

E2FBP1 antagonizes the p16^{INK4A}-Rb tumor suppressor machinery for growth suppression and cellular senescence by regulating promyelocytic leukemia protein stability

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Cellular senescence is an irreversible cell cycle arrest triggered by the activation of oncogenes or mitogenic signaling as well as the enforced expression of tumor suppressors such as p53, p16^{INK4A} and promyelocytic leukemia protein (PML) in normal cells. E2F-binding protein 1 (E2FBP1), a transcription regulator for E2F, induces PML reduction and suppresses the formation of PML-nuclear bodies, whereas the down-regulation of E2FBP1 provokes the PML-dependent premature senescence in human normal fibroblasts. Here we report that the depletion of E2FBP1 induces the accumulation of PML through the Ras-dependent activation of MAP kinase signaling. The cellular levels of p16^{INK4A} and p53 are elevated during premature senescence induced by depletion of E2FBP1, and the depletion of p16^{INK4A}, but not p53 rescued senescent cells from growth arrest. Therefore, the premature senescence induced by E2FBP1 depletion is achieved through the p16^{INK4A}-Rb pathway. Similar to human normal fibroblasts, the growth inhibition induced by E2FBP1 depletion is also observed in human tumor cells with intact p16^{INK4A} and Rb. These results suggest that E2FBP1 functions as a critical antagonist to the p16^{INK4A}-Rb tumor suppressor machinery by regulating PML stability.

Keywords: E2F-binding protein 1; senescence; cell cycle; ubiquitin; promyelocytic leukemia protein

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Introduction

In most human tumors, abnormality in p16^{INK4A}-Rb and/or p14^{ARF}-p53 pathways disturbs cell cycle control. Remarkably, many tumors are p16^{INK4A}-deficient and

have cyclin D overexpression [1]. p16^{INK4A} inhibits the catalytic activity of cyclin D-CDK4/6 complex, and increases hypophosphorylated Rb protein, which result in G1 phase cell cycle arrest by inactive Rb-E2F complex [2-3]. Thus, p16^{INK4A} functions as an important tumor suppressor as it controls cell cycle progression. The expression level of p16^{INK4A} is low in primary human fibroblasts and high in senescent fibroblasts [4-5]. siRNA-mediated p16^{INK4A} knockdown rescues senescent cells from growth arrest [6-7], arguing a critical role

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of p16^{INK4A} in replicative senescence. In senescent cells, p53 is also accumulated and activated by acetylation and phosphorylation [8-9]. In response to oncogenic stimuli, such as ectopic activation of Ras-MAPK pathway, primary fibroblasts exit to premature senescence associated with elevation of p16^{INK4A} and p53 levels [10-15].

E2FBP1/DRIL1/hARID3A is a member of A/T-rich interaction domain (ARID) family protein [16] and an E2F1 associated factor that increases E2F/DP-dependent transcription [17], a human orthologue of mouse B-cell regulator of IgH transcription (Bright) and drosophila dead ringer (Dri) [18-19] expressed ubiquitously in various tissues. More recently, we reported that E2FBP1 acts as a transcriptional suppressor against viral immediate early gene transcription in an intrinsic cellular defense uniquely observed in passage-limited human diploid fibroblast (HDF) cells infected with herpes simplex virus type 1 infection [20]. E2FBP1 also functions as a negative regulator of promyelocytic leukemia protein-nuclear bodies (PML-NBs) [21], which are recognized as gatekeepers of cellular integrity. PML-NBs respond to various cellular events, including the induction of oncogenic transformation and virus infections. The increase in size and numbers of PML-NBs deprive proliferative potential and force cells into premature senescence [22-23] or apoptosis [24]. During the oncogenic Ras-induced premature senescence, PML is up-regulated through the activation of MAPK cascade and contributes ultimately to the cell cycle arrest and senescence mediated by p16^{INK4a}/Rb- and/or p53-pathways [13-15]. E2FBP1 interacts with the components of PML-NBs such as PML, speckled protein (Sp100), and p53, as well as E2F2/4 *in vivo* and *in vitro*. Moreover, siRNA-mediated E2FBP1 knockdown results in increases of PML-NBs in size and number, and subsequently induces PML-dependent premature senescence of HDF cells along with the up-regulation of p16^{INK4A} and p53 [21]. In agreement with this, the ectopic expression of E2FBP1 rescues oncogenic Ras-induced premature senescence [25], and thus E2FBP1 plays an essential role for maintenance of proliferative potential of human cells. However, the mechanism by which E2FBP1 dissociates PML-NBs remains elusive.

