E4orf6 is one of the oncogene products of adenovirus, and it also has an important role for transportation of cellular and viral messenger RNA (mRNA) during the late phase of virus infection. We previously revealed that E4orf6 controls the fate of AU-rich element (ARE) containing mRNA by perturbing the chromosome maintenance region 1-dependent export mechanism. Here, we show that E4orf6 stabilizes ARE–mRNA through the region required for its oncogenic activity and ubiquitin E3 ligase assembly. Cells that failed to stabilize ARE–mRNA after HuR knockdown were unable to produce colonies in soft agar, even when E4orf6 was expressed. Furthermore, the stabilized ARE–mRNA induced the transformation of rodent immortalized cells. These findings indicate that stabilized ARE–mRNA is necessary, if not all, for the oncogenic activity of E4orf6 and has the potential to transform cells, at least under a certain condition.

Keywords: adenovirus; AU-rich element; E4orf6; transformation; stabilization

Introduction

Research on DNA tumor viruses has been conducted in connection with numerous biological events of host cells. These viruses encode proteins that are capable of controlling the messenger RNA (mRNA) transport systems of host cells to replicate their genome and propagate their virus particles. Generally, these proteins achieve their functions by interacting with cellular proteins.

Adenovirus E4orf6 promotes the nuclear export of viral late mRNA and inhibits the bulk of cellular mRNA export during the late phase of adenovirus infection (Döbner and Kzhyshkowska, 2001). It also cooperates with E1A and E1B to transform primary baby rat kidney (BRK) cells, and the expression of E4orf6 markedly enhances the ability of BRK and human 293 cells to form tumors in nude mice (Moore et al., 1996; Nevels et al., 1997). E4orf6 forms a complex with adenovirus E1B55k (Sarnow et al., 1984), and this complex assembles into E3 ubiquitin ligase through the domain of E4orf6 that binds with elongins B and C (Querido et al., 2001). It is suggested that most, if not all, of the functions of the E1B55k/E4orf6 complex, including the control of mRNA export, are achieved through the degradation of target proteins by E4orf6 via its E3 ligase activity (Woo and Berk, 2007; Blanchette et al., 2008).

AU-rich elements (ARE) are present in many proto-oncogene, growth factor and cytokine mRNA, and target ARE–mRNA for rapid degradation (Chen and Shyu, 1995; Brennan and Steitz, 2001). HuR, a member of the embryonic lethal abnormal vision family of RNA-binding proteins, binds to ARE to protect ARE–mRNA from rapid degradation (Brennan and Steitz, 2001; Hinman and Lou, 2008). Although HuR is localized predominantly in the nucleus, it can shuttle between the nucleus and cytoplasm, and the stabilization of ARE–mRNA by HuR is believed to be linked to its localization in the cytoplasm (Lopez de Silanes et al., 2005; Hinman and Lou, 2008).

The export of HuR is mediated by its association with transportin 1 and transportin 2 (Gallouzi and Steitz, 2001b; Rebane et al., 2004) via the shuttling sequence in its hinge region, which is termed HuR nucleocytoplasmic shuttling sequence (HNS) (Fan and Steitz, 1998), and by its association with pp32 and acidic protein rich in leucines (APRIL), which includes the nuclear export signal recognized by the export receptor chromosome maintenance region 1 (Brennan et al., 2000; Gallouzi et al., 2001a). Usually, HuR is exported into the cytoplasm of cancer cells, and cytoplasmic HuR expression has been implicated in the malignancy of several types of carcinomas, such as colon cancer, and has also been postulated to contribute to the cancerous malignant phenotype (Lopez de Silanes, et al., 2005).

