Tumor suppressor p16^INK4a inhibits cancer cell growth by downregulating eEF1A2 through a direct interaction

Mee-Hyun Lee1,2,*, Bu Young Choi3,*, Yong-Yeon Cho1,4, Sung-Young Lee1,2, Zunnan Huang1,‡, Joydeb Kumar Kundu5, Myoung Ok Kim1,6, Dong Joon Kim1,§, Ann M. Bode1, Young-Joon Surh2,∗ and Zigang Dong1,2,†

1The Hormel Institute, University of Minnesota, MN 55912, USA
2WCU Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology and Tumor Microenvironment Global Core Research Center, College of Pharmacy, Seoul National University, Seoul, South Korea
3Pharmaceutical Science and Engineering, School of Convergence Bioscience and Technology, University of SeoWon, Chungju, Chungbuk, 361-742, South Korea
4College of Pharmacy, The Catholic University of Korea, Bucheon, Gyeonggi-do, South Korea
5College of Pharmacy, Keimyung University, Daegu, South Korea
6School of Animal Science, KyungPook National University, Sangju, South Korea
*These authors contributed equally to this work
†Present address: China-America Cancer Research Institute, Guangdong Medical College, Dongguan, Guangdong 523808, People's Republic of China
§Present address: Medical Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea
∗Authors for correspondence (zgdong@hi.umn.edu; surh@plaza.snu.ac.kr)

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

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Tumor suppressor p16\textsuperscript{INK4a} inhibits cancer cell growth by downregulating eEF1A2 through a direct interaction

Mee-Hyun Lee\textsuperscript{1,2,\*}, Bu Young Choi\textsuperscript{3,\*}, Yong-Yeon Cho\textsuperscript{1,4}, Sung-Young Lee\textsuperscript{1,2}, Zunnan Huang\textsuperscript{1}, Joydeb Kumar Kundu\textsuperscript{2,5}, Myoung Ok Kim\textsuperscript{1,5}, Dong Joon Kim\textsuperscript{1,\#}, Ann M. Bode\textsuperscript{1}, Young-Joon Surh\textsuperscript{2,\*} and Zigang Dong\textsuperscript{1,6}

\textsuperscript{1}The Hormel Institute, University of Minnesota, MN 55912, USA
\textsuperscript{2}WCU Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology and College of Pharmacy, Seoul National University, Seoul, South Korea
\textsuperscript{3}C&C Research Labs, Hwasung City, Gyeonggi-do 445-970, South Korea
\textsuperscript{4}College of Pharmacy, The Catholic University of Korea, Bucheon, Gyeonggi-do, South Korea
\textsuperscript{5}School of Animal Science, KyungPook National University, Sangju, South Korea
\textsuperscript{6}Present address: Medical Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea

\*These authors contributed equally to this work
\\#Present address: China-America Cancer Research Institute, Guangdong Medical College, Dongguan, Guangdong 523808, People’s Republic of China

Authors for correspondence (zgdong@hi.umn.edu; surh@plaza.snu.ac.kr)

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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 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 check-point kinases (Chk1 and Chk2) are critical regulators of cell cycle progression (Collins and Garrett, 2005; Galí-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; Ruggiero et al., 2004; Ruggiero and Pandolfi, 2003). Several eukaryotic translation initiation (eIF) and elongation (eEF) factors have been implicated in carcinogenesis (Sonenberg and Hinnebusch, 2009; White-Gilbertson 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 with GTP-dependent 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 (\textit{EEF1A1} in 6q14 and \textit{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