Here we report that the molecular mechanism of E2FBP1-induced PML-NBs dissociation is through ubiquitin-dependent degradation of PML. Down-regulation of E2FBP1, induced by oncogenic Ras expression, induces premature senescence and requires an intact p16^{INK4A}-pathway. Therefore, our study reveals that E2FBP1 antagonizes PML-dependent p16^{INK4A}/Rb-mediated growth suppression and senescence.

Materials and method

Plasmids and synthetic oligonucleotides

HA-E2FBP1 and HA-PML-IV were subcloned into pcDNA3 to construct expression plasmids. Human H-ras^{V12} was cloned in pLenti6 for preparation of recombinant lentiviruses. Coding units for shRNAs in double strands were synthesized and linked to U6 promoter in pLenti6 plasmid according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). Sequences for anti-E2FBP1 shRNA are following; A1: CACCGATGT-AACGTGCGCTTCAAGTGTGCTGTCCTTGGAGTG CACGTTGCATC; A2: CACCGAGATTAACGGTATC-GTGTGTGTGCTGTCCACATGATGCCGTTGATCTC; A3: CACCCTGTGGACCCGATACGTGAACGTGTGC-TGTCCGTTTCATGTATTGGGTCCGCAG Anti-p16^{INK4A} shRNA was prepared as stated by the researchers [26], and the sequence of control shRNA was previously described [27]. shRNA expression plasmid, piGENE-E2FBP1 was generated by inserting anti-E2FBP1 shRNA sequence; CAAGCAGTTCTACGGACTCGATGGGGA-CCCCCTTCCTGTCAGGGGTCCCCGTCGAGTTCGT AGAGCTGCTTGGTAC to piGENE_{etRNA} vector (iGENE Therapeutics, Tokyo, Japan) between Sac I and Kpn I sites.

Cell culture, transfection and infection

TIG-3 human fetal lung derived passage-limited diploid fibroblast cells between population doubling (PD) number 36 and 50, human cancer cell lines such as KB, A431, Saos2, MCF7, and 293FT human embryonic kidney cell line (Invitrogen, Carlsbad, USA) were cultivated in Dulbecco's modified Eagles medium (DME) supplemented with 8% fetal calf serum (FCS). HCT116 was cultivated in McCoy's 5A medium supplemented with 10% FCS. For transfection with plasmid DNA, cells were plated to be 50% confluent, and transformed with FuGene 6 transfection reagent (Roche Applied Science, Indianapolis, USA). For recombinant lentivirus infection, TIG-3 cells plated between 1×10^6 – 2×10^6 cells in a 10 cm dish were incubated with 10 mL of freshly prepared virus stock supernatant that contains 0.5×10^6 – 5×10^6 viruses·mL⁻¹ for 12 h in the presence of 6 µg·mL⁻¹ polybrene. The cells were then overlaid with 10 mL of fresh medium containing 4 µg·mL⁻¹ Brastacidin-S-HCl for 30 h and cultivated for 2 d with fresh medium without the Brastacidin. The cells were subsequently replated at 40%–50% confluence and grown until they were analyzed. For growth assay using lentiviruses, TIG-3 cells at 47 PD were infected with lentiviruses carrying expression unit of indicated shRNAs at total multiplicity of infection (MOI)=2 (e.g. MOI=1 of anti-p16^{INK4A} shRNA-expression

virus plus MOI=1 of A2-expression virus: the sum of the virus mixture contains MOI=2). Infected cells were selected with antibiotics, and counted at the indicated cultivation periods.

Lysate preparation and Western blotting

Cells were washed twice with PBS, lysed in the lysis buffer (400 mmol·L⁻¹ NaCl, 50 mmol·L⁻¹ HEPES-Na, pH 7.0, 1 mmol·L⁻¹ ethylene diamine tetraacetic acid (EDTA), and 0.1% NP-40), and clarified by centrifugation. Protein concentration was determined using the BCA protein assay kit (Sigma-Aldrich, St. Louis, USA). The protein sample (30 µg) was separated on SDS-PAGE gels, and transferred to immobion-P membranes (Millipore, Billerica, USA). To detect the antigen, blots were blocked in TBS-T (25 mmol·L⁻¹ tris-HCl, pH 8.0, 137 mmol·L⁻¹ NaCl, and 2.7 mmol·L⁻¹ KCl, and 0.1% Tween 20) containing 5% skim milk (Difco, Detroit, USA) for 30 min, and incubated with primary antibody for 2 h. The blots were incubated with secondary antibody conjugated with horseradish peroxidase for 1 h. Protein bands were visualized by Lumi-Light^{PLUS} western blotting substrate (Roche Applied Science, Indianapolis, USA). Immunoassays were performed with anti-E2FBP1 polyclonal antibody (BL665, Bethyl Laboratories, Montgomery, USA), Anti-p16^{INK4A} polyclonal (C-20), anti-p21^{CIP1} polyclonal, anti-p53 monoclonal (DO-1), anti-PML monoclonal (PG-M3), anti-Cyclin E polyclonal (M-20) and anti-Daxx polyclonal (C-20) antibodies were obtained from Santa Cruz Bio-technology (Santa Cruz, USA). Anti-Sp100 polyclonal was obtained from Chemicon-Millipore (Watford, UK). Anti-Ras monoclonal (#18), anti-Rb monoclonal (G3-245), anti-ERK1 monoclonal (MK12), anti-phospho-ERK1/2 (pT202/pY204) monoclonal (#20A), anti-p38/SAPK2a monoclonal (#27), and anti-phospho-p38SAPK (pT180/pY182) monoclonal (#30) antibodies were obtained from BD Biosciences (San Jose, USA). Anti-tubulin monoclonal (DM-1A) was obtained from ICN Biomedicals (Irvine, USA).