We previously found that pp32 associates with E4orf6 (Higashino et al., 2005) and that E4orf6 induces the cytoplasmic accumulation of ARE–mRNA in a manner
independent of chromosome maintenance region 1. Here, we show that E4orf6 stabilizes ARE–mRNA via two distinct functional domains, which are required for its oncogenic activity and its assembly into the E3 ligase. HuR-knockdown cells failed to stabilize ARE–mRNA and make colonies in soft agar, even when E4orf6 is expressed in the cells. Furthermore, rodent cells containing the stabilized ARE–mRNA were able to form colonies. These results indicate that E4orf6 stabilizes ARE–mRNA to exert its oncogenic activity, and this study provides the first evidence that the stabilization of ARE–mRNA induces malignant transformation.

Results

Stabilization of ARE–mRNA by E4orf6

The stabilization of ARE–mRNA was examined using transformed BRK cells. The amounts of c-fos, c-myc and COX-2 mRNA in the BRK E1 + E4 cells, which were transformed with the adenovirus E1 (E1A and E1B) and E4orf6 genes, were estimated by quantitative real-time reverse transcriptase PCR and compared with those in BRK E1 cells, which were transformed with the E1 gene. The amount of c-fos mRNA that accumulated in the BRK E1 + E4 cells was about 4.0 times higher than that in the BRK E1 cells (Figure 1a). The BRK E1 + E4 cells also accumulated ~5.5- and 4.2-fold greater levels of c-myc and COX-2 mRNA, respectively, than the BRK E1 cells (Figure 1a).

To eliminate the possibility that the stabilization was induced by the transcriptional activation of each gene, we examined their half-lives. The cells were treated with actinomycin D, and the quantities of each mRNA species in BRK cells was measured by quantitative real-time reverse transcriptase PCR and compared with those in BRK E1 cells, which were transformed with the E1 gene. The amount of c-fos mRNA that accumulated in the BRK E1 + E4 cells was about 4.0 times higher than that in the BRK E1 cells (Figure 1a). The BRK E1 + E4 cells also accumulated ~5.5- and 4.2-fold greater levels of c-myc and COX-2 mRNA, respectively, than the BRK E1 cells (50, 50, and 73 min, respectively; Figure 1b).

To confirm the stabilization of ARE-containing mRNA by E4orf6, we constructed pGL3-based luciferase reporter plasmids with and without the 5′-untranslated region (UTR) of c-fos mRNA including the ARE (luciferase–AREc-fos, Figure 1c left), and these plasmids were introduced into the BRK cells to examine the stabilization of the fusion gene via luciferase activity. Luciferase activity was downregulated in the BRK E1 + E4 cells when the luciferase reporter without AREc-fos (pCMVGL) was used (Figure 1c right). On the other hand, the luciferase activity derived from pCMVGL–AREc-fos was significantly activated in BRK E1 + E4 cells compared with that in BRK E1 cells (Figure 1c right). These results suggest that the luciferase–AREc-fos mRNA was stabilized in the E4orf6-expressing cells in an ARE-dependent manner, indicating that the ARE allows mRNA to be stabilized by E4orf6.

As actinomycin D suppresses the synthesis of all mRNA, the effect of E4orf6 on the ARE–mRNA stabilization described above might be because of the secondary effects. To eliminate this possibility, we constructed 293 cells harboring the luciferase–AREc-fos gene (Figure 1c left), the expression of which was controlled by the tet-off system, that can stop the synthesis of the fusion gene. Constitutively expressed luciferase–AREc-fos mRNA was stopped by adding doxycycline, and the half-life of the mRNA was calculated. The half-life of luciferase–AREc-fos mRNA

Figure 1  E4orf6 stabilizes ARE–mRNA. (a) The accumulation of c-fos, c-myc and COX-2 mRNA in BRK E1 and BRK E1 + E4 cells measured by quantitative real-time reverse transcriptase PCR (RT–PCR). Data are shown as the mean ± s.e.m. of three independent experiments. (b) The same cells were treated with actinomycin D, and the amounts of each mRNA were estimated at 30, 60 and 120 min after treatment by real-time RT–PCR. The plot Shows the mean of three independent experiments. The t1/2 indicates the half-life time (minutes). The half-lives in (E1 + E4) cells are calculated values. (c) pGL3-based luciferase reporters with (pCMVGL–AREc-fos) or without (pCMVGL) the ARE of c-fos mRNA (left) were transfected into BRK E1 or BRK E1 + E4 cells, and luciferase activity was measured 20 h after transfection (right). Data are shown as the mean ± s.e.m. of three independent experiments. The t1/2 indicates half-life time (minutes). The half-life in (+ E4) cells is calculated value.
was extended from 56 to 147 min when E4orf6 was present in 293 cells (Figure 1d). Taken together, we conclude that E4orf6 stabilizes ARE-containing mRNA.

