We carried out antibody staining on fixed spheroplasts from yeast cells, which were labelled with monoclonal antibodies against yeast porin (Molecular Probes) and a rabbit polyclonal antiserum generated against recombinant GFP[12]. Nucleoid bodies were stained with DAPI by standard protocols[29].

Protease protection

Mitochondrion-enriched fractions were prepared as described[30] from wild-type, *RBD1:GFP* and *MGM1:HA* yeast strains. We carried out protease protection assays as described[8] and analysed samples by western blot using the indicated antibodies.

Phenotypic analysis

Growth was tested on plates containing 2% glucose. Respiration competence was tested by growth on plates containing 2% glycerol. We assessed peroxide sensitivity by measuring the zone of cell death around a 5-mm disk containing 5 μl of 30% hydrogen peroxide. All growth assays were done at 30 °C.

Cell culture

We cultured COS cells under standard conditions. The mouse PARL cDNA (a gift from B. De Strooper) was inserted into the expression vector pSGF5 (Stratagene) and was transfected using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). Forty-eight hours after transfection, cells were stained with Mitotracker (Molecular Probes), fixed with 4% formaldehyde and counterstained with a monoclonal antibody against the C-terminal HA tag as described[12].

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Role of ERas in promoting tumour-like properties in mouse embryonic stem cells

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Embryonic stem (ES) cells are pluripotent cells derived from early mammalian embryos[1,2]. Their immortality and rapid growth make them attractive sources for stem cell therapies[3]; however, they produce tumours (teratomas) when transplanted, which could preclude their therapeutic usage[4]. Why ES cells, which lack chromosomal abnormalities, possess tumour-like properties is largely unknown. Here we show that mouse ES cells specifically express a Ras-like gene, which we have named *ERas*. We show that human *HRasp*, which is a recognized pseudogene, does not contain reported base substitutions and instead encodes the human orthologue of ERas. This protein contains amino-acid residues identical to those present in active mutants of Ras[5] and causes oncogenic transformation in NIH 3T3 cells. ERas interacts with phosphatidylinositol-3-OH kinase[6] but not with Raf[7,8]. *ERas*-null ES cells maintain pluripotency but show significantly reduced growth and tumorigenicity, which are rescued by expression of *ERas* complementary DNA or by activated phosphatidylinositol-3-OH kinase. We conclude that the transforming oncogene *ERas* is important in the tumour-like growth properties of ES cells.

To understand mechanisms underlying the pluripotency of ES cells and their propensity to form tumours *in vivo*, we searched for genes that were expressed specifically in murine ES cells by digital differential display. Unigene cluster Mm.249524 was found exclusively in libraries from ES cells. We obtained a full-length cDNA of this gene by the rapid amplification of cDNA ends (RACE). Basic local alignment search tool (BLAST) analysis against mouse genomic databases showed that the gene is located on the X chromosome and contains two exons. The cDNA encodes a protein of 227 amino acids with 43%, 46% and 47% identity to HRas, KRas and NRas, respectively. Five domains essential for small G proteins[9] are highly conserved (Fig. 1a). It contains a CAAX motif[10,11] and is located at cytoplasmic membrane (Fig. 1b). These findings indicate that the

protein, which we designated ERas, is a previously unknown member of the Ras protein family.

By searching human genomic databases, we found that the *ERas* gene was most similar to human *HRasp* (*Ha-Ras2*), which is also located on the X chromosome and is a recognized processed pseudogene with several nonsense and deletion mutations[12]. We re-determined the nucleotide sequence of *HRasp* and found that the previously reported mutations did not exist. *HRasp* has a single open reading frame encoding a polypeptide with 76% identity to mouse ERas (Fig. 1a). Because of the sequence similarity and the common chromosomal localization, we concluded that *HRasp* is the human orthologue of *ERas*.

Northern blot analyses detected a single 1.2-kb *ERas* transcript in undifferentiated RF8 mouse ES cells (ref. 13 and Fig. 1c). ERas was also expressed in three other undifferentiated mouse ES cell lines (Fig. 1d): J1 (ref. 14), CGR8 (ref. 15) and MG1.19 (ref. 16). In contrast, *ERas* was not detected in differentiated ES cells or in 12 somatic tissues from adult mouse (Fig. 1c). Therefore, the specific expression of *ERas* is a common property of ES cells.