Detection of PML ubiquitylation

For ubiquitination assays, 2 d after transfection, cells were treated with 10 µmol·L⁻¹ proteasome inhibitor MG115 for 8 h and harvested by boiling for 5 min in lysis buffer (1% sodium dodecyl sulfate (SDS), 1 mmol·L⁻¹ EDTA in phosphate buffer saline (PBS) for protein analyses.

siRNA-mediated down-regulation

The target sequences for siRNA-Control and E2FBP1 have been described previously [21]. siRNAs were synthesized by Japan Bioservice Co. (Saitama, Japan). Trans-

fection into TIG-3 cells was performed with oligofectamine (Life Technologies, Carlsbad, USA).

SA-β-gal assay

SA-β-gal assay was performed as described [28]. Briefly, cells were fixed with 3% formaldehyde for 5 min, washed with PBS, and incubated at 37 °C with staining buffer (1 mg·L⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mmol·L⁻¹ potassium ferrocyanide, 5 mmol·L⁻¹ potassium ferricyanide, 150 mmol·L⁻¹ NaCl, 2 mmol·L⁻¹ MgCl₂, 40 mmol·L⁻¹ citric acid/sodium phosphate, and pH 6.0) for 12 h.

Colony formation assay

Cancer cell lines were plated at 1×10⁶ per 35-mm dish in triplicate and transfected with piGENE vector or piGENE-E2FBP1. Cells were replanted in 100-mm dishes the next day, cultivated in the presence of 5 µg·mL⁻¹ of puromycin for 48 h, and subsequently developed colonies for two weeks in the presence of 1 µg·mL⁻¹ of puromycin. The puromycin-resistant colonies were stained with 0.1% crystal violet and then calculated using ImageJ software.

Results

E2FBP1 induces degradation of PML through polyubiquitylation

To determine whether E2FBP1-dependent dissociation of PML-NBs is a consequence of degradation of PML, 293FT cells were transfected with expression plasmids for HA-E2FBP1, HA-tagged 633-residue isoform IV of PML (HA-PML-IV) which is a major component of PML-NBs, and humanized green fluorescent protein (hrGFP). To eliminate the influence of squelching of transcription factors, the amount of plasmids were adjusted with the empty vector pcDNA3 that contains a cytomegalovirus (CMV) early gene promoter for common to all expression plasmids. Ectopic expression of E2FBP1 reduced the amount of PML-IV in dose-dependent manner whereas no effect was observed in cells expressing hrGFP (Figure 1A). E2FBP1 also down-regulated another PML-NB-associated 560-residue isoform VI of PML (HA-PML-VI) (Figure 1B). Contrary to these results, siRNA-mediated E2FBP1 depletion [21] resulted in an up-regulation of PML (Figure 1C), and the complementation with exogenous E2FBP1 that overcome siRNA-mediated down-regulation of E2FBP1 resulted in a distinct down-regulation of exogenous PML-IV (Figure 1D). These results confirmed that E2FBP1 down-regulated the expression of PML regardless of its promoter, and thus possibly at the posttranscriptional level, in dose-

dependent manner.

To examine whether E2FBP1 mediates polyubiquitylation of PML-IV, 293FT cells were transfected with plasmids expressing HA-PML-IV and E2FBP1. Forty-eight hours of post transfection, cells were further incubated with a proteasome inhibitor MG115 for 8 h prior to harvest. HA-PML-IV was immunoprecipitated with anti-HA antibody, and the immune complexes were

subjected to immunoblotting. As shown in Figure 2A, an increase of slower migrating PML populations (≥ 100 kDa) that was coupled with high molecular weight polyubiquitylated protein species were evident. Moreover, E2FBP1-induced high molecular weight species of HA-PML were modified with Myc-tagged ubiquitin moiety (Figure 2B). These results revealed that E2FBP1 induces polyubiquitylation of HA-PML-IV *in vivo*.