**The α-helix structure of E4orf6 is required for its interaction with pp32**

We analyzed the region of E4orf6 that is required for the stabilization of ARE–mRNA. In our previous report, we identified that the carboxyl terminal region of E4orf6 (the oncodomain; see Figure 2a) including the arginine-faced amphipathic α-helix is required for its interaction with pp32 and cytoplasmic accumulation of ARE–mRNA (Higashino et al., 2005). This α-helix structure is known to be essential for the oncogenic activities of E4orf6, including cooperative focus formation with E1A and promotion of tumor growth in nude mice (Nevels et al., 2000). In addition, the same structure is also required for E4orf6 to assemble into the E3 ubiquitin ligase complex with E1B55k (Blanchette et al., 2004). To analyze this region further, we produced additional E4orf6 mutants, E4orf6 L245P and E4orf6 R248E (Figure 2a), which possess amino-acid substitutions in their α-helices, and then examined their in vivo interactions with pp32. The E4orf6 L245P mutant has a disrupted α-helical structure (Orlando and Ornelles, 1999), and fails to display oncogenic activity (Nevels et al., 2000). On the other hand, the E4orf6 R248E mutation was reported to interfere with the nuclear retention of E4orf6, but did not affect its oncogenic activity (Dobbelstein et al., 1997; Nevels et al., 2000). As shown in Figure 2b, the E4orf6 L245P mutant failed to interact with pp32, although the E4orf6 R248E mutation did not have any influence on pp32 binding. These findings indicate that the α-helix structure of E4orf6 is necessary for pp32 binding and that the interaction of E4orf6 with pp32 has an important role in the oncogenic activity of the viral gene product.

**The α-helix structure and BC-box motif of E4orf6 are required for stabilization and cytoplasmic localization of ARE–mRNA**

To examine the effect of the α-helix structure of E4orf6 on its ability to stabilize ARE–mRNA, we repeated the same experiment as shown in Figure 1. No increases in the amount of ARE–mRNA were seen in the E4orf6 dl210-294- or E4orf6 L245P-expressing BRK cells (Figure 2c). The half-lives of c-fos, c-myc and COX-2 mRNA expressed in BRK dl210-294 and BRK L245P cells (38, 37 and 56 min, and 39, 46 and 38 min, respectively) were shorter than those in BRK E1 + E4 cells (141, 144 and 165 min; Figure 2d). Thus, these ARE–mRNA were not stabilized in the cells expressing the oncodomain mutants of E4orf6.

The same results were obtained from experiments examining the half-life of luciferase–ARE<sup>c-fos</sup> fusion mRNA using tet-off system. The half-life of luciferase–ARE<sup>c-fos</sup> mRNA in 293 cells expressing the E4orf6 L245P mutant was 68 min (Figure 2e). This is shorter than that in the cells expressing wild-type E4orf6 (147 min). From these results, we conclude that the α-helix structure of E4orf6 is required for the stabilization of ARE–mRNA and that this stabilization is related to the oncogenic activity of E4orf6.