binding affinity for GDP and GTP (Kahns et al., 1998). While eEF1A2 exhibits a stronger affinity for GDP than GTP, eEF1A1 displays the opposite preference. The two isoforms of eEF1A also differ in their tissue distribution. The eEF1A1 isoform is expressed in almost all tissues, whereas eEF1A2 is present only in those tissues, such as brain, heart and skeletal muscle, that are composed of cells locked in a state of nonproliferation (Khalifa et al., 2001; Knudsen et al., 1993; Lee et al., 1992; Lee et al., 1995; Lee et al., 1993). Because eEF1A2 is expressed specifically in certain cells, such as neurons, cardiomyocytes and myocytes, that have permanently deviated from the cell cycle (Kahns et al., 1998; Knudsen et al., 1993; Lee et al., 1994), eEF1A2 is suggested to be involved in pathways of protein synthesis that are preferentially for nonproliferating cells or play non-translational roles. Beyond its defined role in the protein translation process, eEF1A2 has been reported to be involved in other non-translational functions of cells and has been identified as a putative oncogene (Amiri et al., 2007; Anand et al., 2002; Kulkarni et al., 2007; Lee and Surh, 2009; Li et al., 2010; Tomlinson et al., 2005). Anand and colleagues reported that p16INK4a interacts with eukaryote elongation factor 1A2 in mammalian cells.

The ectopic overexpression of eEF1A2 provides mouse fibroblasts (NIH3T3) with oncogenic potential and accelerates the growth of ovarian carcinoma (ES-2) cells as xenografts in nude mice. In addition, the results of tissue microarray for the assessment of eEF1A2 protein abundance in 500 primary ovarian tumors showed high expression levels in about 30% of all primary ovarian tumors (Pinke et al., 2008). In the present study, we demonstrate a novel mechanism whereby p16INK4a interacts with eEF1A2 and exerts its tumor suppressor function by downregulating eEF1A2 in ovarian cancer cells.

Fig. 1. eEF1A2 is identified as a p16INK4a-interacting protein.

(A) The interaction of proteins expressed from pM-BD-p16INK4a and pVP-eEF1A2 was determined using the mammalian two-hybrid assay. The luciferase activity indicates the change in relative luminescence units normalized to a negative control (average %). (B) COS-7 cells were co-transfected with p16INK4a and eEF1A2. The p16INK4a protein was immunoprecipitated with a p16INK4a monoclonal antibody. The eEF1A2 proteins in the immunoprecipitates were detected by immunoblotting with a polyclonal antibody against V5-tagged eEF1A2. (C) A pull-down assay was used to confirm the interaction between GST–p16INK4a and eEF1A2 or CDK4 in whole cell lysates, and proteins were detected by western blotting. (D) Whole cell lysates prepared from serum-starved (24 hours) PA-1 cells were incubated for an additional 12 hours with or without 1% fetal bovine serum (+/− serum). p16INK4a and eEF1A2 proteins were co-immunoprecipitated from PA-1 cells. Cell lysates were immunoprecipitated with a p16INK4a antibody and the immunoprecipitate was subjected to immunoblot analysis using an eEF1A2 rabbit polyclonal antibody.
transfection in 293 cells, and the cell lysates were pulled down using the Sepharose-4B-bead-conjugated GST fusion protein, p16\textsuperscript{INK4a}. Results of the GST pull-down assay showed that p16\textsuperscript{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\textsuperscript{INK4a} (Fig. 2B). The binding between p16\textsuperscript{INK4a} and eEF1A2 was also predicted by the Fast Fourier transform-based docking algorithm ZDOCK. The model of the p16\textsuperscript{INK4a}–eEF1A2 complex obtained from the protein–protein docking experiment suggested that p16\textsuperscript{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\textsuperscript{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\textsuperscript{INK4a} are expected to be crucial in the binding of p16\textsuperscript{INK4a} with eEF1A2. Arg24 in p16\textsuperscript{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\textsuperscript{INK4a} and those from domain I of eEF1A2 would also contribute to the stability of the p16\textsuperscript{INK4a}–eEF1A2 complex (supplementary material Table S1; Fig. S3C,D). Arg131 in p16\textsuperscript{INK4a} forms two hydrogen bonds with Glu217 in eEF1A2. These hydrogen bonds would explain the \textit{in vitro} and \textit{ex vivo} experimental findings regarding the p16\textsuperscript{INK4a} interaction with eEF1A2 (328–463 aa) and eEF1A2 (1–249 aa). To confirm this idea, we constructed a p16\textsuperscript{INK4a} protein with mutations at Arg24 and Arg131. Double mutation of Arg24 (R24A) and Arg131 (R131E) disrupted the interaction of p16\textsuperscript{INK4a} with eEF1A2 as expected (Fig. 2D). This result indicated that Arg24 and Arg131 of p16\textsuperscript{INK4a} are important for interaction with eEF1A2 domain I/III.