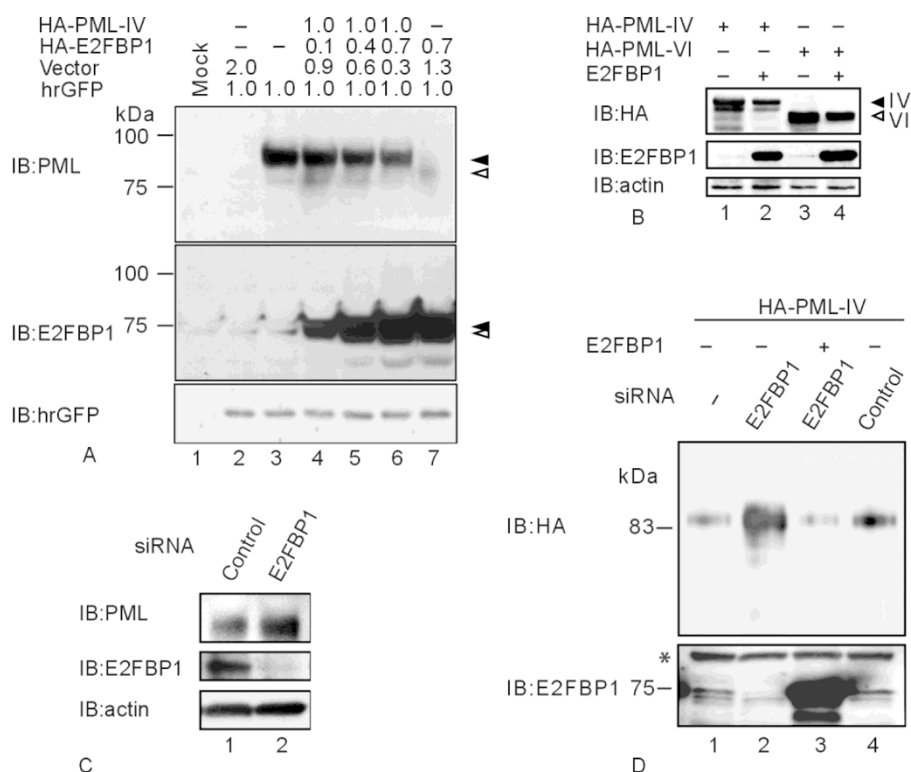


Figure 1 E2FBP1 induces down-regulation of PML. (A) E2FBP1 induces down-regulation of PML-IV in dose dependent manner. 293FT cells (1.7×10^6) plated in 6-cm dishes were transfected with 3 μ g of expression plasmids mixtures composed of pcDNA3 (vector), pIRES-hrGFP-2, pcDNA-HA-E2FBP1 and pcDNA-HA-PML-IV at indicated ratio. Cell lysates were prepared after two days of cultivation. Thirty-three μ g of each lysate was then subjected to immunoblot analyses with antibody for PML (PG-M3), E2FBP1 (BL665) and hrGFP. Open arrowheads and closed arrowheads show endogenous and exogenous antigens, respectively. (B) Both major PML components of PML-NBs are down-regulated under the ectopic expression of E2FBP1. Expression plasmids for either of major PML component of PML-NBs, pcDNA3-HA-PML-IV or pcDNA3-HA-PML-VI was transfected to 293FT cells with or without pcDNA3-HA-E2FBP1, and the cell lysates were similarly subjected to immunoblot analyses. (C) siRNA-mediated down-regulation of endogenous E2FBP1 stabilizes endogenous PML in TIG-3 cells. TIG-3 cells were transfected with siRNA against either E2FBP1 or nonsense control. Two days later, cell lysates were collected, and 15 μ g of the lysate protein was subjected to immunoblot analysis for PML, E2FBP1 and actin. (D) siRNA-mediated down-regulation of endogenous E2FBP1 stabilizes exogenous HA-PML-IV, and this stabilization is canceled by simultaneous expression of exogenous E2FBP1. 293FT cells were transfected with siRNA against either E2FBP1 or nonsense control. Two days later, the cells were simultaneously transfected with indicated mixtures containing same siRNA and either of following expression plasmid mixtures; HA-PML-IV plus empty vector (1 : 1) or HA-PML-IV plus non-tagged E2FBP1 (1 : 1), and cultivated for in a further two days. Fifteen μ g of cell lysate proteins were subjected to immunoblotting for HA and E2FBP1. Asterisk indicates a cross-reactive protein and serves as a loading control.

