Tumor suppressor p16INK4a inhibits cancer cell growth by downregulating eEF1A2 through a direct interaction

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doi: 10.1242/jcs.137521

There was an error published in J. Cell Sci. 126, 1744-1752.

The affiliations of Zunnan Huang, Joydeb Kumar Kundu and Myoung Ok Kim were given incorrectly. The correct affiliations are as listed above.

We apologise for this error.
Tumor suppressor p16\textsuperscript{INK4a} inhibits cancer cell growth by downregulating eEF1A2 through a direct interaction

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Accepted 21 January 2013
Journal of Cell Science 126, 1744–1752
© 2013. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.113613

Summary

The tumor suppressor protein p16\textsuperscript{INK4a} is a member of the INK4 family of cyclin-dependent kinase (Cdk) inhibitors, which are involved in the regulation of the eukaryotic cell cycle. However, the mechanisms underlying the anti-proliferative effects of p16 \textsuperscript{INK4a} have not been fully elucidated. Using yeast two-hybrid screening, we identified the eukaryotic elongation factor (eEF)1A2 as a novel interacting partner of p16\textsuperscript{INK4a}. eEF1A2 is thought to function as an oncogene in cancers. The p16\textsuperscript{INK4a} protein interacted with all but the D2 (250–327 aa) domain of eEF1A2. Ectopic expression of p16\textsuperscript{INK4a} decreased the expression of eEF1A2 and inhibited cancer cell growth. Furthermore, suppression of protein synthesis by expression of p16\textsuperscript{INK4a} \textit{ex vivo} was verified by luciferase reporter activity. Microinjection of p16\textsuperscript{INK4a} mRNA into the cytoplasm of \textit{Xenopus} embryos suppressed the luciferase mRNA translation, whereas the combination of p16\textsuperscript{INK4a} and morpholino-eEF1A2 resulted in a further reduction in translational activity. We conclude that the interaction of p16\textsuperscript{INK4a} with eEF1A2, and subsequent downregulation of the expression and function of eEF1A2 is a novel mechanism explaining the anti-proliferative effects of p16\textsuperscript{INK4a}.

Key words: p16\textsuperscript{INK4a}, Anti-proliferative effects, eEF1A2, Ovarian cancer, Translational activity

Introduction

Malignant progression of transformed cells involves inappropriate cell division. Eukaryotic cell division occurs through a tightly regulated cell cycle, which comprises four different phases: gap I (G1), synthesis (S), gap 2 (G2) and mitosis (M) phases. Two cell-cycle checkpoints maintain genetic stability by ensuring an authentic chromosome replication and separation (Swanton, 2004). Failure of these checkpoints is a hallmark of cancer (Hartwell et al., 1994; Hartwell and Kastan, 1994). One of the checkpoints, the restriction check-point, occurs in mid-G1, after which cells become independent of growth factors and commit to cell division (Pardee, 1974). Intracellular signaling pathways comprising various cyclins, cyclin-dependent kinases (Cdks), Cdk inhibitors, and checkpoint kinases (Chk1 and Chk2) are critical regulators of cell cycle progression (Collins and Garrett, 2005; Gali-Muhtasib and Bakkar, 2002). The tumor suppressor protein p16\textsuperscript{INK4a} causes cell cycle arrest and inhibits tumor cell proliferation, at least partially, by acting as a specific inhibitor of Cdk4 and Cdk6 (Serrano et al., 1993). The regulation of the Cdk4/ cyclin D complex by p16\textsuperscript{INK4a} occurs in the nucleus (Ruas and Peters, 1998; Sherr and Roberts, 1999). However, subcellular localization of p16\textsuperscript{INK4a} has been documented in both the cytoplasm and nucleus (Evangelou et al., 2004). Choi and colleagues demonstrated that cytoplasmic p16\textsuperscript{INK4a} suppressed UV-induced cell transformation by interacting with c-Jun N-terminal kinase (JNK)-1 (Choi et al., 2005). Thus, cytoplasmic p16\textsuperscript{INK4a} may play other functional roles in cells.

Recent studies demonstrated that deregulation of translational machinery is associated with malignant transformation of cells (Bilanges and Stokoe, 2007; Ruggero et al., 2004; Ruggero and Pandolfi, 2003). Several eukaryotic translation initiation (eIF) and elongation (eEF) factors have been implicated in carcinogenesis (Sonenberg and Hinnebusch, 2009; White-Gilberston et al., 2009). Eukaryotic elongation factor 1 alpha (eEF1A), a member of the G protein family, is one of four subunits that constitute the eukaryotic elongation factor 1 (Browne and Proud, 2002; Ejiri, 2002) and catalyzes the binding of aminoacyl-tRNA to the A-site of the ribosome in a GTP-dependent manner (Ejiri, 2002; Moldave, 1985). Although the biological roles of mammalian eEF1A, which exists as two isoforms, eEF1A1 and eEF1A2, have been extensively investigated, the necessity of the existence of two isoforms of eEF1A still remains unexplained. Despite sharing 98% amino acid homology and playing a similar role in protein synthesis, these two isoforms differ in their chromosomal location (EEF1A1 in 6q14 and EEF1A2 in 20q13) (Lund et al., 1996) and their
Results

Tumor suppressor p16INK4a interacts with eukaryote elongation factor 1A2 in vitro and ex vivo

In order to identify proteins that interact with p16INK4a, we performed a yeast two-hybrid screening of a human HeLa cDNA-GAL4 expression plasmid library using a full-length p16INK4a fused to the GAL4 DNA-binding domain as the ‘bait’. We selected the clone encoding eEF1A2 (supplementary material Fig. S1) for further investigation. The full-length eEF1A2 clone consists of 1392 nucleotides, encoding a protein of 463 amino acids. The predicted molecular mass of the protein is 53 kDa. For further study, we cloned the eEF1A2 gene, which was extracted from total mRNA of HeLa cells, into mammalian vectors.