\textbf{p16\textsuperscript{INK4a} inhibits translational activity \textit{in vitro} and \textit{in vivo}: role of eEF1A2}

We then examined the functional significance of the interaction between p16\textsuperscript{INK4a} and eEF1A2. Because eEF1A2 functions as an elongation factor in peptide chain elongation during protein synthesis, the antiproliferative protein p16\textsuperscript{INK4a} might attenuate the translational activity of eEF1A2. To explore this possibility, \textit{in vitro} protein synthesis was assessed in the presence of varying concentrations (0, 100, 200, 400 and 800 nM) of recombinant p16\textsuperscript{INK4a}. The \textit{in vitro} transcription and translation experiments were performed using luciferase cDNA as the reporter gene and
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\textsuperscript{INK4a} (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\textsuperscript{INK4a} on eEF1A2 translational activity. When Xenopus embryos were injected with mRNAs of luciferase alone or with luciferase plus p16\textsuperscript{INK4a}, the luciferase translational activity was significantly decreased in the presence of p16\textsuperscript{INK4a}, suggesting that the expression of p16\textsuperscript{INK4a} inhibits the translation of luciferase mRNA (Fig. 3C). Moreover, the expression of eEF1A2 was decreased with injection of luciferase (1 ng) plus p16\textsuperscript{INK4a} (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 μM; Fig. 3E). Injection of MO-eEF1A2 (5 μM) in Xenopus decreased the luciferase activity (Fig. 3F). To clarify the effect of p16\textsuperscript{INK4a} on eEF1A2 translational activity, Xenopus embryos were separately injected with luciferase (500 pg) alone, luciferase plus p16\textsuperscript{INK4a} (100 pg), luciferase plus p16\textsuperscript{INK4a} plus MO-eEF1A2 (2.5 μM), 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\textsuperscript{INK4a} (Fig. 3G). These data suggest that p16\textsuperscript{INK4a} inhibited the translational activity of eEF1A2 in vitro and in vivo.

**Fig. 3.** p16\textsuperscript{INK4a} 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 INK4a 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\textsuperscript{INK4a}. 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\textsuperscript{INK4a} after injection of p16\textsuperscript{INK4a} 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\textsuperscript{INK4a}. Antibodies against eEF1A2 and p16\textsuperscript{INK4a} were used for the detection of endogenous eEF1A2 and exogenous p16\textsuperscript{INK4a}, respectively. The p16\textsuperscript{INK4a} 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. β-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\textsuperscript{INK4a}, luciferase plus p16\textsuperscript{INK4a} 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.
p16^{INK4a} inhibits the expression of eEF1A2

The finding that p16^{INK4a} modulates the expression of eEF1A2 was confirmed by using the stably transfected p16^{INK4a} Tet-off system in HeLa cells (Fig. 4A). After removal of doxycycline, p16^{INK4a} 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 p16^{INK4a} and a unique concentration of eEF1A2 substantially reduced the expression of eEF1A2 with increasing concentrations of p16^{INK4a} (Fig. 4B). Furthermore, when eEF1A2-overexpressing and p16^{INK4a}-deficient SKOV3 cells were transfected with pcDNA-p16^{INK4a}, the level of eEF1A2 was decreased with increasing amounts of p16^{INK4a} (Fig. 4C). To rule out the involvement of the Rb pathway, Rb^{-/-} MEFs were co-transfected with V5-eEF1A2 and pcDNA-p16^{INK4a}. Immunoblot analysis showed that the expression of eEF1A2 was decreased with increasing concentrations of p16^{INK4a} (Fig. 4D). Furthermore, decreased eEF1A2 expression level was overcome by additional transfection of eEF1A2 (Fig. 4E). To identify the effect of mutant R24A/R131E p16^{INK4a} on expression of eEF1A2, we transfected SKOV3 cells with wild-type p16^{INK4a} or mutant R24A/R131E p16^{INK4a}. Wild-type p16^{INK4a} decreased expression of eEF1A2. However, the mutant R24A/R131E p16^{INK4a} had no effect on the expression level of eEF1A2 (Fig. 4F).

