4 and E2F6 onto the p15PAF promoter.

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p15PAF expression is negatively regulated by the Rb/E2F pathway

The Rb/E2F pathway is the major regulatory mechanism for genes that are required for S-phase entry, such as DNA polymerase subunits, cyclin A, and cyclin E [12]. Therefore, we hypothesized that p15PAF is a direct transcriptional target of the Rb/E2F pathway. We analyzed the p15PAF promoter sequence using the TFSEARCH website, identifying 3 putative E2F binding motifs located at -94 to -87, -29 to -22, and 17 to 25 bp of the transcriptional start site (Fig. 5A). HeLa cells have a defective Rb function caused by the binding of human papilloma virus protein E7 to Rb [13]; therefore, we used the breast cancer cell line MCF7 to evaluate Rb/E2F pathway-mediated p15PAF regulation. As shown in Fig. 5B, following Rb attenuation by siRNA, p15PAF expression was upregulated. This observation indicated that Rb is a negative regulator of p15PAF expression. To validate the biological functionality of the 3 putative E2F motifs, we cloned the promoter sequence containing the 3 putative E2F-binding sites into a position upstream of the luciferase reporter gene in the plasmid pGL3-basic. Results from the luciferase assay showed the promoter activity was repressed by Rb (Fig. 5C). Rb also inhibited the promoters of 2 known Rb/E2F targets AURKA and MCL1 [14,15]. To determine which E2F motifs are functional, we mutated each site using site-directed mutagenesis. When analyzing the effects of each mutation, we found that mutations in all 3 sites resulted in increased luciferase activity (Fig. 5D), indicating that all...
3 sites are functional and that the Rb/E2F pathway exerts inhibitory effects on the p15PAF promoter. Rb interacts with several E2F family members. Of these, E2F1–3 are transcriptional activators, whereas E2F4–8 are transcriptional repressors [16]. Cotransfection of E2F4 or E2F6 with the p15PAF promoter reporter resulted in repressed promoter activity (Fig. 3E). We also performed chromatin immunoprecipitation (ChIP) to confirm the binding of E2F4 and E2F6 with the p15PAF promoter in vivo (Fig. 3E). Overall, these results support the hypothesis that p15PAF is a target of the Rb/E2F pathway.

Discussion

p15PAF is overexpressed in many types of solid tumor [2–6]. Previous studies have identified several functions for p15PAF, including DNA repair, cell proliferation, and tumor invasion [5–7,17]. Using immunohistochemical staining, we found that p15PAF is specifically expressed in proliferative cells in normal adult organs. Besides, p15PAF is a PCNA-binding protein, so we propose that it is a component of cell proliferation machinery. In this study, we found that p15PAF is expressed predominantly in the S phase, and is essential for S-phase progression and DNA synthesis.

The hypothesis that p15PAF is involved in cell proliferation is not totally new. Consistent with our results, Mizutani et al. showed that p15PAF knockdown markedly inhibited the growth of anaplastic thyroid cancer cells [18]. However, the distribution of p15PAF during the cell cycle and its functional role in cell cycle progression remain controversial. Emanuele et al. concluded that p15PAF expression peaks in the G2/M phase of the cell cycle and declines rapidly at the mitotic exit [19]. In contrast, we observed the predominant expression of p15PAF during the S phase. We consider the discrepancies to be caused by differences in the interpretation of the results. In the figure 1C of their report, p15PAF expression remained at high levels from 0 h to 7 h after release from the double thymidine block, and declined 8 h after release, coinciding with the appearance of the G2/M marker phospho-serine 10 on histone 3. This expression pattern is more consistent with that of an S-phase protein than that of a G2/M protein. In our study, we used immunofluorescence and western blotting to evaluate the cell cycle distribution. We identified that p15PAF expression is highly concordant with BrdU incorporation, p15PAF knockdown upregulated p21 expression and increased the levels of p21 in the chromatin-bound fraction, indicating that p15PAF is able to compete with p21 for binding to PCNA. Consistent with our results, Yu et al. observed that p21 overexpression reduced p15PAF binding to PCNA, and that p15PAF upregulation inhibited the binding of p21 to PCNA [21]. Therefore, equilibrium between p21 and p15PAF expression might be an important mechanism in the determination of S-phase entry.

Our study results from luciferase assays, ChIP experiments, and transient transfection assays clearly show that E2F4 and E2F6 repressed transcription from the p15PAF promoter by binding to the proximal promoter region. These observations supported our hypothesis that p15PAF expression varies during cell cycle progression. Many E2F target genes, such as p15PAF, have complex promoter structures that include 2 or more E2F consensus sites [22,23]. In some E2F target genes, the activating and repressing E2Fs bind to the same or different E2F-binding motifs to regulate gene expression [24]. In p15PAF, we observed that all 3 E2F-binding motifs repressed p15PAF expression. However, whether each site has a distinct function in the control of p15PAF expression remains unknown.

In summary, our results indicate that p15PAF is an S-phase protein tightly regulated by the Rb/E2F complex, and that loss of Rb/E2F-mediated inhibition during the G1/S transition leads to upregulated p15PAF expression. p15PAF then competes with p21 for binding to PCNA. Therefore, the presence of p15PAF is essential for DNA synthesis and S-phase progression.

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Author Contributions

Conceived and designed the experiments: YMJ YLC RHY. Performed the experiments: MJF CNC. Analyzed the data: YMJ YLC. Wrote the paper: YMJ.

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