Gene encoding protein elongation factor EEF1A2 is a putative oncogene in ovarian cancer

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Published online: 10 June 2002, doi:10.1038/ng904

We have found that *EEF1A2*, the gene encoding protein elongation factor EEF1A2 (also known as eEF-1α2), is amplified in 25% of primary ovarian tumors and is highly expressed in approximately 30% of ovarian tumors and established cell lines. We have also demonstrated that EEF1A2 has oncogenic properties: it enhances focus formation, allows anchorage-independent growth and decreases the doubling time of rodent fibroblasts. In addition, EEF1A2 expression made NIH3T3 fibroblasts tumorigenic and increased the growth rate of ES-2 ovarian carcinoma cells xenografted in nude mice. Thus, EEF1A2 and the process of protein elongation are likely to be critical in the development of ovarian cancer.

The genetic amplification of growth-enhancing genes has a key role in the development of human malignancy. An important task in understanding oncogenesis is the identification of those genes whose copy number and expression increase during tumorigenesis. An increase in the copy number of the 20q13 locus (refs 1–3) occurs in 20–30% of ovarian tumors, implicating one or more genes at 20q13 in tumor genesis and progression. But the 20q13 gene(s) relevant to ovarian cancer are not yet identified.

One of the genes found at 20q13 is *EEF1A2*, which maps to 20q13.3 (ref. 4) and encodes protein elongation factor EEF1A2 (eEF-1α2). During protein translation, eukaryotic elongation factors control the recruitment of amino-acylated tRNA to the ribosome and regulate the translocation of the growing polypeptide from the ribosome A to P sites[5]. Human EEF1A2 is one of two isoforms of eukaryotic elongation factor 1α (EEF1A1 and EEF1A2), which share greater than 90% DNA sequence and amino-acid identity. EEF1A proteins bind and hydrolyze GTP and catalyze the association of tRNAs to the ribosome during protein elongation[5]. In addition to their role in protein translation, EEF1A proteins from a variety of sources bind to F-actin[6,7] and depolymerize α-tubulin microtubules[8], characteristics that are consistent with the idea that these proteins regulate cytoskeletal organization.

To determine whether *EEF1A2* was part of the 20q13 amplicon in ovarian cancer, we used fluorescence *in situ* hybridization (FISH) to measure *EEF1A2* copy number in primary ovarian tumors. We found that 25% of primary ovarian tumors (14 of 53) had *EEF1A2* amplifications. Three representative primary ovarian tumor samples with amplifications are shown (Fig. 1*a–c*). Amplifications of *EEF1A2* were visualized as an increased number of loci hybridizing to an *EEF1A2* BAC probe. The BAC probe contained the 3′ untranslated region of *EEF1A2* as determined by PCR (data not shown). Hybridization of a control 20p11 probe to the same samples indicated that the increase in *EEF1A2*-hybridizing loci did not result from polyploidy of chromosome 20. A representative ovarian tumor with normal *EEF1A2* copy number is shown in Fig. 1*d*. The BAC clone used for FISH hybridized to a metaphase spread of chromosome 20q13 (Fig. 1*e*). Thus, *EEF1A2* copy