To confirm the interaction between p16INK4a and eEF1A2 in a cell system, we performed the mammalian two-hybrid assay using COS-7 cells transfected with the GAL4 binding domain containing pM-BD-p16 and VP16 containing pVP-eEF1A2. These results indicated that GAL4-p16INK4a interacted with VP16-eEF1A2 in cells (Fig. 1A). The interaction between p16INK4a and eEF1A2 was further confirmed by a co-immunoprecipitation assay. Plasmids encoding eEF1A2 fused to N-terminal V5 were transiently co-transfected with pcDNA-p16INK4a into COS-7 cells. Immunoprecipitation of V5-eEF1A2 resulted in the co-precipitation of p16INK4a (Fig. 1B). To demonstrate a direct interaction between p16INK4a and eEF1A2, we performed a GST pull-down assay using a whole lysate and GST-conjugated- or GST–p16-conjugated Sepharose 4B beads. Immunoblot analysis revealed that p16INK4a interacted with serum-induced eEF1A2 (Fig. 1C). Serum-induced CDK4 was used as a positive control to show the p16INK4a interaction. Furthermore, endogenous p16INK4a was also co-immunoprecipitated by eEF1A2 in human ovarian cancer (PA-1) cells (Fig. 1D). These results indicate that p16INK4a physically interacts with eEF1A2 in mammalian cells.

To identify the interacting domains of p16INK4a and eEF1A2, various deletion constructs of p16INK4a and eEF1A2 were designed. Plasmids encoding the corresponding fragments of eEF1A2 were constructed and used to identify the p16INK4a binding domain of eEF1A2. The GST fusion full-length p16 protein, p16 (1–80 aa) and p16 (77–156 aa) interacted with eEF1A2, but not with eEF1A1 (Fig. 2A; supplementary material Fig. S2). In addition, each eEF1A2 fragment was produced by
transfection in 293 cells, and the cell lysates were pulled down using the Sepharose-4B-bead-conjugated GST fusion protein, p16\(^{INK4a}\). Results of the GST pull-down assay showed that p16\(^{INK4a}\) interacted with full-length eEF1A2, and FLAG-tagged eEF1A2 (1–249 aa) or eEF1A2 (328–463 aa) proteins. However, FLAG-tagged eEF1A2 (250–327 aa) did not bind to p16\(^{INK4a}\) (Fig. 2B). The binding between p16\(^{INK4a}\) and eEF1A2 was also predicted by the Fast Fourier transform-based docking algorithm ZDOCK. The model of the p16\(^{INK4a}\)-eEF1A2 complex obtained from the protein–protein docking experiment suggested that p16\(^{INK4a}\) bound to eEF1A2 (1–249 aa) and eEF1A2 (328–463 aa), but not to eEF1A2 (250–327 aa) (Fig. 2C).

In the predicted p16\(^{INK4a}\)/eEF1A2 complex, the hydrogen-bond networks between β-strands in domain III of eEF1A2 and the first, second and third ankyrin repeats of p16 INK4a are expected to be crucial in the binding of p16\(^{INK4a}\) with eEF1A2. Arg24 in p16 INK4a forms two hydrogen bonds with Glu374 and one hydrogen bond with Glu403 in eEF1A2 (supplementary material Table S1; Fig. S3A,B). In addition, the hydrogen bonds between the residues from the fourth repeat of p16\(^{INK4a}\) and those from domain I of eEF1A2 would also contribute to the stability of the p16\(^{INK4a}\)-eEF1A2 complex (supplementary material Table S1; Fig. S3C,D). Arg131 in p16\(^{INK4a}\) forms two hydrogen bonds with Glu217 in eEF1A2. These hydrogen bonds would explain the in vitro and ex vivo experimental findings regarding the p16\(^{INK4a}\) interaction with eEF1A2 (328–463 aa) and eEF1A2 (1–249 aa). To confirm this idea, we constructed a p16\(^{INK4a}\) protein with mutations at Arg24 and Arg131. Double mutation of Arg24 (R24A) and Arg131 (R131E) disrupted the interaction of p16\(^{INK4a}\) with eEF1A2 as expected (Fig. 2D). This result indicated that Arg24 and Arg131 of p16\(^{INK4a}\) are important for interaction with eEF1A2 domain I/III.

p16\(^{INK4a}\) inhibits translational activity in vitro and in vivo: role of eEF1A2

We then examined the functional significance of the interaction between p16\(^{INK4a}\) and eEF1A2. Because eEF1A2 functions as an elongation factor in peptide chain elongation during protein synthesis, the antiproliferative protein p16\(^{INK4a}\) might attenuate the translational activity of eEF1A2. To explore this possibility, in vitro protein synthesis was assessed in the presence of varying concentrations (0, 100, 200, 400 and 800 nM) of recombinant p16\(^{INK4a}\). The in vitro transcription and translation experiments were performed using luciferase cDNA as the reporter gene and