As shown previously, stabilized ARE–mRNA was detected in the cytoplasm of cells that had been transformed by E4orf6 with its associated proteins, such as HuR and pp32 (Higashino et al., 2005). If the export of ARE–mRNA is a prerequisite for its stabilization, the oncodomain of E4orf6 including the α-helix may be required for the cytoplasmic accumulation of ARE–mRNA. To confirm this, we observed the interaction of c-fos mRNA in the cytoplasm with its associated proteins using an ribonucleoprotein (RNP)-immunoprecipitation assay. Bands for c-fos mRNA appeared in both the nucleus and cytoplasmic fractions precipitated with anti-HuR and -pp32 antibodies using the lysate from BRK cells expressing wild-type E4orf6 (Figure 2f). This data indicate that c-fos mRNA bound to HuR and pp32 exists in the nucleus and cytoplasm of cells expressing wild-type E4orf6. On the other hand, there was no such band in the cytoplasm of the cells expressing the E4orf6 oncodomain mutants, whereas mRNA for these proteins was present in the nucleus (Figure 2f). The stabilization of the ARE–mRNA in the cytoplasm of the E4orf6 derivative expressing 293 cells was confirmed using luciferase–ARE<sup>c-fos</sup> mRNA. In the cells expressing E4orf6 mutants, the half-life of the mRNA (34 min) was shorter than that of the wild-type E4orf6 expressing 293 cells (65 min; Figure 2g). In this case, the half-lives of luciferase–ARE<sup>c-fos</sup> mRNA were different from those in Figures 1d and 2f, which were calculated by the data obtained from tet-off system. These data strongly suggest that HuR and pp32 are associated with ARE–mRNA and that the complex is exported from the nucleus and stabilized in the cytoplasm in cells that express E4orf6.

As the α-helix structure is also thought to be important for E3 ubiquitin ligase activity mediated by E4orf6 (Orlando and Ornelles, 2002; Blanchette et al., 2004), we examined whether another E4orf6 mutant that has lost the ability to assemble into the E3 ligase complex is able to stabilize ARE–mRNA. To confirm this, we constructed the E4orf6 (BC<sup>−</sup>) mutant by introducing specific point mutations in BC boxes 1 and 2 (Figure 2a), which are necessary for E4orf6 to associate with Cullin5, a complex containing E3 ubiquitin ligase bound with E1B55k, using mutant adenovirus H5pm4139 (a generous gift from T Dobner) as a template (Blanchette et al., 2008). We confirmed whether this mutant is functional by measuring its ability to degrade p53 (Supplementary Figure 1). The levels of accumulated c-fos and COX-2 mRNA expressed in the cytoplasm of 293 cells expressing wild-type E4orf6 were upregulated compared with those of the control 293 cells, which were transfected with a parent plasmid. On the other hand, their levels were not increased in the cells possessing E4orf6 L245P or BC<sup>−</sup> mutants (Figure 3a). The level of the control GAPDH mRNA was not increased by the expression of the wild-type E4orf6. These results suggest that the ability
of E4orf6 to assemble into E3 ligase is required for the cytoplasmic expression of ARE–mRNA. Similar results were obtained when we examined the stabilization of ARE–mRNA using total cell lysates from each cell type (Figure 3b). As in the case of the cytoplasmic mRNA, the E4orf6 L245P and BC (−) mutants were not able to stabilize c-fos or COX-2 mRNA. These findings suggest that E4orf6-mediated E3 ligase activity is important for the export and stabilization of ARE–mRNA.
The BC-box motif of E4orf6 is required for the stabilization of ARE–mRNA. The accumulation of c-fos, COX-2 and GAPDH mRNA in the cytoplasm (a) and the (b) total of 293 cells expressing indicated E4orf6 derivatives were measured by quantitative real-time reverse transcriptase PCR. Data show the mean ± s.e.m. of three independent experiments.

**Discussion**

We demonstrated here that the adenovirus E4orf6 stabilizes ARE–mRNA via an HuR-mediated pathway. The z-helix structure and the BC-box motif within the E4orf6 protein are required for ARE–mRNA stabilization. Furthermore, E4orf6-mediated transformed cells could not produce colonies in soft agar when the stabilization of ARE–mRNA was inhibited by HuR knockdown. The stabilized ARE–mRNA appeared to have the potential to induce the formation of transformed colonies. These findings indicate that E4orf6 transforms cells, at least in part, by the stabilizing system of ARE–mRNA.