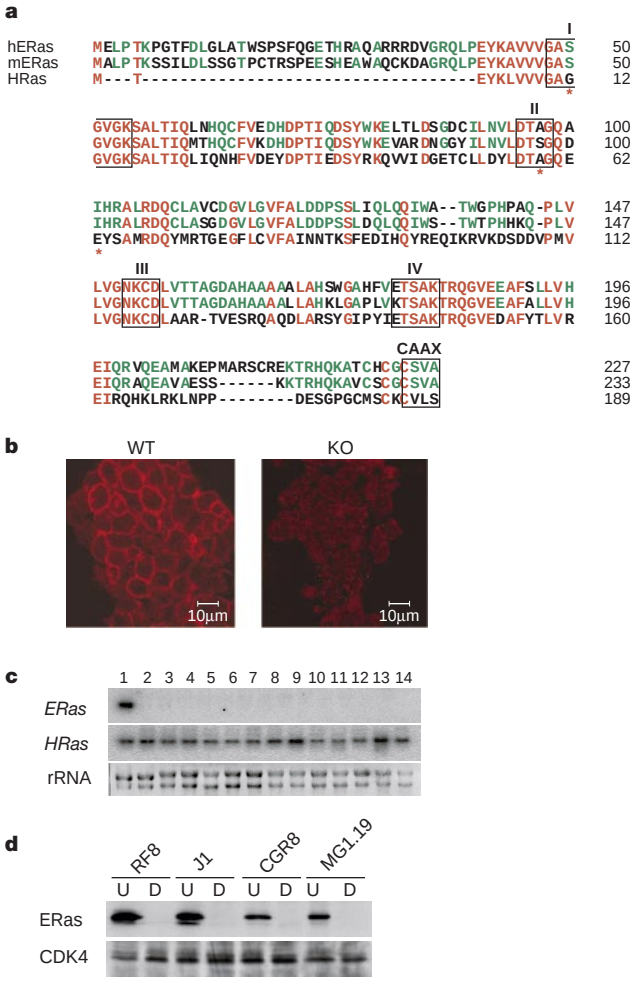

Figure 1 Identification of ERas. **a**, Amino acid sequences of murine and human ERas and HRas. Boxes indicate conserved domains and the CAAX motif. Asterisks indicate three amino acid residues that are often mutated in oncogenic HRas mutants. Amino acids conserved among all three proteins are red, and those conserved between human and mouse ERas are green. **b**, Immunostaining of ERas in wild-type (WT) and *ERas* knockout (KO) ES cells. **c**, Northern blot. lane 1, undifferentiated ES cells; lane 2, retinoic-acid-treated ES cells; lane 3, testis; lane 4, lung; lane 5, heart; lane 6, liver; lane 7, stomach; lane 8, kidney; lane 9, brain; lane 10, spleen; lane 11, thymus; lane 12, small intestine; lane 13, skin; lane 14, muscle. **d**, Western blot analysis of ERas expression in four ES cell lines. U, undifferentiated; D, differentiated.

Point mutations of several amino acids of HRas, including Gly 12, Ala 59 or Glu 63, render the protein constitutively active[17]. We found that mouse ERas has serine, serine and isoleucine (and human ERas has serine, alanine and asparagine) at the positions corresponding to Gly 12, Ala 59 and Glu 63 of HRas (Fig. 1a), suggesting that both were constitutively active. To explore this possibility, we analysed the association of GTP and GDP with the proteins (Fig. 2a). As expected, 95% of mouse and human ERas was in a GTP-bound form. In contrast, about 90% of wild-type HRas existed in a GDP-bound form, whereas about 80% of HRasV12 (a constitutively active mutant in which Gly 12 is mutated to valine) was in a GTP-bound form. These data show that ERas is indeed constitutively active.

To examine whether ERas has the ability to transform cells, we used an efficient retroviral gene transfer system to generate a polyclonal NIH 3T3 cell population expressing ERas or HRasV12 (Supplementary Fig. S1). Both mouse and human ERas induced morphological changes indicative of transformation: high refractivity and spindle-like shape (Fig. 2b) and loss of contact inhibition (Fig. 2c). When we cultured 5,000 NIH 3T3 cells expressing mouse ERas, human ERas or HRasV12 in soft agar, we obtained similar numbers (992 ± 160, 934 ± 49 and 989 ± 86) of colonies showing anchorage-independent growth (Fig. 2d).

By contrast, from control cells transfected with the parent vector or ERas inactivated by deletion of the CAAX motif (ERas-ΔC), we obtained a few (2 ± 1 and 1 ± 1) colonies that were much smaller than those from wild-type ERas and HRasV12. When transplanted into nude mice, cells expressing ERas effectively produced tumours (Fig. 2e). ERas was more potent than HRasV12 in promoting cell growth in culture (Fig. 2c, d) but produced smaller tumours (Fig. 2e). Their difference was more evident in mouse embryonic fibroblasts (MEFs): HRasV12 caused premature senescence (ref. 18 and Fig. 2f), whereas ERas significantly increased growth rate. These results show that ERas induces transformation as effectively as HRasV12, but with a different mode of action.