Fig. 2. Identification of the interacting domains of eEF1A2 and p16\(^{INK4a}\). (A,B) Full-length eEF1A1 and eEF1A2 proteins were synthesized and incubated with equal amounts of GST–p16 and GST, GST–p16-full-length (1–156 aa), GST–p16D1 (1–80 aa) and GST–p16D2 (77–156 aa) (A, top). (B) FLAG-tagged full-length eEF1A2 and various deletion fragments (top) were incubated with equal amounts of GST–p16\(^{INK4a}\). Bound proteins were analyzed by 15% SDS-PAGE and detected by autoradiography (A) or western blotting (B). (C) Predicted model of the p16\(^{INK4a}\) and eEF1A2 complex. A cross-eyed stereo view of the complex is shown where p16\(^{INK4a}\) is in orange and eEF1A2 in cyan. The α-helices are drawn as spirals and the β-strands as arrows. These figures were generated using VMD (visual molecular dynamics) (Humphrey et al., 1996). (D) The amino acids Arg24 and Arg131 of p16\(^{INK4a}\) interact with eEF1A2. Results of a pull-down assay between GST–eEF1A2 and wild-type p16\(^{INK4a}\) or mutant R24A/R131E p16\(^{INK4a}\) eEF1A2 did not interact with the double mutant p16 (R24A/R131E), but did interact with each individual p16 mutant (R24A or R131E). Representative blots are shown and the results of three replications are quantified in the graph. There is a significant decrease in eEF1A2 expression (P<0.05) in wild-type p16\(^{INK4a}\) compared to the double mutant R24A/R131E p16\(^{INK4a}\).
transcription and translation were detected by the luciferase activity assay and autoradiography. Results of the in vitro translation assay showed a significant decrease in protein synthesis with the addition of recombinant p16\(^{\text{NK4a}}\) (400 or 800 nM) (Fig. 3A,B). Analogous experiments performed with the same concentrations of GST as a negative control, did not show noticeable changes in the synthesis of 35S-luciferase (Fig. 3A,B).

The micro-injection of mRNAs into Xenopus embryos constitutes a very sensitive method for the identification of mRNAs and the study of protein translation mechanisms (Richter et al., 1982). Therefore, we performed an in vivo Xenopus translation assay to explore the effect of p16\(^{\text{NK4a}}\) on eEF1A2 translational activity. When Xenopus embryos were injected with mRNAs of luciferase alone or with luciferase plus p16\(^{\text{NK4a}}\), the luciferase translational activity was significantly decreased in the presence of p16\(^{\text{NK4a}}\), suggesting that the expression of p16\(^{\text{NK4a}}\) inhibits the translation of luciferase mRNA (Fig. 3C). Moreover, the expression of eEF1A2 was decreased with injection of luciferase (1 ng) plus p16\(^{\text{NK4a}}\) (500 pg) compared to embryos injected with luciferase alone or left untreated (Fig. 3D). We optimized a morpholino-based eEF1A2 siRNA (MO-eEF1A2) by injecting into Xenopus embryos at different concentrations. Western blot results showed that MO-eEF1A2, but not MO-control, specifically inhibited the translation of endogenous eEF1A2. Results indicated that the expression of eEF1A2 was decreased by MO-eEF1A2 (5 or 10 \(\mu\)M; Fig. 3E). Injection of MO-eEF1A2 (5 \(\mu\)M) in Xenopus decreased the luciferase activity (Fig. 3F). To clarify the effect of p16\(^{\text{NK4a}}\) on eEF1A2 translational activity, Xenopus embryos were separately injected with luciferase (500 pg) alone, luciferase plus p16\(^{\text{NK4a}}\) (100 pg), luciferase plus p16\(^{\text{NK4a}}\) plus MO-eEF1A2 (2.5 \(\mu\)M), or luciferase plus MO-eEF1A2. The luciferase plus MO-eEF1A2 group, but not the MO-control (data not shown), showed moderate inhibition of the luciferase activity and this inhibition was further decreased in the presence of p16\(^{\text{NK4a}}\) (Fig. 3G). These data suggest that p16\(^{\text{NK4a}}\) inhibited the translational activity of eEF1A2 in vitro and in vivo.

Fig. 3. p16\(^{\text{NK4a}}\) inhibits the translational activity of eEF1A2. (A) The luciferase activity of an in vitro translated luciferase protein was determined by adding 0, 100, 200, 400, or 800 nM GST or GST–p16\(^{\text{NK4a}}\) into a rabbit reticulocyte system. (B) Autoradiograph of the in vitro translated 35S-Met-labeled luciferase proteins. The proteins produced were analyzed by 15% SDS-PAGE and detected by autoradiography. Equal lane loading was confirmed by Coomassie Blue (CB) staining. The lower panel shows the quantification (means ± s.d.) of three experiments. (C) Luciferase activity in Xenopus embryos in the presence and absence of p16\(^{\text{NK4a}}\). All experiments were repeated at least three times using independent samples and data are shown as means ± s.d. (D) The abundance of eEF1A2 and p16\(^{\text{NK4a}}\) after injection of p16\(^{\text{NK4a}}\) and luciferase mRNA into Xenopus embryo was examined by western blotting. Embryos were injected with synthetic mRNAs of luciferase alone or in combination with p16\(^{\text{NK4a}}\). Antibodies against eEF1A2 and p16\(^{\text{NK4a}}\) were used for the detection of endogenous eEF1A2 and exogenous p16\(^{\text{NK4a}}\), respectively. The p16\(^{\text{NK4a}}\) protein effectively repressed the expression of eEF1A2. The expression of eEF1A1 was not changed. (E) Lysates from Xenopus embryos injected with the indicated concentrations of MO-eEF1A2 or MO-control were subjected to western blot analysis. \(\beta\)-Actin served as a control to verify equal protein loading. (F) The luciferase activity of the translated luciferase protein after injection of MO-control or MO-eEF1A2 into Xenopus embryos. (G) The luciferase activity of the translated luciferase protein after injection of luciferase, luciferase plus p16\(^{\text{NK4a}}\), luciferase plus p16\(^{\text{NK4a}}\) plus MO-eEF1A2, or luciferase plus MO-eEF1A2 mRNAs into Xenopus embryos. All experiments were repeated at least three times using independent samples and data are shown as means ± s.d.
p16NK4a inhibits the expression of eEF1A2