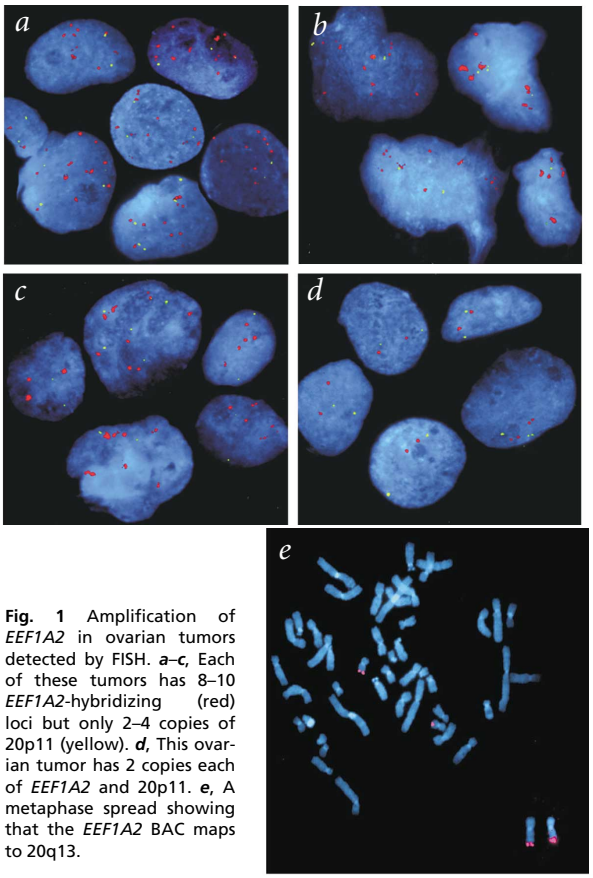

Fig. 1 Amplification of *EEF1A2* in ovarian tumors detected by FISH. ***a–c***, Each of these tumors has 8–10 *EEF1A2*-hybridizing (red) loci but only 2–4 copies of 20p11 (yellow). ***d***, This ovarian tumor has 2 copies each of *EEF1A2* and 20p11. ***e***, A metaphase spread showing that the *EEF1A2* BAC maps to 20q13.

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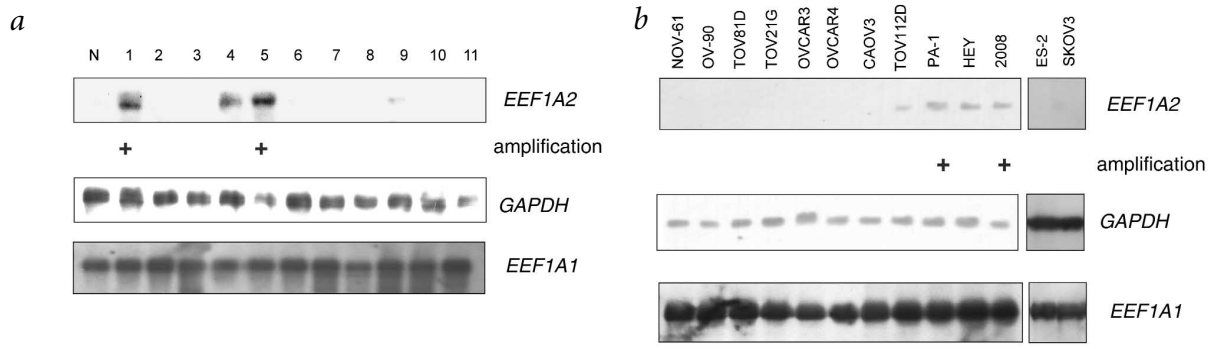

Fig. 2 Increased expression of *EEF1A2* mRNA in ovarian tumors and cell lines ***a***, We readily detected *EEF1A2* mRNA in 3 of 11 ovarian tumor samples but not in normal ovary mRNA (N). We stripped the membrane and reprobed with *GAPDH* and *EEF1A1* probes to show that loading was approximately equal. We also tested each of the tumors for *EEF1A2* amplifications (+). ***b***, We detected expression of *EEF1A2* in the ovarian cancer cell lines TOV112D, PA-1, HEY and 2008 but not in the normal ovarian cell lines NOV-61 or OV-90, TOV81D, TOV21G, OVCAR3, OVCAR4, CAOV3, ES-2 or SKOV3. Hybridization to *GAPDH* and *EEF1A1* probes was used as a loading control.

number was higher in a substantial subset of ovarian tumors and was part of the 20q13 amplicon. Amplification of the 20q13 locus is a marker for late stages of ovarian cancer[1], and the presence of four or more copies of 20q13 is associated with decreased five-year survival after diagnosis[3].

To determine whether there was an increase in *EEF1A2* expression in ovarian tumors, we used northern blotting to measure *EEF1A2* mRNA amounts in primary ovarian tumors and in established ovarian carcinoma cell lines. Although the tissue-specific expression pattern of human *EEF1A2* is unknown, rat and mouse *Eef1a2* RNA are expressed only in normal brain, heart and skeletal muscle[9,10]. Normal ovarian tissue did not have detectable amounts of *EEF1A2* mRNA, whereas 3 of 11 primary ovarian tumors had readily detectable *EEF1A2* mRNA (Fig. 2*a*). Expression of *GAPDH*, which was included as a loading control, and *EEF1A1* were similar among the samples. Previously, *EEF1A1* has been mapped to 6q14 (ref. 4), a locus not previously known to be involved in ovarian cancer. Two of three tumor samples with elevated *EEF1A2* mRNA also had an elevated *EEF1A2* copy number (Fig. 2*a*). One of the tumor samples with elevated *EEF1A2* expression did not have detectable *EEF1A2* amplification (Fig. 2*a*), indicating that *EEF1A2* expression may increase independently of changes in *EEF1A2* copy number.