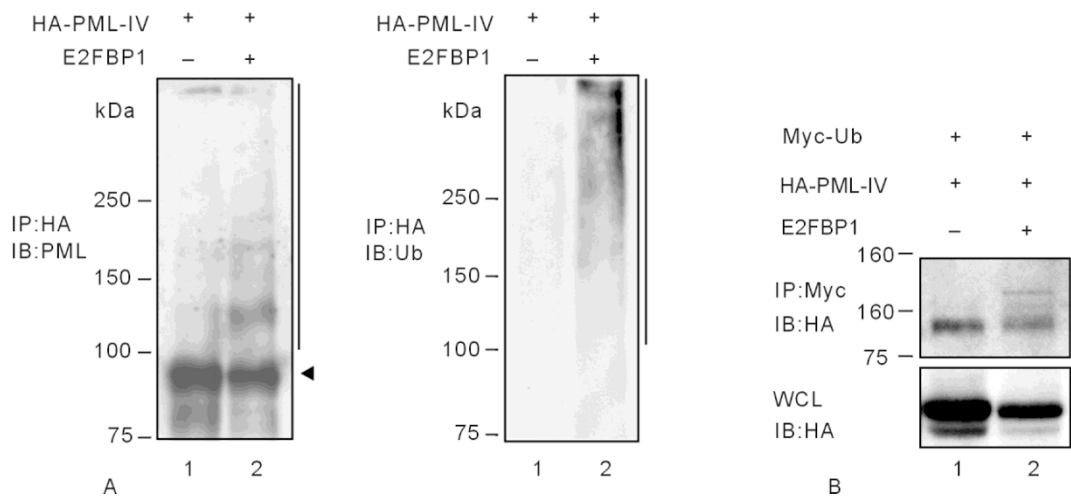


Figure 2 E2FBP1 induces polyubiquitylation of PML. **(A)** Overexpression of E2FBP1 increases polyubiquitylation of PML-IV *in vivo*. 293FT cells were transformed with expression plasmids for HA-PML-IV and/or non-tagged E2FBP1 and incubated for two days. The cells were then treated with $10 \mu\text{mol}\cdot\text{L}^{-1}$ MG115 for an additional 8 h before cell lysate preparation. HA-PML-IV from 2 mg of lysate was immunoprecipitated with $0.5 \mu\text{g}$ of anti-HA antibody 3F10, and the precipitate was thoroughly washed and subjected to immunoblotting with antibody to PML (PG-M3, upper panel). The blot was then stripped and reprobed with anti-ubiquitin antibody (1B3, lower panel). **(B)** Overexpression of E2FBP1 increases polyubiquitylation of PML-IV. 293FT cells were transformed with indicated expression plasmids and treated with $10 \mu\text{mol}\cdot\text{L}^{-1}$ of MG115. After a further 8 h of cultivation, the cell lysates were subjected to immunoprecipitation with anti-Myc-tag antibody (4A6) followed by immunoblotting with anti-HA antibody (3F10). WCL: whole cell lysate. Bar shows bands of modified HA-PML-IV.

E2FBP1 down-regulation induces premature senescence

Since siRNA-mediated E2FBP1 knockdown induced PML-dependent premature senescence in human primary fibroblast TIG-3 cells, we tested whether down-regulation of E2FBP1 activates Ras-MAPK pathway that results in up-regulation of PML and oncogene-induced premature senescence. TIG-3 cells infected with either lentivirus expressing efficient anti-E2FBP1 short hairpin RNA (shRNA) A2 or A3 exhibited flat enlarged morphology, a characteristic phenotype of senescence and the expression of SA- β -gal (Figure 3A). SA- β -gal positive cells were $2.9\% \pm 0.15\%$, $94\% \pm 8.5\%$, and $96\% \pm 1.8\%$ for A1, A2, and A3, respectively. As expected, the PML expression level was increased in both A2 and A3-infected cells, but not A1-infected cells (Figure 3B). The expression levels of Sp100 and Daxx that are major components of PML-NBs were also increased significantly (Figure 3B). Moreover, up-regulation of $p16^{\text{INK4A}}$ and both phosphorylated ERK1/2 (p-ERK1/2) and p38/SAPK (p-p38), and down-regulation of phosphorylated RB were evident in A2 and A3-infected cells but not in A1-infected cells (Figure 3B).

Next we tested the effect of oncogenic Ras^{V12}, a premature senescence inducer, on the E2FBP1 expression.