The finding that p16NK4a modulates the expression of eEF1A2 was confirmed by using the stably transfected p16NK4a Tet-off system in HeLa cells (Fig. 4A). After removal of doxycycline, p16NK4a expression was induced, whereas the expression level of eEF1A2 was decreased in a time-dependent manner. Moreover, co-transfection of CHO-K1 cells with varying amounts of p16NK4a and a unique concentration of eEF1A2 substantially reduced the expression of eEF1A2 with increasing concentrations of p16NK4a (Fig. 4B). Furthermore, when eEF1A2-overexpressing and p16NK4a-deficient SKOV3 cells were transfected with pcDNA-p16NK4a, the level of eEF1A2 was decreased with increasing amounts of p16NK4a (Fig. 4C). To rule out the involvement of the Rb pathway, Rb−/− MEFS were co-transfected with V5-eEF1A2 and pcDNA-p16NK4a. Immunoblot analysis showed that the expression of eEF1A2 was decreased with increasing concentrations of p16NK4a (Fig. 4D). To identify the effect of mutant R24A/R131E p16NK4a on expression of eEF1A2, we transfected SKOV3 cells with wild-type p16NK4a or mutant R24A/R131E p16NK4a. Wild-type p16NK4a decreased expression of eEF1A2. However, the mutant R24A/R131E p16NK4a had no effect on the expression level of eEF1A2 (Fig. 4F).

Ectopic expression of p16NK4a attenuates the growth of eEF1A2-overexpressing ovarian cancer cells

We examined the physiological significance of the p16NK4a interaction with eEF1A2 and subsequent inhibition of the expression and translational activity of eEF1A2. When PA-1 cells, which have constitutively high expression of eEF1A2, were transfected with pcDNA-p16NK4a (0, 50, 100 or 200 ng), their growth was significantly retarded by the presence of p16NK4a (100 or 200 ng; Fig. 5A). In addition, PA-1 cells stably transfected with pcDNA-p16NK4a showed significantly decreased colony formation compared to mock-transfected cells (Fig. 5B). We also generated stable cell lines (PA-1, SKOV3 and OVCAR8) transfected with short hairpin RNA against eEF1A2 (sh-eEF1A2) and examined the anchorage-independent colony formation ability of these cells. Reduced colony formation was observed in PA-1 (Fig. 5C), SKOV3 (Fig. 5D) and OVCAR8 (Fig. 5E) cells harboring sh-eEF1A2 compared to cells transfected with control sh-RNA (sh-c). Moreover, cell cycle analysis revealed that sh-eEF1A2-transfected PA-1 cells underwent G1 phase cell cycle arrest (Fig. 5F), whereas sh-eEF1A2-transfected SKOV3 (Fig. 5G) cells exhibited G2/M phase cell cycle arrest. In contrast, cell cycle was not affected in sh-eEF1A2-transfected OVCAR8 cells (Fig. 5H). Different phases of cell cycle arrest might be due to the different characteristics of each cell line. For example, PA-1 cells express Rb, p16NK4a and eEF1A2, SKOV3 cells express Rb and eEF1A2, but are deficient in p16NK4a. OVCAR8 cells express p16NK4a and eEF1A2, but are deficient in Rb. This suggests that p16NK4a can inhibit cell growth by downregulation of eEF1A2 in a cell-cycle-independent manner.

Discussion

In this study, we showed that the tumor suppressor protein, p16NK4a, interacted with a novel binding partner, eukaryotic translation elongation factor, eEF1A2, and the binding caused a downregulation of cancer cell growth by suppressing the expression and subsequent translational activity of eEF1A2. Tumor suppressor p16NK4a is a well-known cell cycle inhibitor of the Cdk4 or Cdk6/cyclin D complexes in the early check-point of the G1 phase. A dysfunctional p16NK4a protein results in the deregulation of the Rb and p53 tumor-suppressor signaling pathways in a wide variety of human cancers (Caldas et al., 1994; Krimpenfort et al., 2001; Sherr, 2001). The eEF1A1 mRNA and