Ectopic expression of p16^{INK4a} attenuates the growth of eEF1A2-overexpressing ovarian cancer cells

We examined the physiological significance of the p16^{INK4a} 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-p16^{INK4a} (0, 50, 100 or 200 ng), their growth was significantly retarded by the presence of p16^{INK4a} (100 or 200 ng; Fig. 5A). In addition, PA-1 cells stably transfected with pcDNA-p16^{INK4a} 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, p16^{INK4a} and eEF1A2. SKOV3 cells express Rb and eEF1A2, but are deficient in p16^{INK4a}. OVCAR8 cells express p16^{INK4a} and eEF1A2, but are deficient in Rb. This suggests that p16^{INK4a} 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, p16^{INK4a}, 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 p16^{INK4a} 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 p16^{INK4a} 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
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\(^{\text{INK4a}}\) but not associated with p15\(^{\text{INK4b}}\) or p21 expression. Thus, p16\(^{\text{INK4a}}\) deficiency seems to be associated with an increased expression level of eEF1A2 in cancer cells. We observed interaction of p16\(^{\text{INK4a}}\) and eEF1A2 in vitro and ex vivo (Fig. 1). These results strongly indicate that p16\(^{\text{INK4a}}\) regulates eEF1A2 expression through a direct interaction. Although eEF1A1 and eEF1A2

Fig. 5. Implications of p16\(^{\text{INK4a}}\)-mediated downregulation of eEF1A2 in the anchorage-independent growth and cell-cycle regulation of PA-1, SKOV3 and OVCAR8 cells. (A) PA-1 ovarian cancer cell growth was assessed by MTT assay in the presence of increasing amounts of p16\(^{\text{INK4a}}\). (B) Anchorage-independent cell growth was decreased by transfection of PA-1 cells with p16\(^{\text{INK4a}}\). (C–E) Anchorage-independent colony formation was reduced by transfection with sh-eEF1A2, but not by sh-control, in (C) PA-1, (D) SKOV3, and (E) OVCAR8 cells. (F–H) Cell cycle analysis was performed using propidium iodide staining of (F) PA-1, (G) SKOV3 and (H) OVCAR8 cells transfected with either control-shRNA or eEF1A2 sh-RNA. All experiments were repeated at least three times using independent samples and data are shown as means ± s.d. Asterisks (*) indicate a significant difference.
exhibit a high sequence homology, the smaller binding cavity of eEF1A1 compared to eEF1A2 might prevent a strong binding interaction with p16\(^{INK4a}\) (Soares et al., 2009). In the threedimensional structure of eEF1A2 (Fig. 2C), domain II is located behind domain I/III and therefore p16\(^{INK4a}\) interacts with domain I/III. Even though results indicate a low binding affinity between p16\(^{INK4a}\) and eEF1A2, this does not necessarily reflect activity (Marles et al., 2004). Our computational docking model (supplementary material Table S1) indicates that 12 hydrogen bonds are formed between p16\(^{INK4a}\) and eEF1A2. If each hydrogen bond cooperatively affects the activity of eEF1A2, the regulatory effect might become quite significant, as we showed. For the translation activity regulation of eEF1A2 by p16\(^{INK4a}\), we used an in vitro rabbit reticulocyte translational system in a limited time frame (Fig. 3.A,B). Because the sequence of human eEF1A2 coincides with rabbit eEF1A2 and p16\(^{INK4a}\) binds only to eEF1A2, the downregulatory translational activity of eEF1A2 was probably due to the inhibition by p16\(^{INK4a}\) in the time measured. On the other hand, whether all eEF1A isoforms correspond with eEF1A2 is not clear. However, the formation of a heterodimer of eEF1A1 and eEF1A2 has been reported (Sanges et al., 2012). This idea supports our data showing that selective regulation of eEF1A2 by p16\(^{INK4a}\) causes a decrease in the translational activity with the exclusion of a functional eEF1A1, which cannot act alone. If eEF1A2 and eEF1A1 work in the elongation phase of translation as a heterodimer, downregulation of eEF1A2 by p16\(^{INK4a}\) will affect the activity of the heterodimer. Therefore, translational activity could be decreased. To provide direct evidence supporting the regulation of the translational activity of eEF1A2 by p16\(^{INK4a}\) in vivo, we injected Xenopus embryos with combinations of p16\(^{INK4a}\) mRNA, luciferase mRNA, and MO-eEF1A2 and the results confirmed that p16\(^{INK4a}\) regulated eEF1A2 activity (Fig. 3). Specifically, we injected one half of the amount of MO-eEF1A2 in Xenopus embryos (Fig. 3G). Thus, the rest of eEF1A2 activity was inhibited by p16\(^{INK4a}\) thereby decreasing the synthesis of luciferase. In contrast, p16\(^{INK4a}\) had no effect on the expression of eEF1A1 under the same conditions (Fig. 3D,E).