Ras^{V12} induced the down-regulation of E2FBP1 along with up-regulations of PML, $p16^{\text{INK4A}}$, and hypophosphorylation of Rb (Figure 3C). These results suggest that the Ras-ERK-p38 signaling axis induces PML-dependent premature senescence at least partly through the down-regulation of E2FBP1.

p16^{INK4A} plays a critical role in growth suppression initiated by E2FBP1 down-regulation

To investigate the role of $p16^{\text{INK4A}}$ -Rb pathway on the growth arrest induced by down-regulation of E2FBP1, we depleted $p16^{\text{INK4A}}$ simultaneously with E2FBP1. Depletion of E2FBP1 showed both growth arrest and the characteristic morphology of senescence [21] (Figure 4A, shControl vs shE2FBP1-A2); however, cells transfected with both shp16^{INK4A} and shE2FBP1 showed recovery in the growth rate and acquired normal morphology after 10 days of lag phase (Figure 4A, shp16^{INK4A} + shE2FBP1-A2). The depletion of both p53 and E2FBP1 increased cell numbers more than four-times greater than that of cells depleted only E2FBP1; however, these double-depleted cells were eventually arrested around Day 15 (Figure 4B). These results suggest that $p16^{\text{INK4A}}$ has a critical role in the growth arrest induced by the

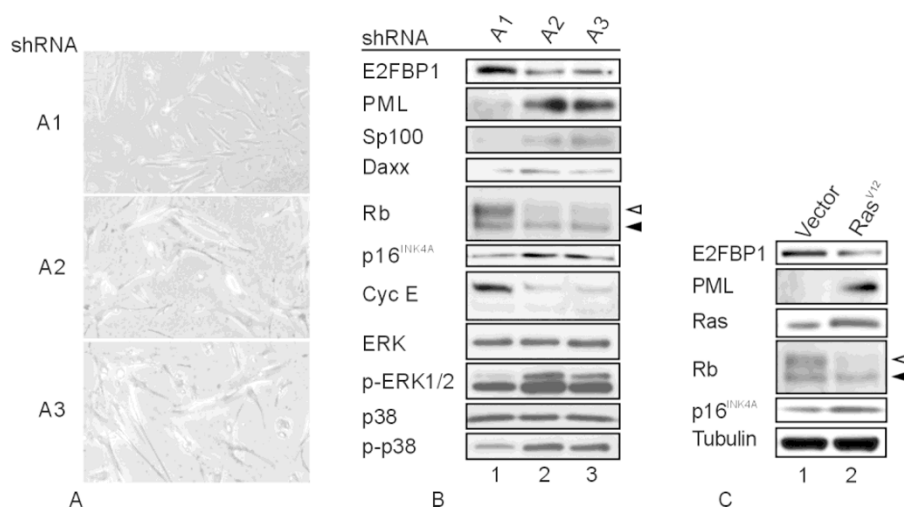


Figure 3 Down-regulation of E2FBP1 accompanies with cellular features of premature senescence. **(A)** shRNA-mediated down-regulation of E2FBP1 induces premature senescence of TIG-3 cells. Either functional (A2 and A3) or nonfunctional (A1; serves as a negative control) anti-E2FBP1 shRNA linked to the U6-promoter was introduced to cells by infection with recombinant lentiviruses (multiplicity of infection (MOI) = 2). SA- β -gal activity was assessed after 14 d of selection with $4 \mu\text{g}\cdot\text{mL}^{-1}$ brastacidin-S-HCl. **(B)** Down-regulation of E2FBP1 induced accumulation of PML and up-regulation of phosphorylation of ERK1/2 and p38/SAPK accompanied with premature senescence. TIG-3 cells were infected with the indicated lentiviruses, and selected for 10 d with $4 \mu\text{g}\cdot\text{mL}^{-1}$ brastacidin-S-hydrochloride. Cell lysates were subjected to immunoblot analyses with indicated antibodies. The open and closed arrows show hyper- and hypo-phosphorylated Rb, respectively. Pan-ERK1/2 and pan-p38 serve as loading controls. **(C)** Ras^{V12} down-regulates E2FBP1 expression. Lysates from cells infected with the control (lane 1) or Ras^{V12} (lane 2) expressing lentiviruses were subjected to immunoblot analyses with indicated antibodies. The open and closed arrows show hyper- and hypo-phosphorylated Rb, respectively.