![Fig. 4. p16NK4a regulates eEF1A2 expression. (A–D) The protein level of eEF1A2 is decreased (A) by increasing the expression of p16NK4a in the Tet-off system; (B) upon co-transfection of p16NK4a and eEF1A2 into COS-7 cells; (C) when transfected with p16NK4a in p16NK4a-deficient, eEF1A2-overexpressing SKOV3 cells; and (D) when co-transfected with p16NK4a and eEF1A2 in Rb−/− MEFS. The expression of p16NK4a and eEF1A2 was detected by immunoblot analysis of protein lysates prepared from the respective cells. (E) Rescue of eEF1A2 expression. Rb−/− MEFS were co-transfected with p16NK4a and eEF1A2. (F) The effect of wild-type p16NK4a or mutant R24A/R131E p16NK4a on SKOV3 cells. All experiments were repeated at least three times using independent samples and data are representative of similar experimental results.](image-url)
protein are expressed ubiquitously in many cancer cell types (Joseph et al., 2004; Scaggiante et al., 2012) (supplementary material Fig. S4). An anchorage-independent cell growth assay was performed to compare the oncogenic properties of eEF1A1 and eEF1A2. Results indicated that eEF1A2 expression induced more colony formation compared to eEF1A1 expression (supplementary material Fig. S5). The expression of eEF1A2 appears to be inversely related to the expression of p16\textsuperscript{INK4a} but not associated with p15\textsuperscript{INK4b} or p21 expression. Thus, p16\textsuperscript{INK4a} deficiency seems to be associated with an increased expression level of eEF1A2 in cancer cells. We observed interaction of p16\textsuperscript{INK4a} and eEF1A2 \textit{in vitro} and \textit{ex vivo} (Fig. 1). These results strongly indicate that p16\textsuperscript{INK4a} regulates eEF1A2 expression through a direct interaction. Although eEF1A1 and eEF1A2
Materials and Methods

**Chemicals and reagents**

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was procured from Sigma (St. Louis, MO). Minimum essential medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640 and fetal bovine serum (FBS) were purchased from Invitrogen ( Gibco, Grand Island, NY). Primary antibodies for p16^INK4a^ were from Pharmingen, Becton Dickinson Co. (Franklin Lakes, NJ). The eEF1A2 antibody was constructed by Takara Korea by changing the peptide sequence from mouse to human (Khalyfa et al., 2001). The eEF1A2 antibody does not detect eEF1A1 expression. The secondary horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies were purchased from Zymed Laboratory (San Francisco, CA).

**Plasmid construction**

pGBK-T7-p16 and pcDNA3.1-v5-His-A-eEF1A2 were generated by PCR using the pcDNA3.1-v5 and HeLa cDNA as templates. Plasmids were filtered and purified and digested with EcoRI/BamHI and EcoRI/XbaI, respectively, and cloned into the EcoRI/BamHI and EcoRI/XbaI sites of pGBK-T7 (Clontech, Mountain View, CA) and pcDNA3.1-v5-His-A (Invitrogen, Carlsbad, CA). The pM-BD-p16 plasmid was constructed by releasing p16 from pGBK-T7 by EcoRI/BamHI digestion. The cDNA insert was subcloned into the EcoRI/BamHI site of the pMD-B vector (Clontech). pVP16-AD/eEF1A2 was generated by PCR using pcDNA3.1-v5-HisA-eEF1A2 as a template. The PCR fragment was purified, digested with EcoRi/XbaI and cloned into the EcoRI/XbaI site of the pVP16-AD vector (Clontech). eEF1A2 (full-length), eEF1A2-D1 (1–249 aa), eEF1A2-D2 (250–327 aa), eEF1A2-D3 (328–463 aa), eEF1A2-D1+D2, eEF1A2-D2+D3 and eEF1A2-D1+D3 were subcloned into the pcDNA3-FLAG expression vector (Invitrogen) for the 5’ EcoRI and 3’ Xhol sites. p16^INK4a^ (159 aa), p16^INK4a^ (1–159 aa), p16^INK4a^ (1–180 aa) and p16^INK4a^ (81–156 aa) were subcloned into the pGEX-4T-2 expression vector (Amersham, Piscataway, NJ) at the 5’ BamHI and 3’ Xhol sites. The R24A and R31E mutations were produced using the QuickChange Lightning site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Various expression vectors were amplified in *Escherichia coli*. XL-Blue bacteria and the plasmids were purified using the Jetstar midi kit (Genomed GmbH, Löhne, Germany). The DNA sequences of all plasmids were confirmed by sequencing (Dye Terminator ABI Type Seq., Bioneer, Seoul, South Korea).

**Yeast two-hybrid screening**

Saccharomyces cerevisiae strain AH109 was transformed with 0.1 μg of pGBK-T7-p16 as bait using a lithium acetate method and was plated on medium lacking –Trp DP supplement. The yeast strain AH109 (Clontech) containing pGBK-T7-p16 was mated with the pre-transformed human HeLa cDNA library in Y187 (Clontech) and plated on medium lacking –Leu–Trp–His–Leu–Trp. –Ads–His–Leu–Trp–Trp DP supplements (Clontech). Colonies were detected with X-gal. To recover library plasmids, total DNA from Ads–His–Leu–Trp+ colonies was isolated by the lyticase method and used to transform *E. coli* (XL-1 blue strain from Stratagene, Santa Clara, CA). DNA/As were identified through DNA sequencing (Dye Terminator ABI Type Seq., Bioneer, Seoul, South Korea).