EEF1A2 mRNA expression was also higher in some established ovarian cancer cell lines relative to normal ovarian epithelial cells. A normal ovarian epithelial cell line, NOV-61 (ref. 11), had undetectable *EEF1A2* mRNA, whereas 4 of 12 ovarian tumor cell lines (TOV112D, PA-1, HEY, 2008) expressed *EEF1A2* (Fig. 2*b*). The OV-90, TOV81D, TOV21G, OVCAR3, OVCAR4, CAOV3, SKOV3

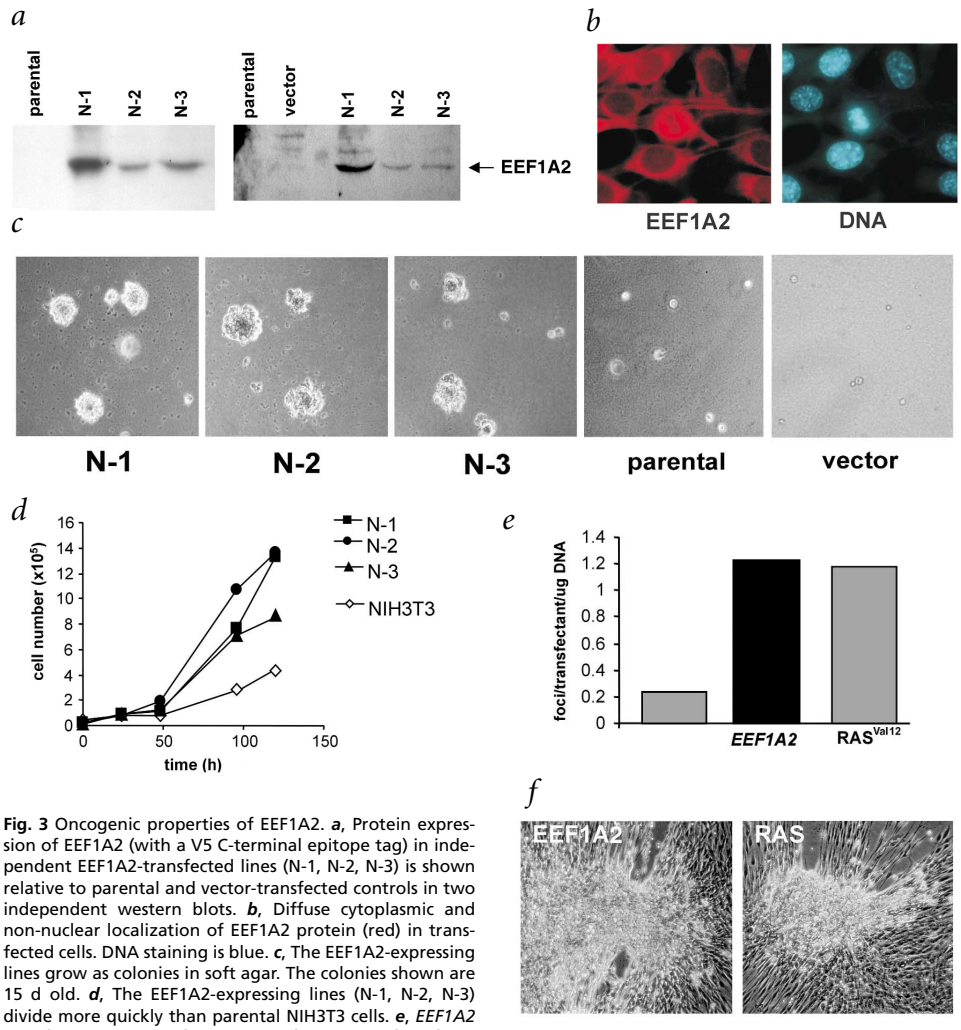

Fig. 3 Oncogenic properties of EEF1A2. ***a***, Protein expression of EEF1A2 (with a V5 C-terminal epitope tag) in independent EEF1A2-transfected lines (N-1, N-2, N-3) is shown relative to parental and vector-transfected controls in two independent western blots. ***b***, Diffuse cytoplasmic and non-nuclear localization of EEF1A2 protein (red) in transfected cells. DNA staining is blue. ***c***, The EEF1A2-expressing lines grow as colonies in soft agar. The colonies shown are 15 d old. ***d***, The EEF1A2-expressing lines (N-1, N-2, N-3) divide more quickly than parental NIH3T3 cells. ***e***, *EEF1A2* transfection induces foci in Rat1 fibroblasts. ***f***, A focus induced by EEF1A2 expression has a similar morphology to a focus induced by RAS^{Val12} expression.

and ES-2 cell lines, like the normal NOV-61 cell line, did not express *EEF1A2* mRNA. Expression of *GAPDH* and *EEF1A1* were similar among the cell lines. When these results are taken together, *EEF1A2* expression was higher in ~30% of ovarian tumor samples and cell lines.

We next determined whether human EEF1A2 had oncogenic properties. We established NIH3T3 rodent fibroblast cell lines that stably expressed EEF1A2 under the control of the CMV promoter. The EEF1A2 used to generate the cell lines had been tagged at its carboxy terminus with the V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) to facilitate detection by western blotting. Protein expression of exogenous EEF1A2 in three independent NIH3T3 clones (N-1, N-2, N-3) is shown (Fig. 3*a*). EEF1A2 in interphase cells is nonnuclear and diffusely cytoplasmic (Fig. 3*b*), corresponding to the wildtype localization of the protein[12]. The EEF1A2-expressing clones grew as colonies in soft agar (Fig. 3*c*), a property not observed in the parental NIH3T3 cells or in NIH3T3 cells transfected with the empty vector. Moreover, the EEF1A2-expressing clones had an accelerated growth rate relative to the parental NIH3T3 controls (Fig. 3*d*). Four days after equal numbers of cells were plated, there were approximately four times as many EEF1A2-expressing cells as parental cells, indicating that EEF1A2 expression may enhance cell growth rate.