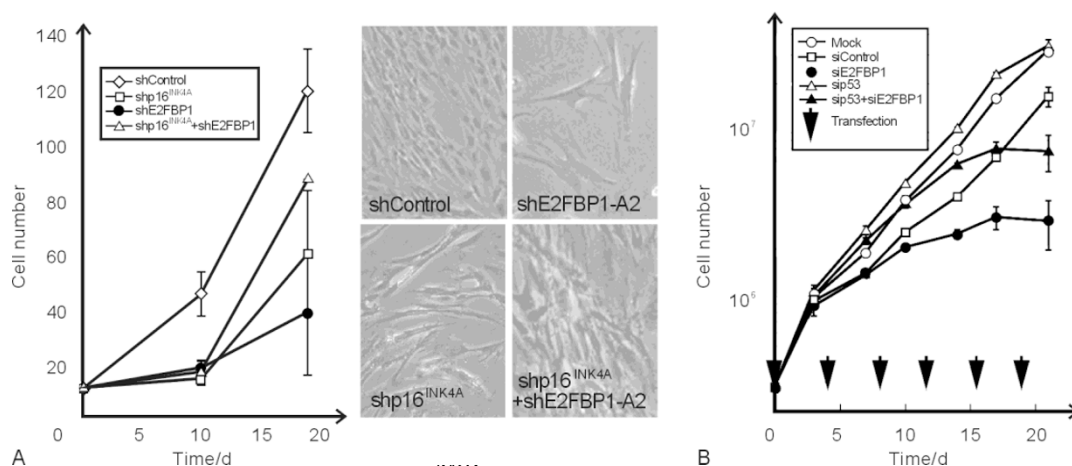


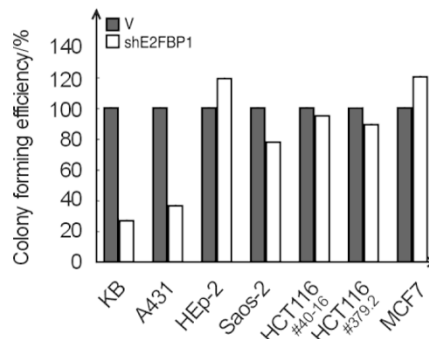
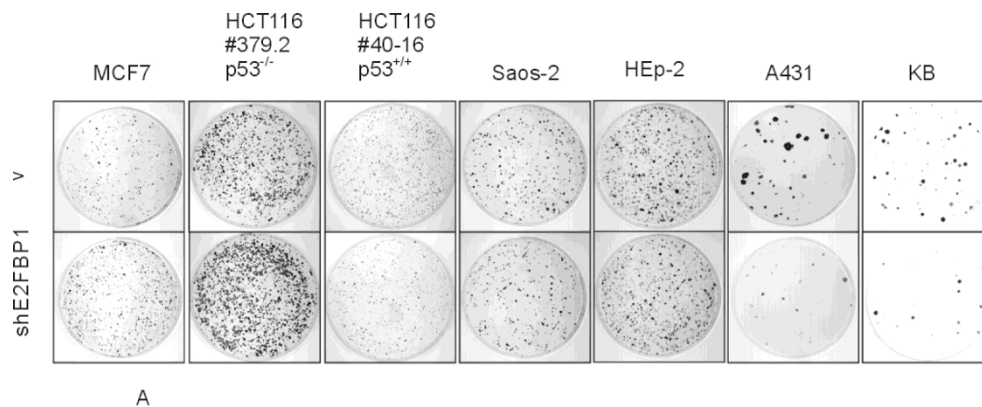
Figure 4 Simultaneous down-regulation of p16^{INK4A} rescues premature senescence induced by the down-regulation of E2FBP1. **(A)** The role of p16^{INK4A} in cell growth suppression induced by down-regulation of E2FBP1 (left). TIG-3 cells were infected with lentiviruses carrying indicated shRNA expression unit, and replated in the presence of brastacidin-S-hydrochloride. Live cell numbers were determined by trypan blue exclusion assay. The values and error bars represent averages and standard deviations of three independent assays. Representative photoimages. Cells infected with lentiviruses expressing indicated shRNA were photographed under phase contrast optics (right). **(B)** Cell growth suppression induced by down-regulation of E2FBP1 was partly rescued by simultaneous down-regulation of p53. TIG-3 cells at 36 PD were treated with indicated siRNAs or mock reagent (M) at every passage (arrow) and counted. The values and error bars represent averages and standard deviations of three independent assays.

down-regulation of E2FBP1, whereas p53 has only a minor role.