The exact mechanism explaining the decreased expression of eEF1A2 in the presence of p16\(^{INK4a}\) was not associated with ubiquitination and degradation (data not shown). On the hand, the downregulation of the translational activity of eEF1A2 by p16\(^{INK4a}\) might be due to decreased expression of eEF1A2 (supplementary material Fig. S6). After transfection of p16\(^{INK4a}\) in SKOV3 and OVCAR8 cells, we examined the mRNA level of eEF1A2, eEF1A1, and p16\(^{INK4a}\). The results showed that the mRNA level of eEF1A2 was decreased, but that of eEF1A1 was not changed (supplementary material Fig. S6). However, further studies, such as direct interruption of eEF1A2 and other interacting proteins, are needed to determine the specific mechanism for this relationship (Panasyuk et al., 2008) with p16\(^{INK4a}\). Although many questions remain to be answered, the interaction between p16\(^{INK4a}\) and eEF1A2 does induce cell cycle arrest and inhibition of colony formation in various cancer cell types, which suggests a novel function of the tumor suppressor p16\(^{INK4a}\).
against p16NK4a (4 µg/ml plasmid, Becton Dickinson Co., Franklin Lakes, NJ) in NET-NR 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. Immunoprecipitates 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 selenite, 1 mM MgCl2, 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, Piscatway, NJ).

GST pull-down assay

For expression of deletion mutant eEF1A2, the plasmids (pcDNA-FLAG-full-length eEF1A2, pcDNA-D1 (1-249 aa), pcDNA-D2 (250-327 aa), pcDNA-D3 (328-463 aa), pcDNA-D1 (D1-D2), pcDNA-D2 (D2-D3), pcDNA-D1 (D1+D3) and mutant p16INK4a (R24A, R131E, R24A/R131E) were transfected into 293 cells or pcDNA-FLAG-full-length eEF1A2 and pcDNA-D1 were translated in vitro with the TNT Quick coupled transcription/translation system (Promega). GST fusion proteins were collected on glutathione-Sepharose beads (Amersham Pharmacia Biotech) incubated at 4°C for 4 hours with 30 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), and protein–protein interactions were detected by the TNT Quick coupled transcription/translation system (Promega). GST fusion proteins were collected on glutathione-Sepharose beads (Amersham Pharmacia Biotech) incubated at 4°C for 4 hours with 30 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.

Chemical Mfg. Corp., New Brunswick, NJ). Embryos at the one-or-two cell stage were injected with mRNA and morpholinos as described in the figure legends and incubated for 7–8 hours. Luciferase activities were measured using a luciferase assay system according to manufacturer’s instructions. Four or five embryos per group were pooled and homogenized in 15 µl of lysis buffer per embryo. All experiments were repeated at least three times using independent samples. All statistical analyses were performed by using Student’s t-test or ANOVA. The Student’s t-test was used for microinjection were produced by in vitro transcription. Each of the cDNAs (luciferase and p16INK4a) was linearized and used for the in vitro synthesis of capped mRNAs using an in vitro transcription kit (Ambion, Austin, TX) in accordance with the manufacturer’s instructions. The morpholino antisense oligonucleotide directed against Xenopus eEF1A2 (MO-eEF1A2) was 5'-ATGTTGTTCTCTTCTTCCCCATTCC (GeneTools, Philomath, OR). Control MO (5'-CCCTTTAACGTTACATTTATATA-3′; Gene Tools) was used as a toxicity control. Oligos were re-suspended in sterile water and injected into embryos. The embryo lysates were homogenized in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 50 mM NaF and 1 mM NaVO4) containing of 1 mM PMSF, 15 mM β-glycerophosphate, 1× protease inhibitor cocktail (Calbiochem, Darmstadt, Germany) and then used for western blotting.