To further explore the capacity of EEF1A2 to enhance cell growth, we measured the ability of EEF1A2 to induce focus formation in Rat1 fibroblasts. The ability to form foci in cell culture is a marker of cell transformation and is considered one of the general properties of an oncogene such as *RAS*[13]. *EEF1A2* induced focus formation in Rat1 cells (Fig. 3*e*). The constitutively active and transforming RAS^{Val12} allele[14] was used as a positive control. The morphology of EEF1A2-induced foci was similar to that of RAS^{Val12}-induced foci (Fig. 3*f*).

To determine whether EEF1A2 could enhance tumorigenicity, we subcutaneously injected the EEF1A2-expressing NIH3T3 cells into nude mice. Expression of EEF1A2 in NIH3T3 cells was sufficient to induce *in vivo* tumorigenicity (Fig. 4*a*). No tumor growth was observed in the parental or vector-transfected NIH3T3 cells. Although the N-1 line expressed more EEF1A2 than either N-2 or N-3 did, it did not seem to form larger tumors in the mice and it was not more efficient at forming colonies in soft agar (Fig. 3*a*). This indicates that N-1, N-2 and N-3 may have expressed enough EEF1A2 so that its abundance was not the limiting factor in either anchorage-independent growth or *in vivo* tumorigenesis.

To determine the effect of EEF1A2 on a cell derived from the ovaries, we generated independent ES-2 ovarian cell lines that expressed EEF1A2 (E-1, E-2, E-3, E-4). ES-2 cells are ovarian clear cell carcinoma cells that do not express detectable EEF1A2 mRNA (Fig. 2*a*). Protein expression of EEF1A2 in four independent ES-2 derivatives is shown (Fig. 4*b*). A nonspecific background band of slightly higher molecular weight than EEF1A2 can be seen in the parental and vector lanes and can also be discerned in the E-1, E-2 and E-3 lysates. The cell lines expressing EEF1A2 all had an accelerated rate of tumor formation in nude mice relative to the ES-2 controls (Fig. 4*c*). Thus, EEF1A2