As elucidating the mechanisms of action of viral oncoproteins has often led to insights into human oncogenesis, we hypothesize that the stabilization of ARE–mRNA has an important role in the generation of human cancer. The export of ARE–mRNA and HuR are evident in a number of human cancers, and cytoplasmic HuR expression has been implicated in many large colonies in soft agar, whereas the BRK E1 cells expressing only the E1 (E1A and E1B) gene did not produce many colonies (Figure 4d). This is reasonable because the BRK cells transformed with the E1 and E4orf6 genes produced tumors in nude mice, whereas BRK E1 cells did not (Moore et al., 1996). On the other hand, the HuR-silenced BRK cells produced almost no colonies in soft agar (Figure 4d). These results suggest that the HuR-silenced cells lost their ability for anchorage-independent growth in conditions in which ARE–mRNA was not stabilized, even when E4orf6 was present in the cells. Taken together, these results suggest that the HuR-mediated stabilization of ARE–mRNA is one of the major causes of the oncogenic activity of E4orf6.
the malignancy of these carcinomas (Lopez de Silanes et al., 2005). Indeed, the stabilization of ARE–mRNA is reported in several cancers such as oral carcinoma (Hasegawa et al., 2009). Furthermore, in oral cancer cells, ARE–mRNA was exported to the cytoplasm of cells in a manner independent of chromosome maintenance region 1 just like the case of transformed cells mediated by E4orf6. These data indicate further that the oncogenic mechanism through stabilization of ARE–mRNA shown in this report is general oncogenic process.

Although the stabilization of ARE–mRNA has been observed in cancer cells, there was no evidence that ARE–mRNA stabilization has the potential to induce oncogenic transformation. This is because of the fact that there was no constitutive system for exporting HuR or ARE–mRNA into the cytoplasm, whereas some stresses like heat shock can export them to the cytoplasm transiently. As confirmed in this report, ARE–mRNA was constitutively stabilized in the cells transformed with E4orf6, such as the BRK E1 + E4 cells; we think this is one of the best systems for examining the effects of ARE–mRNA stabilization on cell transformation. Indeed, the cells expressing c-fos, c-myc and COX-2 mRNA with E4orf6 produced a lot of colonies compared with the control (Figure 4e).

Figure 4  Stabilized ARE–mRNA is required for the oncogenic activity of E4orf6. (a) HuR short hairpin RNA was introduced into BRK E1 + E4 cells, the amounts of HuR and β-actin in each BRK cells were estimated by western blot analysis. (b) HuR-knockdown BRK cells were treated with actinomycin D, and amounts of c-fos mRNA were estimated at 30, 60 and 120 min after treatment by quantitative real-time reverse transcriptase PCR. The plot shows the mean of three independent experiments. The $t_{1/2}$ indicates half-life time (minutes). (c) The half-life of c-fos mRNA was estimated using 293 cells that had been transfected with pcDNA-E4orf6 and HuR small interfering RNA as described in b. (d) The BRK cells described in a were subjected to a soft agar colony formation assay. Photographs of the stained colonies were taken at 3 weeks after transfection. (e) Rat embryonal fibroblast cells were transfected with the expression constructs of the indicated genes. The number of colonies was counted at 2 weeks after transfection and is shown as a histogram. Data represent the mean ± s.e.m. of three independent experiments.
of ARE–mRNA can transform cells, at least in certain conditions. Again, these results support our hypothesis that the stabilization of ARE–mRNA is general oncogenic mechanism.