Statistical analysis

Values are expressed as means ± s.e.m. of at least three independent experiments. Statistical significance was determined by the Student’s t-test and a P-value of less than 0.05 was considered to be statistically significant.

Author contributions


Funding

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Supplementary material available online at http://jcs.biologists.org lookup/supplid/10.1242/jcs.113613/-/DC1

References


Dawes, A. J., 52-62.


References


Fig. S1. Candidate clones and sequence alignments. (A) Approximately $2.0 \times 10^6$ 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.

Human eEF1A1 MKGEKTHINIVGHVDGSKSTTTGTHLYKCGGIDKRTIEKFEKEAAEMKGSFKFAYWVL
Human eEF1A2 MKGEKTHINIVGHVDGSKSTTTGTHLYKCGGIDKRTIEKFEKEAAEMKGSFKFAYWVL
Mouse eEF1A2 MKGEKTHINIVGHVDGSKSTTTGTHLYKCGGIDKRTIEKFEKEAAEMKGSFKFAYWVL
Yeast eEF1A MKGEKSHINVVGHVDGSKSTTTGTHLYKCGGIDKRTIEKFEKEAAELGKGSFKFAYWVL
INT SE MKGEKTHINIVGHVDGSKSTTTGTHLYKCGGIDKRTIEKFEKEAAEMKGSFKFAYWVL
Human eEF1A1 DKLKAERGIDISLWFKETKYI11 TIDAPRDFIKNMTIGTSQACAVLIIAAGV
Human eEF1A2 DKLKAERGIDISLWFKETKYI11 TIDAPRDFIKNMTIGTSQACAVLIIAAGV
Mouse eEF1A2 DKLKAERGIDISLWFKETSKYTVI11 TIDAPRDFIKNMTIGTSQACAVLIIAAGV
Yeast eEF1A1 DKLKAERGIDISLWFKETPKYQVTVIDAPRDFIKNMTIGTSQACAVLIIAAGV
INT SE DKLKAERGIDISLWFKETSKYTVI11 TIDAPRDFIKNMTIGTSQACAVLIIAAGV
Human eEF1A1 GFEAGISKNGQTREHALAYTLGVKQVLVGNKMDSTEPYPSQKRYEEYIKVESTYIKK
Human eEF1A2 GFEAGISKNGQTREHALAYTLGVKQVLVGNKMDTEPAYSEKRYDEVKESAYIKK
Mouse eEF1A2 GFEAGISKNGQTREHALAYTLGVKQVLVGNKMDTEPAYSEKRYDEVKESAYIKK
Yeast eEF1A1 GFEAGISKDOQTREHALATLQVRLQILAVKNMDSKWDERFQIEIKETSIFN IFK
INT SE GFEAGISKNGQTREHALAYTLGVKQVLVGNKMDSTEPYPSQKRYEEYIKVESTYIKK
Human eEF1A1 IGYNPDTPAVFPVPSWGNGDMLLEPSANMPWFKGWVTRKDGNASGTTLLEALDCILPPT
Human eEF1A2 IGYNPDTPAVFPVPSWGNGDMLLEPSANMPWFKGWVTRKDGNASGTTLLEALDCILPPT
Mouse eEF1A2 IGYNPDTPAVFPVPSWGNGDMLLEPSANMPWFKGWVTRKDGNASGTTLLEALDCILPPT
Yeast eEF1A1 VGYNPVTPAVPFVPSWGNGDMLLEPSANMPWFKGWVTRKDGNASGTTLLEALDCILPPT
INT SE IGYNPDTPAVFPVPSWGNGDMLLEPSANMPWFKGWVTRKDGNASGTTLLEALDCILPPT
Human eEF1A1 PTDLKPLRLPDVYKIGGGTVGTVPVRGVTGVLKPGMVTFAPVNVTEVSVMHEALS
Human eEF1A2 PTDLKPLRLPDVYKIGGGTVGTVPVRGVTGVLKPGMVTFAPVNVITEVSVMHEALS
Mouse eEF1A2 PTDLKPLRLPDVYKIGGGTVGTVPVRGVTGVLKPGMVTFAPVNVITEVSVMHEALS
Yeast eEF1A1 PTDLKPLRLPDVYKIGGGTVGTVPVRGVTGVLKPGMVTFAPVNVITEVSVMHEALS
INT SE PTDLKPLRLPDVYKIGGGTVGTVPVRGVTGVLKPGMVTFAPVNVITEVSVMHEALS
Human eEF1A1 EALPGDNVFKNVSNVSVKDRVRGNGAGSDKNNDPEAAGTFAVIIILNHGPSISAYAPV
Human eEF1A2 EALPGDNVFKNVSNVSVKDRVRGNGAGSDKNNDPEAAGTFAVIIILNHGPSISAYAPV