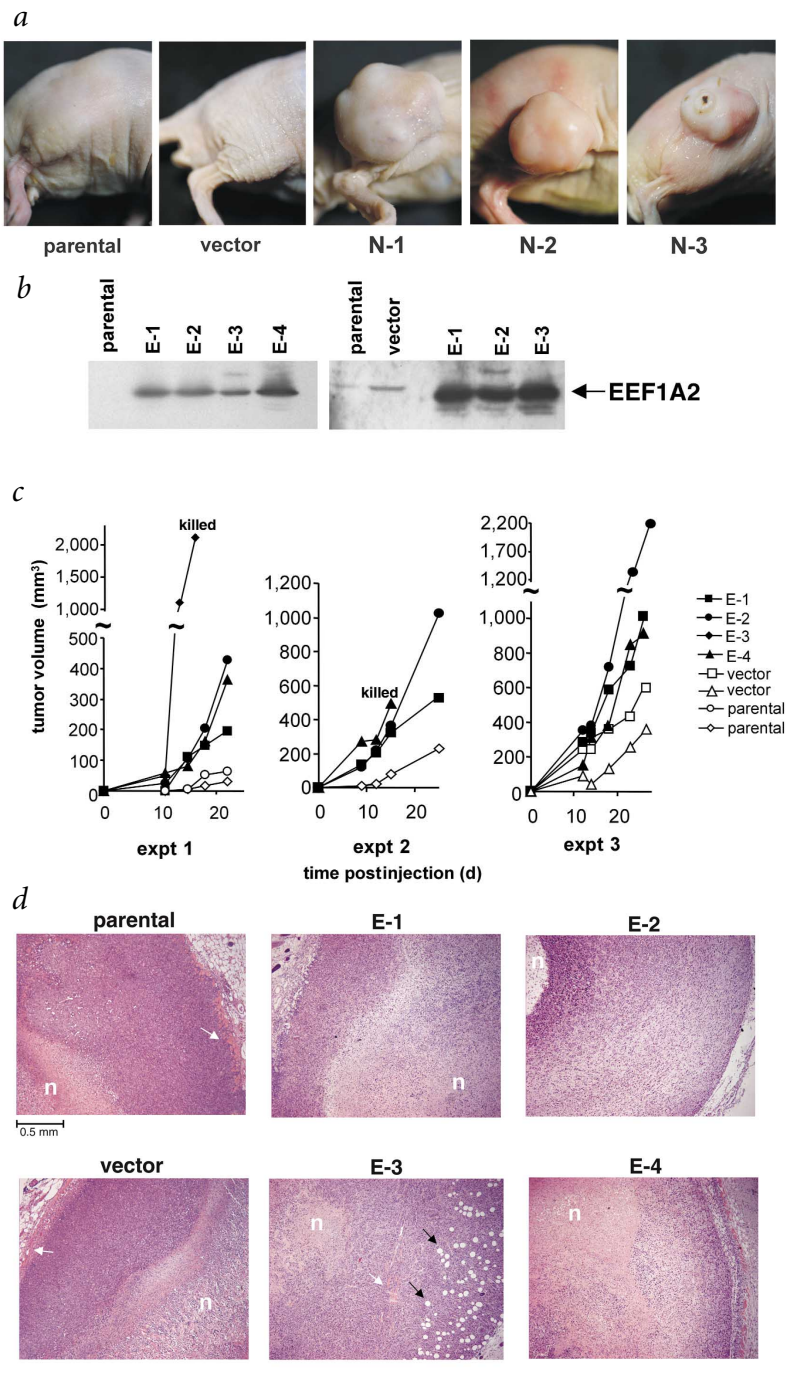

Fig. 4 EEF1A2 expression enhances tumorigenicity. ***a***, EEF1A2-expressing NIH3T3 cells grow as tumors in nude mice, although parental NIH3T3 cells do not. Tumors shown are 21 d postinjection. ***b***, Expression of EEF1A2 protein (with a V5 C-terminal epitope tag) in independent ES-2 lines (E-1, E-2, E-3, E-4) is shown relative to parental and vector-transfected controls in two independent western blots. ***c***, EEF1A2-expressing cell lines grow faster than controls. We injected the ES-2 lines into nude mice and measured tumor volume as a function of time (killed, the animal was killed because of ulceration of the primary tumor). ***d***, Histology of ES-2-derived tumors stained with hematoxylin and eosin. Ischemic necrosis (n) and invasion of subcutaneous adipose (black arrows) and muscle (white arrows) tissues are indicated.