E2FBP1 depletion suppresses tumor cell growth in the presence of intact p16^{INK4A} and Rb

To further confirm the roles of p16^{INK4A} and p53 in the growth arrest induced by E2FBP1 depletion, we examined seven human tumor cell lines (KB, A431, Hep-2, Saos2, two HCT116 derivatives and MCF7) by colony forming assay. Cells were transfected with either anti-E2FBP1 shRNA or control shRNA expressing plas-

mid and selected by puromycin. Colonies were detected by staining with crystal violet (Figure 5A and B). E2FBP1 depletion clearly reduced the colony formation of KB and A431 cells that retain intact p16^{INK4A} and Rb. In contrast, other cell lines defective in either p16^{INK4A} and/or Rb showed no significant effect of E2FBP1-depletion on colony formation regardless of p53 status. These observations suggest that p16^{INK4A}-Rb pathway plays a critical role on growth arrest of tumor cells induced by E2FBP1 depletion, whereas the p53 status is insignificant.



	KB	A431	HEp-2	Saos-2	HCT116 #40-16	HCT116 #379.2	MCF7
p16 ^{INK4A}	+	+	+	+	-	-	-
Rb	+	+	D*	-	+	+	+
p53	+	m	D**	-	+	-	+

+: wild type; +*: up-regulated; -: null; m: mutated.
D*: disturbed by human papilloma virus (HPV) E7.
D**: disturbed by HPV E6.

B

Figure 5 Down-regulation of E2FBP1 suppresses growth of human cancer cells through the p16^{INK4A}-Rb pathway. **(A)** Colony formation of human cancer cells under shRNA-mediated down-regulation of E2FBP1. Indicated human cancer cells were transfected with either piGENE-shControl (V) or piGENE-shE2FBP1 (shE2FBP1). Drug-resistant cells were selected with puromycin for two weeks, and colonies were stained with crystal violet. **(B)** Colony-forming efficiencies of human tumor cells under shRNA-mediated down-regulation of E2FBP1 is dependent on the status of p16^{INK4A}/Rb pathway. The table beneath the chart indicates the status of relevant genes in the respective cell lines. In the chart, vector control was set as 100% and error bars represent standard deviations of three independent assays.

Discussion

Several studies have revealed that functional PML-NBs play significant roles in the induction of both replicative and premature senescence in human fibroblasts and primary mouse embryo fibroblasts [15, 29-33]. Under oncogenic stimulation such as the expression of oncogenic Ras^{V12}, a transcription factor Ets1 is activated by MAPK, which results in the up-regulation of p16^{INK4A} for preventing hyperphosphorylation of Rb and S-phase entry [34]. p16^{INK4A}-Rb pathway cooperates with mitogenic signals to enforce irreversible cellular senescence through the production of reactive oxygen species (ROS) [35]. In addition, the oncogenic Ras^{V12} increases the number of PML-NBs and activates p53 by post-translational modification to induce premature senescence [23]. Our previous report showed that E2FBP1 dissociates PML-NBs through interactions with their components and thus, the down-regulation of E2FBP1 induced the accumulation of PML, Sp100, Daxx and p53 followed by PML-dependent growth arrest in passage-limited HDF cells [21]. Here we describe the molecular mechanism of E2FBP1-dependent dissociation of PML-NBs. E2FBP1 induces ubiquitin-dependent degradation of PML although the mechanism how E2FBP1 induces polyubiquitylation of PML remains elusive. It is possible that E2FBP1 recruits an endogenous ubiquitin ligase that stimulates the degradation of PML.

Since the depletion of E2FBP1 has a significant inhibitory effect on the growth of human cells in the presence of functional p16^{INK4A}/Rb-pathway regardless of the functional p53, our results together place E2FBP1 as a crucial regulator for the MAPK-p16^{INK4A}-Rb cascade and the formation of PML-NBs during the induction of premature senescence. It has been demonstrated that the accumulated p53 induces premature senescence in cells under certain conditions activated by post-translational modification such as acetylation and phosphorylation [8-9, 23]. Depletion of E2FBP1 accumulates p53; however, we did not see the phosphorylation of Ser 15 in p53 or the induction of p14^{ARF} and p21 (data not shown). Thus, it is likely that the accumulated p53 is transcriptionally inactive and therefore, p16^{INK4A}-Rb-dependent senescence plays a major role in the E2FBP1 depletion-dependent induction of premature senescence.

Since our results show that oncogenic Ras^{V12} down-regulates the amount of E2FBP1, it is assumed that a kinase(s) in the Ras-MAPK pathway affect the stability of E2FBP1 by phosphorylation since E2FBP1 carries several potential phosphorylation target motifs for casein kinase II (CKII) and cAMP-dependent kinase (PKA) [17]. Studies presented in this manuscript establish an important role of E2FBP1 in antagonizing p16^{INK4A}/Rb-

mediated growth suppression and senescence through regulating the protein stability of PML by ubiquitination and PML-NBs dissociation.

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