We found that the BC boxes 1 and 2 mutant of E4orf6 failed to export and stabilize ARE–mRNA (Figure 3). This indicates that not only the β-helix structure, which is the region essential for the pp32 binding, but also the BC-box motif is required for stabilizing ARE–mRNA. As BC box is binding site of elongin C (Blanchette et al., 2004), which is one of the subunits of E3 ubiquitin ligase, the data suggest that the E3 ubiquitin ligase complex including E4orf6 has an important role for the stabilization of ARE–mRNA. As far as we know, this is a new function of the E4orf6 ubiquitin ligase. So far, several proteins were identified as a target of this ubiquitin ligase, such as p53 (Querido et al., 2001), Mre11 (Stracker et al., 2002), DNA ligase IV (Baker et al., 2007) and integrin α3 (Dallaire et al., 2009); we have no evidence about which protein or additional another protein is required as a substrate for the stabilization of ARE–mRNA. As the E4orf6 E3 ubiquitin ligase activity was reported to be necessary for the export of viral late mRNAs (Woo and Berk, 2007; Blanchette et al., 2008), this activity is involved in the regulation of both cellular and viral mRNAs.

The stabilization of ARE–mRNA is also interesting with regard to viral infection. So far, several virus gene products, for example HSV UL41 (Esclatine et al., 2004) and KSHV KaposinB (McCormick and Ganem, 2001), have been shown to have the potential to control the fate of ARE–mRNA. In the late phase of adenovirus infection, although the export of the bulk of cellular mRNA is inhibited, ARE–mRNA is exported into the cytoplasm (Higashino et al., 2005). As viral late mRNA is exported into the cytoplasm at the same time, it is possible that ARE–mRNA and late mRNA share the same export pathway.

Materials and methods

Cells and plasmids

Human embryonal kidney 293 cells, BRK cells (BRK E1 + E4 and BRK E1; Higashino et al., 2005) and rat embryonic fibroblast cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum with antibiotics. To produce BRK L245P and BRK dl210–294 cells, BRK E1 cells were transfected with pcDNA-E4orf6 L245P and pcDNA-E4orf6 dl210–294 (see below), respectively, and the clones were selected in medium containing 300 μg/ml G418. To create E4orf6-substituted mutants (pcDNA-E4orf6 L245P, pcDNA-FALG-E4orf6 L245P, pcDNA-E4orf6 R248E and pcDNA-FLAG-E4orf6 R248E), the leucine and the arginine at amino acids 245 and 248, respectively, were replaced by glutamine at amino acids 245 and 248, respectively, and the clones were selected in medium containing 300 μg/ml G418.

To create E4orf6-substituted mutants (pcDNA-E4orf6 L245P, pcDNA-FALG-E4orf6 L245P, pcDNA-E4orf6 R248E and pcDNA-FLAG-E4orf6 R248E), the leucine and the arginine at amino acids 245 and 248, respectively, were changed to proline and glutamic acid by site-directed mutagenesis (Quickchange, Stratagene, La Jolla, CA, USA), as described previously (Dobbelstein et al., 1997; Orlando and Ornelles, 1999), using pcDNA3-E4orf6 and pcDNA3-FLAG-E4orf6 (Higashino et al., 2005) as templates. E4orf6 dl210–294, pcMVGL and pCMVGL–ARE–are were described previously (Higashino et al., 2005). The complementary DNA of the luciferase–ARE–ARE–fusion gene was inserted into the pTRE vector to produce a tet-off system according to the manufacturer’s (Clontech, Palo Alto, CA, USA) instructions. pcDNA-E4orf6 BC– was made by amplifying segments of the E4orf6-coding region using the primer 5′-CGGGATCCC ATGACTAGTCCGGCGTT-3′, together with a second primer 5′-GAATTCTACATGGGGGATGACCTA-3′ using mutant adenovirus H5pm4139 (a generous gift from T Döbner) as a template (Blanchette et al., 2008). The PCR-amplified fragments were inserted into the BamHI/EcoRI site of pcDNA3. All mutants were confirmed by nucleotide sequencing. COX-2 expression plasmid (pCMV-sport-COX-2) was purchased from Invitrogen (Carlsbad, CA, USA) (NIH Mammalian Gene Collection).

mRNA stability analysis

To examine the accumulation of ARE–mRNA in total cell lysate, the cells were treated with TRI Reagent (Sigma) to acquire total RNA, and then the cells were treated with actinomycin D (final concentration 10 μg/ml) or doxycycline (1 μg/ml) to elucidate the half-life of ARE–mRNA. After treatment, total RNA was isolated and subjected to reverse transcription using Rever Tra Ace (TOYOBO, Osaka, Japan).