enhanced their *in vivo* tumorigenicity. All ES-2-derived tumors showed high-grade malignancy with an ischemic necrotic core (Fig. 4*d*), which is indicative of rapid tumorigenesis. We observed infiltration of subcutaneous muscle and adipose soft tissue in some tumors (Fig. 4*d*), but we saw this in control as well as in EEF1A2-expressing cells.

Several genes that may have a role in ovarian cancer map to 20q13. Other than *EEF1A2*, 20q13 genes include *ZNF217*, *BCAS1* (*NABC1*; ref. 15), *CYP24* (ref. 16) and *STK15* (or *BTAK*)[17]. Although *ZNF217* promotes the immortalization of mammary epithelial cells[18] and *STK15* is transforming[17], their roles in ovarian tumor development are unknown. Neither *CYP24* nor *NABC1* is known to have tumorigenic properties. It is possible that *EEF1A2* may cooperate with other 20q13 genes to promote ovarian oncogenesis. Mapping of the 20q13 amplicon found in breast cancers by comparative genomic hybridization indicates that the DNA amplifications may center on an ~2-Mb region around 20q13.2 and *CYP24* (ref. 16), the gene for vitamin D 24-hydroxylase[19], implicating this gene as the 'amplicon driver' for 20q13 in breast cancer. The strong transforming and tumorigenic properties of *EEF1A2* indicate that it is likely to be important in oncogenesis over and above any potential role as a 20q13 amplicon driver.

A homozygous deletion of the first intron and promoter of *Eef1a2*, termed *Eef1a2*^{wst}, occurs in the wasted mouse, a spontaneous HRS/J variant[20,21]. The deletion prevents *Eef1a2* transcription[21]. Eef1a2-deficient wasted mice suffer a B- and T-cell immunodeficiency and neuromuscular abnormalities[20] and die of unknown causes by 30 days of age. Wasted mice showed an increase in lymphocyte apoptosis relative to *Eef1a2*$^{+/-}$ animals[22], raising the intriguing possibility that Eef1a2 may be an inhibitor of apoptosis.

How does a protein translation factor enhance tumorigenesis? Protein initiation factor EIF4E is highly expressed in primary breast carcinomas and established cell lines[23,24] and is thought to regulate tumorigenesis by enhancing the translation of genes promoting cellular growth[25]. EEF1A2 may function in the same manner. Alternatively, the ability of EEF1A proteins to shorten microtubules and bind to F-actin[6,7] raises the possibility that cytoskeletal alteration may facilitate tumorigenesis. The PTI oncogene is a gene identified by PCR in prostate cancer cell lines that consists of a truncated and mutated form of human *EEF1A1* fused to a *Mycoplasma* ribosomal RNA gene[26]. PTI can transform rodent fibroblasts and make them tumorigenic in nude mice, but because of the unusual nature of the PTI sequence, its role in carcinogenesis is unclear. Understanding the mechanism by which EEF1A2 regulates cell transformation and tumorigenesis will be an important part of our understanding of ovarian cancer.

Methods

Fluorescence hybridization and microscopy. We carried out FISH as described[27]. Briefly, we labeled *EEF1A2* and 20p11 BAC clones with fluorescein isothiocyanate–dUTP and digoxygenin-dUTP, respectively, and hybridized them at 37 °C to interphase nuclei from frozen ovarian carcinoma tissue samples. We counter stained the slides with 4,6-diamidino-2-phenylindole and used a Zeiss Axioplan 2 microscope to view them. We used a Photometrics PXL 1400 CCD camera to capture images of representative interphase nuclei and Electronic Photography version 1.3 Biological Detection software to align the images. We used Adobe Photoshop to add pseudocolor to fluorescein isothiocyanate– and digoxygenin-labeled probes. A V5 antibody (Invitrogen) diluted 1:500 in PBS followed by an Alexa 546–conjugated secondary antibody (1:200 in PBS) were used to determine EEF1A2 localization.