To study the functions of ERas in ES cells, we transfected either the sense or antisense cDNA of *ERas* into MG1.19 ES cells. When the sense cDNA was transfected, expression of ERas increased roughly fourfold (Fig. 3a) and cell growth was significantly enhanced (Fig. 3b). Transfection of ERas-ΔC did not produce such growth-promoting effects. In contrast, transfection of HRasV12 resulted in differentiation and growth retardation (Fig. 3b), as reported previously[19,20]. After transfection of the antisense cDNA, ERas expression decreased to a fifth of the normal level (Fig. 3a) and cell growth was significantly repressed (Fig. 3b). These data suggest that ERas, unlike HRasV12, promotes the growth of ES cells.

To clarify further the *in vivo* functions of ERas, we disrupted the *ERas* gene by homologous recombination in another ES cell line, RF8. We obtained two clones in which the coding region was replaced with a fusion comprising the β-galactosidase and neomycin resistance genes (β-geo; Fig. 3c). As expected from its location on the X chromosome, the wild-type *ERas* allele was absent in both clones, as assessed by Southern blot analysis (Fig. 3d). Northern blot (Fig. 3e), western blot (Fig. 3f) and immunohistochemical assessment (Fig. 1b) confirmed the absence of ERas expression. In addition, we established two clones, one from each knockout clone, in which ERas expression was partially recovered by transfection with *ERas* cDNA (Fig. 3f).

ERas-null ES cells were normal in both morphology (data not shown) and expression of *Oct3/4* (Fig. 3e). Blastocyst injection of these cells resulted in germline transmission of the mutation. Mutant mice did not show gross abnormalities or infertility (data not shown). These results indicate that ERas is not required for pluripotency and normal mouse development. However, *ERas*-null cells grew significantly more slowly than wild-type cells (Fig. 3g).

The slow growth phenotype was more evident when ES cells were grown without feeder cells. Wild-type ES cells expanded 6×10^5 times under these suboptimal conditions over 16 d, whereas the *ERas*-null cells expanded only 1×10^3 times. When transplanted into nude mice, *ERas*-deficient cells produced markedly smaller tumours than did wild-type cells (Fig. 3h). Re-expression of *ERas* led to a partial recovery of growth and tumorigenicity (Fig. 3g, h). These results show the important role of ERas in the tumour-like growth properties of ES cells.

We thought that the functional differences between ERas and HRasV12 might be due to different effector preferences. To test this hypothesis, we determined whether ERas could activate Raf[7,8]. Co-immunoprecipitation experiments showed that HRasV12, but not ERas, associates with Raf1 (Fig. 4a) and BRaf (Supplementary Fig. S2a). In reporter assays, AP1 enhancer activity was activated by HRasV12 and repressed by the dominant-negative mutant HRasN17 (Supplementary Fig. S2b). In contrast, ERas did not affect AP1 activity. These data indicate that ERas cannot bind to Raf or activate the mitogen-activated protein kinase (MAPK) cascade.

We next examined whether ERas binds to phosphatidylinositol-3-OH kinase (PI(3)K), another effector of Ras[6] that is important in both transformation[21] and ES cell propagation[22,23]. Co-immunoprecipitation experiments showed that ERas, as well as HRasV12, associated with PI(3)K p110δ (Fig. 4a). We also confirmed interaction between endogenous ERas and PI(3)K p85 (Supplementary Fig. S2c). Phosphorylation of Akt, a downstream molecule of PI(3)K, was decreased in *ERas*-null cells (Fig. 4b)[24,25]. Transgenic re-expression of ERas partially recovered this reduction