For quantitative real-time reverse transcriptase PCR analysis, PCR amplification was performed in DNA Engine Opticon 2 (MJ Research, Waltham, MA, USA) with SYBR Green PCR Master Mix (DyNaMo SYBR Green qPCR kit, MJ Research). The primers used in this assay were as follows: for c-fos, 5′-TGACCTGTCGTCGCTTC3′ and 5′-CT CGGGATCGTGAAGACAA3′; for c-myc, 5′-ATCAGTAC CGCCGAGAACACGAC-3′ and 5′-CTTCTGGGTGAAACACGAC ACTT-3′; for COX-2, 5′-CTGAGGGGATGACCTCACTGCA-3′ and 5′-TGAGCGAAGCTCGGGTTCAAG3′; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CACTCATCGGTCAACTTCA-3′ and 5′-GCCAGTGACTACCC ACGC-3′. The Student’s t-test was used to assess statistically significant differences.

To examine the cytoplasmic transportation of ARE–mRNA, the cells were separated into nuclear and cytoplasmic fractions (Higashino et al., 2005), and amounts of mRNA in the cytoplasm were measured by quantitative real-time reverse transcriptase PCR.

For the luciferase assay, BRK cells were transfected with the pcCMVGL or pCMVGL-ARE–ARE–reporter plasmid using the FuGENE6 transfection reagent (Roche, Basel, Switzerland), and luciferase assays were performed 20 h later according to the manufacturer’s instructions.

Protein analysis

Western blot analysis and immunoprecipitation were performed, as described previously (Aoyagi et al., 2003). The antibodies used were specific to HuR, pp32 (Santa Cruz, Santa Cruz, CA, USA), β-actin and M2 (anti-FLAG) (Sigma). The secondary antibody was horseradish peroxidase-conjugated IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

RNP-immunoprecipitation assay

BRK cells were treated with phosphate-buffered saline containing 1% formamide (Higashino et al., 2005). After the cells had been separated into nuclear and cytoplasmic fractions, each lysate was incubated with mouse immunoglobulin G, anti-HuR or -pp32 antibody. The pellet and supernatant were incubated at 70 °C for 45 min to reverse cross-linking; the isolated RNA was subjected to reverse
transcription, and PCR amplification for c-fos was performed using primers as described above.

**HuR knockdown**

pSUPER (Brummelkamp et al., 2002) including the sequence for HuR short hairpin RNA (5'-UUACCAGUUUCAUUGG UCAUUUUCAAGAGAAAGACCAUUGAAAUCUGGU AA-3') was introduced into BRK cells by calcium phosphate-mediated transfection, and stable transfectants were selected with 300 μg/ml G418.

**Soft agar colony formation assay**

The soft agar colony formation assay was performed as described previously (Kakuguchi et al., 2010). Single-cell suspensions of 3 × 10⁴ cells were plated in 60-mm culture dishes in 3 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 0.36% agar on a layer of 5 ml of the same medium containing 0.75% agar. After 3 weeks of plating, the colonies were stained with 0.04% crystal violet-2% ethanol in PBS, and photographs of the stained colonies were taken.

**Transformation assay**

Rat embryonal fibroblast cells on 6-well dishes were transfected with a total 1.5 ml of pEF-c-myc, pSG5-c-fos or pCMV-sport-COX-2, and 0.5 ml of pBlanchette P, Cheng CY, Yan Q, Ketner G, Ornelles DA, Dobner T infected with a total 1.5 ml of pXhoIC, 0.5 μg of pCMV-COX2, and 0.5 μg of plasmids encoding E4orf6 variants; the total amount of DNA was adjusted with pUC118) using Hilymax (DOHINDO Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions, and maintained in Dulbecco’s modified Eagle’s medium with 7.5% fetal bovine serum. After 2 weeks of transfection, the plates were stained with crystal violet, and the number of foci was counted.

**Conflict of interest**

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)