RNA purification and northern blotting. We obtained fresh-frozen ovarian tumor samples from the Gynecology and Oncology Group of the Cooperative Human Tissue Network. We prepared RNA from 100–200 mg frozen tumor homogenized in 2 ml of TRIzol (Gibco) as per the manufacturer's directions. We obtained RNA from cell lines by lysing a 60-mm plate with 1 ml of TRIzol. We loaded 10 μg total RNA per lane and transferred the RNA to a GeneScreen membrane. We obtained normal ovary mRNA from Stratagene. We prehybridized membranes at 63 °C in 25 ml Church's buffer, hybridized in 15 ml Church's at 59 °C overnight and washed at 62 °C. The *EEF1A2* probe consisted of a 598-bp *Bam*HI/*Pst*I fragment of the human *EEF1A2* cDNA.

Cell culture and western blotting. We grew NIH3T3 and ES-2 cells in 10% FBS/DMEM and 10% FBS/McCoy's 5A, respectively. We transfected NIH3T3 and ES-2 cells with 5 μg *EEF1A2* plasmid and 15 μl SuperFect (Qiagen) per 60-mm dish to generate EEF1A2-expressing NIH3T3 and ES-2 cells, respectively. We used 0.4 mg ml^{-1} Zeocin (Invitrogen) to select transfectants, and we isolated independent clones by limiting-dilution cloning. We used an α-V5 antibody (Invitrogen; 1:500 in TBST (100 mM Tris-Hcl, 0.9% NaCl, 0.1% Tween-20, pH 7.5)) followed by a horseradish peroxidase–conjugated goat anti-mouse antibody (Bio-Rad; 1:1,000 in TBST) and ECL+ (Amersham Pharmacia Biotech) to detect EEF1A2 expression. We measured cell growth by Coulter counting triplicate independent platings from a NUNC 6-well plate. For focus-forming assays, we transfected with *EEF1A2* and *RAS*^{Val12}, both under the control of the CMV promoter, into Rat1 fibroblasts using calcium phosphate according to the manufacturer's directions (Gibco). We used the pCDNA3 empty vector as a control. We grew transfected cells in 2% FBS/DMEM at 37 °C for 14 d and changed the medium every 3 d. We determined transfection efficiency by counting colonies that arose in selective media (Zeocin for EEF1A2 and G418 for RAS cells). We counted foci by washing plates in PBS, fixing in 10% acetic acid and staining with 0.4% crystal violet. Counts are the mean of triplicate experiments, each containing triplicate independent transfections. For soft agarose assays, we placed 2×10^4 NIH3T3 cells in 3 ml of 0.35% low gelling temperature agarose (Sigma) in 10% FBS/DMEM and overlaid them on 5 ml of 0.8% agarose/10% FBS/DMEM in a 60-mm dish. We grew cells at 37 °C for 14 d to allow colony formation.

Tumor xenografts. We injected 1×10^6 NIH3T3 or ES-2 cells subcutaneously into the hind leg of a nude mouse and killed the animals 21 d postinjection. We estimated tumor volume (V) from the length (l) and width (w) of the tumor using the formula: $V = (\pi/6) \times ((l + w)/2)^3$. We fixed tumors in formalin overnight at 4 °C and embedded them in paraffin. We de-waxed and stained the sections with hematoxylin and eosin. Animal experiments were carried out using protocols approved by the Central Animal Facility at McMaster University.

Acknowledgments

We thank A. Al Abadi, R. Austin, J. Hanlon, S. Innocente, S. Lhotak, S. Popovic and E. Seidlitz for help with many of the assays and A. Bernstein, H. Ghosh, J. Hassell, H. Hirte, B. Muller, M. Rozakis-Adcock, G. Singh and P. Whyte for discussion and critical reading of the manuscript. We thank K. Dougherty and H. Blackborrow for secretarial assistance. We acknowledge the sharing of cell lines and information by P. Tonin, T. Hudson, D. Provencher and A.-M. Mes-Masson. This work was supported by funding from the National Cancer Institute of Canada and the Hamilton Regional Cancer Centre Foundation.

Competing interests statement

The authors declare that they have no competing interests.

Received 29 March; accepted 1 May 2002

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