**Cell culture and transfection**

Human ovarian cancer (PA-1, SKOV3), human cervical cancer (HeLa), CHO-K1 and COS-7 cells (Chinese hamster ovary and African green monkey kidney, respectively) were purchased from American Type Culture Collection (Manassas, VA). Rb^−^ mouse embryonic fibroblasts (MEFs) and OVCAR8 cells were a kind gift from Dr Anton Berns (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Dr Shridar Vijayalakshmi (Mayo Clinic College of Medicine, Rochester, MN), respectively. PA-1, OVCAR3 and HeLa cells were cultured in MEM containing penicillin (100 units/ml), streptomycin (100 μg/ml) sodium pyruvate (1 mM) and 10% FBS (GIBCO). SKOV3 cells were cultured in RPMI 1640 containing penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% FBS. COS-7 and Rb^−^ MEFs were grown in DMEM containing neomycin (100 μg/ml), streptomycin (100 μg/ml) and 10% FBS. Cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO2. We transfected SKOV3 and COS-7 cells with the pcDNA3-1-p60^INK4a^ (4 μg) or pcDNA3.1-v5-His-eEF1A2 (4 μg) plasmid and jetPEI poly transfection reagent (84 μl; Polyplus-transfection SAS, Saint Quentin Yvelines, France) in 60-mm dishes to generate SKOV3 cells transiently expressing p16^INK4a^ or COS-7 cells expressing p16^INK4a^ and eEF1A2. For the mammalian two-hybrid assay, we seeded COS-7 cells in 24-well plates for 24 hours and then cells were transfected with pl-MB-p16^INK4a^ (0.4 μg), pVP16-AD/eEF1A2 (0.4 μg), pcDNA3.1-Flag (0.4 μg) plasmids, and pRL-TK internal control reporter (0.5 μg) plasmid and jetPEI poly transfection reagent according to the manufacturer’s instructions. Cells were harvested 24 hours after transfection and disrupted with Sx lysis buffer. Protein–protein interaction activity was determined by luciferase activity and was normalized to pRL-TK activity (Promega, Madison, WI).

**Immunoprecipitation**

Transfected PA-1 and COS-7 cells were harvested in NET-NL lysis buffer containing 50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, 0.2 mM PMSF, and a mixture of protease inhibitors (Complete; Roche, Mannheim, Germany). Cell lysates (1000 μg) were clarified by centrifugation before incubation overnight at 4°C with a monoclonal antibody...
against p16INK4a (4 µg/μl) (Chemicon, Becton Dickinson Co., Franklin Lakes, NJ) in NET-NT 150 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, 2 µg/ml BSA, 0.2 mM PMSF, and a mixture of protease inhibitors (Complete, Roche). An aliquot of 50 µl pre-washed protein G-agarose (Roche; 50% slurry) was then added and the incubation continued for 2 h at 4°C. Immunecomplexes were recovered by centrifugation, washed three times in NETW buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, and 0.2 mM PMSF) and resolved by western blotting.

**Western blot analysis**

Cells were disrupted on ice for 30 min in cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 20,817 g for 15 minutes, the supernatant fraction was harvested as the total cellular protein extract. The protein concentration was determined using the Bio-Rad protein assay reagent (Richmond, CA). The total cellular protein extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes in 20 mM Tris-HCl (pH 8.0), containing 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 5% nonfat dry milk in 1× TBS containing 0.05% Tween 20 (TBS-T) and incubated with antibodies against p16, eEF1A2 or actin. Blots were washed three times in 1× TBS-T buffer, followed by the incubation with the HRP-linked IgG. The specific proteins in the blots were visualized using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**GST pull-down assay**

For expression of deletion mutant eEF1A2, the plasmids (pcDNA-FLAG-full-length eEF1A2, pcDNA1Δ1-124a, pcDNA1Δ2-250-327 aa, pcDNA1Δ2-328-463 aa, pcDNA1Δ1+Δ2, pcDNA1Δ2+Δ3, pcDNA1Δ1+Δ3) and mutant p16INK4a (R24A, R131E, R24A/R131E) were transfected into 293 cells or pcDNA-expression vectors were incubated at 30°C for 4 hours with 300 µl of cell lysate or 20 µl of translated proteins. The bound proteins were washed three times and boiled with 2.5× sample buffer for 3 minutes, centrifuged, and then the supernatant fraction was examined by 15% SDS-PAGE. The binding was detected by autoradiography or western blotting.

**Protein–protein docking**

The docking structures of p16INK4a and eEF1A2 were generated by the rigid-body global search algorithm and further refined by molecular dynamics simulations. Rigid-body docking was performed by the Fast Fourier Transform-based docking algorithm ZDOCK (Chen et al., 2003; Chen and Weng, 2003; Mintseris et al., 2007), where p16INK4a and eEF1A2 were treated as solid objects. Subsequent energy-minimization and molecular dynamics simulations of the p16INK4a/eEF1A2 complex were performed by using the Impref and Impact modules from the Schrödinger software package (Maestro v 9.0; Schrödinger; L.L.C.: New York, NY, 2010).

**Cell proliferation assay**

To determine growth of PA-1 cells, p16INK4a-transfected cells were plated at a density of 5×103 cells per well in 96-well plates for 24 hours prior to the assay. Cells were transfected with 0, 50, 100, 200 ng of pcDNA3.1-p16INK4a and jetPEI polytransfection reagent to generate p16INK4a-transient expression. Cells were then incubated for 6, 12, 24, 48 or 72 hours at 37°C in a humidified atmosphere of 95% air/5% CO2. At the various time points indicated, 10 µl of MTT (5 mg/ml) were added to each well. After 2 hours of incubation, 100 µl of DMSO were added and the optical density (O.D.) was read at 570 nm with a Microplate reader (Molecular Devices, Downingtown, PA). For the anchorage-independent colony formation assay, each cell line (8×103) was suspended in 1 ml of 0.33% basal medium Eagle (BME) agar with 10% FBS and plated over a layer of solidified BME/10% FBS/0.5% agar (3 ml). Cultures were maintained in a 5% CO2 incubator at 37°C for ~7 days. Colonies were then counted using a microscope and the Image-Pro PLUS computer software program (v. 6.2, Media Cybernetics, Bethesda, MD).