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Yeast eEF1A1 QCGVPDNVFKNVSNVSVKDRVRGNGAGSDKNNDPEAAGTFAVIIILNHGPSISAYAPV
INT SE EALPGDNVFKNVSNVSVKDRVRGNGAGSDKNNDPEAAGTFAVIIILNHGPSISAYAPV
Human eEF1A1 LDCTHAIHCFAELKEKIDRSSKKELEDPLKFDGKLSGAAIVDMVPGKPCMCEFSDYYP
Human eEF1A2 LDCTHAIHCFAELKEKIDRSSKKELEDPLKFDGKLSGAAIVDMVPGKPCMCEFSDYYP
Mouse eEF1A2 LDCTHAIHCFAELKEKIDRSSKKELEDPLKFDGKLSGAAIVDMVPGKPCMCEFSDYYP
Yeast eEF1A1 LDCTHAIHCFAELKEKIDRSSKKELEDPLKFDGKLSGAAIVDMVPGKPCMCEFSDYYP
INT SE LDCTHAIHCFAELKEKIDRSSKKELEDPLKFDGKLSGAAIVDMVPGKPCMCEFSDYYP
Human eEF1A1 LGRAFADMRQTVAVGVKAVDKKAAGGKVTSAQAKQA
Human eEF1A2 LGRAFADMRQTVAVGVKAVDKKAAGGKVTSAQAKQA
Mouse eEF1A2 LGRAFADMRQTVAVGVKAVDKKAAGGKVTSAQAKQA
Yeast eEF1A1 LGRAFADMRQTVAVGVKAVDKKAAGGKVTSAQAKQA
INT SE LGRAFADMRQTVAVGVKAVDKKAAGGKVTSAQAKQA
**Fig. S2. Interaction between **p16<sup>INK4a</sup>** and eEF1A2 or eEF1A1.** A Flag-tagged eEF1A2 or eEF1A1 full-length or mock plasmid was incubated with an equal amount of GST-p16<sup>INK4a</sup>. 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<sup>INK4a</sup>-eEF1A2 complex.** (A) Hydrogen bonds between the residues from the first ankyrin repeat of p16<sup>INK4a</sup> and those from Domain III of eEF1A2. (B) Hydrogen bonding between the residues from the second ankyrin repeat of p16<sup>INK4a</sup> and those from Domain III of eEF1A2. (C) Hydrogen bonding between the residues from the third ankyrin repeat of p16<sup>INK4a</sup> and those from Domain III of eEF1A2. (D) Hydrogen bonding between the residues from the fourth ankyrin repeat of p16<sup>INK4a</sup> 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 p16INK4a in cancer cell lines. The seven upper panels show protein expression of eEF1A2 and p16INK4a and the four lower panels show mRNA levels of eEF1A2 and p16INK4a.
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 p16INK4a in SKOV3 and OVCAR8 cells. Cells were transfected with mock or p16INK4a and harvested after 48 h for preparing total RNAs. mRNA expression of eEF1A2, eEF1A1 and p16INK4a 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).
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<th>Element (Atom-A)</th>
<th>Distance (&lt;2.5 Å)</th>
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<th>HAB angle (&gt;90°)</th>
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The ankyrin repeats of p16\textsuperscript{INK4a} and domains of eEF1A2 are also labeled in the table below. 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 \( \varepsilon \)-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.