**Translational activity assay**

An aliquot (1 µg) of luciferase DNA (Promega) was added to 100 µl of a rabbit reticulocyte lysate in vitro translation reaction (Promega) in the presence of eEF1A2 or GST purified recombinant p16INK4a protein at different concentrations (100, 200, 400 or 800 nM). The reaction mixture was incubated at 30°C for 2 hours. Luciferase protein activity was measured by adding substrate (Promega). The newly synthesized [35S]labeled luciferase protein was analyzed by a 12% SDS-PAGE and detected by autoradiography. Coomassie staining was performed to verify equal loading. To measure the translational activity, Xenopus laevis embryos were obtained by artificial fertilization. Vitelline membranes were removed by immersing embryos in 2% cysteine solution (pH 8.0, Spectrum...


J. Mol. Graph. 14, 33-38.


Fig. S1. Candidate clones and sequence alignments. (A) Approximately 2.0×10^6 yeast transformants were screened and 70 primary candidates were identified as follows: Group I: >1000 bp, 16 clones; Group II: ≤1000 bp (>500 bp) 33 clones; Group III: <500 bp, 21 clones. The corresponding prey plasmids were isolated and sequenced. (B) Amino acid sequence of eEF1A2 and INT SE shows 96% similarity. The candidate p16^INK4a binding partners were then identified by NCBI BLAST searches of the nucleotide and protein databases.
**Fig. S2. Interaction between p16^{INK4a} and eEF1A2 or eEF1A1.** A Flag-tagged eEF1A2 or eEF1A1 full-length or mock plasmid was incubated with an equal amount of GST-p16^{INK4a}. Bound proteins were analyzed by 12% SDS-PAGE and detected by western blotting.

**Fig. S3. Hydrogen bonding between the interface residues in the computational model of the p16^{INK4a}-eEF1A2 complex.** (A) Hydrogen bonds between the residues from the first ankyrin repeat of p16^{INK4a} and those from Domain III of eEF1A2. (B) Hydrogen bonding between the residues from the second ankyrin repeat of p16^{INK4a} and those from Domain III of eEF1A2. (C) Hydrogen bonding between the residues from the third ankyrin repeat of p16^{INK4a} and those from Domain III of eEF1A2. (D) Hydrogen bonding between the residues from the fourth ankyrin repeat of p16^{INK4a} and those from Domain I of eEF1A2 (with one exception of Arg382 from Domain III). These figures were generated using the Schrödinger Maestro software program.
**Fig. S4. Expression of eEF1A2 and p16^{INK4a} in cancer cell lines.** The seven upper panels show protein expression of eEF1A2 and p16^{INK4a} and the four lower panels show mRNA levels of eEF1A2 and p16^{INK4a}. 

<table>
<thead>
<tr>
<th>Ovary</th>
<th>Breast</th>
<th>Prostate</th>
<th>Skin</th>
<th>Sarcoma</th>
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<tr>
<td>PA-1</td>
<td>SK-OV-3</td>
<td>MCF-7</td>
<td>MDA-MB-231</td>
<td>LNCaP-FGC</td>
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<table>
<thead>
<tr>
<th>Protein level</th>
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<tr>
<td>eEF1A2</td>
</tr>
<tr>
<td>p16^{INK4a}</td>
</tr>
<tr>
<td>p15^{INK4b}</td>
</tr>
<tr>
<td>p21</td>
</tr>
<tr>
<td>eEF1A1</td>
</tr>
<tr>
<td>eEF1A</td>
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<tr>
<td>actin</td>
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</table>

<table>
<thead>
<tr>
<th>mRNA level</th>
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<tbody>
<tr>
<td>eEF1A2</td>
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<tr>
<td>p16^{INK4a}</td>
</tr>
<tr>
<td>eEF1A1</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
</tbody>
</table>
Fig. S5. Anchorage-independent cell growth in NIH3T3 cells transfected with mock, eEF1A1 or eEF1A2. Cells were stably transfected with mock, eEF1A1 or eEF1A2. Similar results were obtained from three independent experiments. Data are shown as means ± s.d. of 3 independent experiments and the asterisk (*) indicates a significant difference ($P<0.01$).
Fig. S6. The mRNA levels of eEF1A2, eEF1A1 and p16^INK4a in SKOV3 and OVCAR8 cells. Cells were transfected with mock or p16^INK4a and harvested after 48 h for preparing total RNAs. mRNA expression of eEF1A2, eEF1A1 and p16^INK4a were analyzed by reverse transcriptase-PCR. Similar results were obtained from three independent experiments. Data are shown as means ± s.d. of 3 independent experiments and the asterisk (*) indicates a significant difference (P<0.01).
Table S1. Hydrogen bonds in the computational model of the p16\textsuperscript{INK4a}-eEF1A2 complex

<table>
<thead>
<tr>
<th>Element (Atom-H)</th>
<th>Element (Atom-A)</th>
<th>Distance (&lt;2.5 Å)</th>
<th>DHA angle (&gt;120º)</th>
<th>HAB angle (&gt;90º)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eEF1A2(III): Lys376-3HZ</td>
<td>P16(1\textsuperscript{st}): Arg22-O</td>
<td>1.666</td>
<td>150.6</td>
<td>162.3</td>
</tr>
<tr>
<td>P16(1\textsuperscript{st}): Arg22-HE</td>
<td>eEF1A2(III):Glu442-OE2</td>
<td>2.322</td>
<td>126.4</td>
<td>140.8</td>
</tr>
<tr>
<td>P16(1\textsuperscript{st}): Arg24-1HH1</td>
<td>eEF1A2(III):Glu374-OE1</td>
<td>1.535</td>
<td>154.2</td>
<td>102.1</td>
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<td>P16(1\textsuperscript{st}): Arg24-2HH2</td>
<td>eEF1A2(III):Glu374-OE2</td>
<td>1.907</td>
<td>157.8</td>
<td>107.1</td>
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<td>P16(1\textsuperscript{st}): Arg24-2HH1</td>
<td>eEF1A2(III):Glu403-OE2</td>
<td>1.686</td>
<td>150.7</td>
<td>139.3</td>
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<tr>
<td>eEF1A2(III):Gln335-2HE2</td>
<td>P16(2\textsuperscript{nd}): Tyr44-OH</td>
<td>2.356</td>
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<td>96.9</td>
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<tr>
<td>P16(2\textsuperscript{nd}): Tyr44-HH</td>
<td>eEF1A2(III): Lys443-O</td>
<td>2.008</td>
<td>143.8</td>
<td>121.3</td>
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<tr>
<td>P16(2\textsuperscript{nd}): Arg46-1HH2</td>
<td>eEF1A2(III):Asp332-OD1</td>
<td>1.698</td>
<td>173.9</td>
<td>109.4</td>
</tr>
<tr>
<td>eEF1A2(III):Asn440-1HD2</td>
<td>P16(2\textsuperscript{nd}):Met52-O</td>
<td>1.732</td>
<td>166.8</td>
<td>107.5</td>
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<tr>
<td>eEF1A2(III):Glu377-HE2</td>
<td>P16(2\textsuperscript{nd}):Gly55-O</td>
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<td>161.9</td>
<td>113.1</td>
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<tr>
<td>P16(2\textsuperscript{nd}): Arg58-2HH1</td>
<td>eEF1A2(III): Asp389-OD2</td>
<td>1.812</td>
<td>155.3</td>
<td>162.5</td>
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<tr>
<td>eEF1A2(III):Gln417-2HE2</td>
<td>P16(3\textsuperscript{rd}): Asp74-OD2</td>
<td>1.954</td>
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<td>144.9</td>
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<tr>
<td>P16(3\textsuperscript{rd}): Arg87-1HH1</td>
<td>eEF1A2(III): Ser416-O</td>
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<td>130.0</td>
<td>120.9</td>
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<tr>
<td>eEF1A2(III):Ser416-HG</td>
<td>P16(3\textsuperscript{rd}): Glu88-OE1</td>
<td>1.739</td>
<td>155.5</td>
<td>119.8</td>
</tr>
<tr>
<td>eEF1A2(III): Lys439-1HZ</td>
<td>P16(3\textsuperscript{rd}): Glu88-OE2</td>
<td>1.530</td>
<td>176.1</td>
<td>119.3</td>
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<tr>
<td>eEF1A2(III): Lys385-1HZ</td>
<td>P16(3\textsuperscript{rd}): Asp92-OD2</td>
<td>1.492</td>
<td>169.8</td>
<td>144.6</td>
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<tr>
<td>P16(4\textsuperscript{th}): Trp110-HE1</td>
<td>eEF1A2(I): Asp243-OD2</td>
<td>1.966</td>
<td>136.1</td>
<td>103.6</td>
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<tr>
<td>P16(4\textsuperscript{th}): Arg112-1HH1</td>
<td>eEF1A2(I): Thr239-OG1</td>
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<tr>
<td>eEF1A2(I): Lys219-3HZ</td>
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<td>1.608</td>
<td>172.3</td>
<td>157.4</td>
</tr>
<tr>
<td>eEF1A2(I): Lys146-2HZ</td>
<td>P16(4\textsuperscript{th}): Glu120-OE1</td>
<td>1.447</td>
<td>171.2</td>
<td>121.6</td>
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</table>
The ankyrin repeats of p16INK4a and domains of eEF1A2 are also labeled in the table below.

<table>
<thead>
<tr>
<th>eEF1A2(I): Tyr141-HH</th>
<th>P16(4th): Gly122-O</th>
<th>1.690</th>
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<th>127.1</th>
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<tr>
<td>eEF1A2(III): Arg382-1HH1</td>
<td>P16(4th): Asp125(OD1)</td>
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<tr>
<td>P16(4th): Arg131-2HH2</td>
<td>eEF1A2(I): Glu217-OE2</td>
<td>1.714</td>
<td>151.5</td>
<td>143.9</td>
</tr>
</tbody>
</table>

The hydrogen bonds are defined by default when the following distances and angle cut-offs were satisfied: 2.5 Å for H-A distance; D-H-A angle greater than 120°; and H-A-B angle greater than 90° where H is the hydrogen, A is the acceptor, D is the donor, and B is a neighbor atom bonded to the acceptor.

Note: HH is the hydrogen of the phenolic hydroxyl group of tyrosine. 2HE2 is one hydrogen in the amide side chain group of glutamine; HE2 is one carboxylic hydrogen of glutamic acid; 1HZ, 2HZ and 3HZ are the three hydrogens in the ε-ammonium group of lysine; 1HH1, 1HH2, 2HH2, 2HH1 and HE are the five hydrogens in the guanidinium side chain of arginine; O is the oxygen in the backbone amide group; OE1, OE2 are the two carboxylic oxygens of glutamic acid; OD1 and OD2 are the two carboxylate oxygens of aspartic acid.