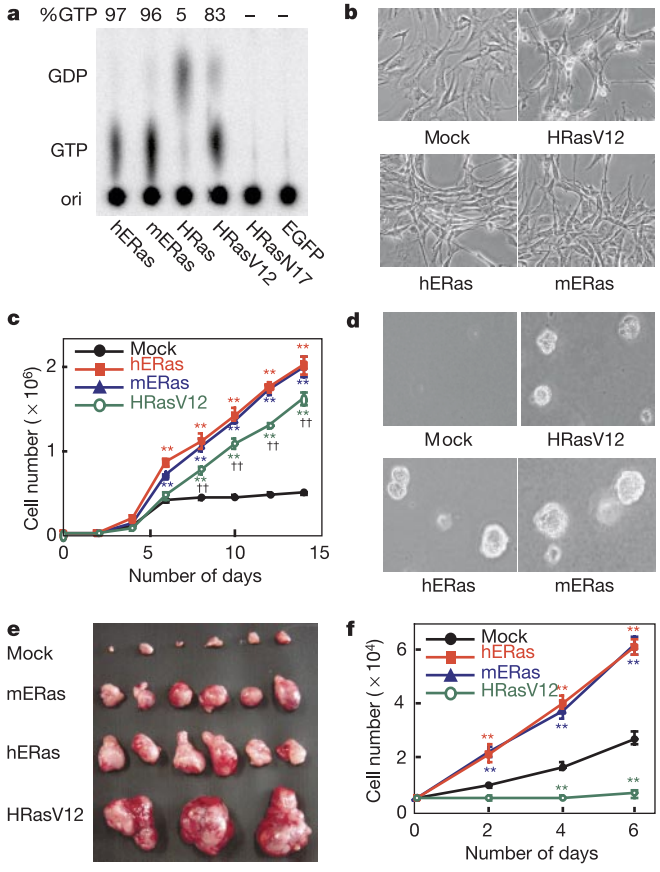

Figure 2 Transformation by ERas. **a**, Thin-layer chromatography showing protein association with GTP and GDP. **b**, Morphology of NIH 3T3 cells expressing HRasV12 or ERas. **c**, Cell growth. Ten thousand cells were plated and counted every other day for 14 d. Double asterisk, $P < 0.01$ versus mock transfected; double dagger, $P < 0.01$ versus ERas ($n = 4$). **d**, Anchorage-independent growth in soft agar. **e**, Tumours in nude mice. One million cells were injected subcutaneously and tumours were dissected after 14 d. **f**, Growth of MEFs expressing HRasV12 or ERas. Five thousand cells were plated and counted after 2, 4 and 6 d. Double asterisk, $P < 0.01$ versus mock transfected ($n = 5$).

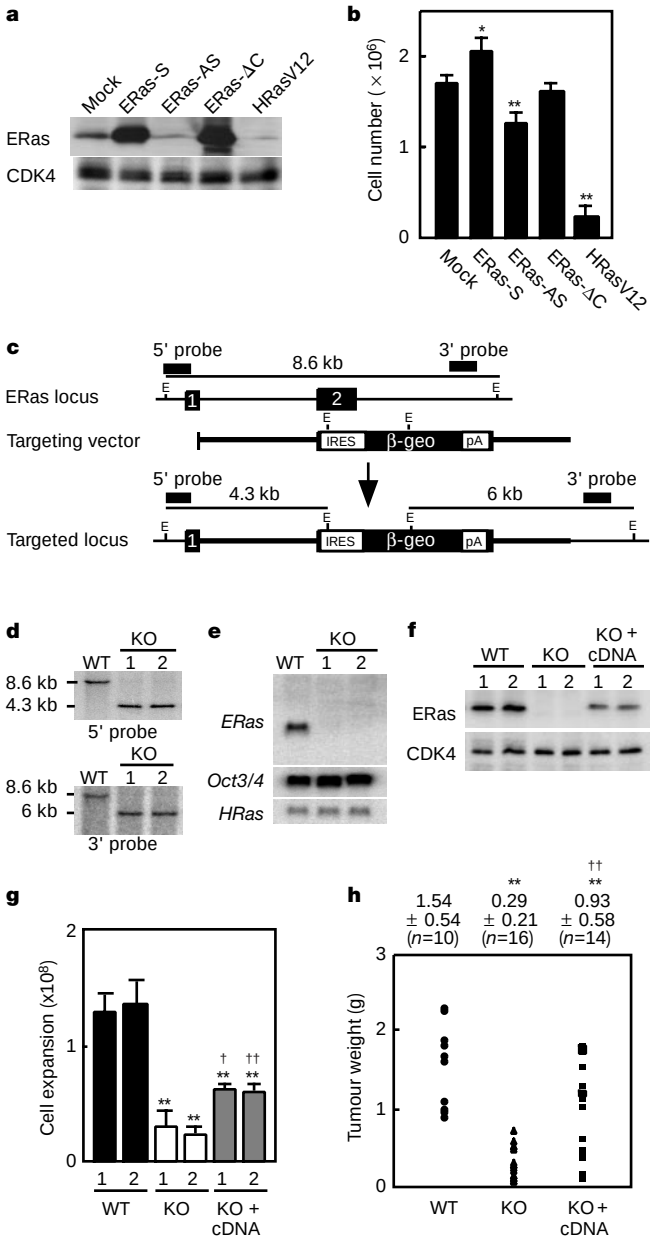

Figure 3 Role of ERas in ES cell growth. **a**, Western blot analysis of ERas expression in MG1.19 ES cells expressing sense cDNAs of ERas, ERas without the CAAX motif (ERas-ΔC) or HRasV12, or an antisense cDNA of ERas. **b**, Cell growth. Ten thousand of the cells in **a** were plated and counted after 6 d. Asterisk, $P < 0.05$ and double asterisk, $P < 0.01$ versus mock transfected ($n = 4$). **c**, Targeted disruption of *ERas*. Filled boxes indicate exons. The length of diagnostic *Eco*RI (E) restriction fragments and the locations of the 5′ and 3′ flanking probes are shown. **d**, Southern blot analyses. WT, wild-type ES cells; KO, *ERas* knockout cells. **e**, Northern blot analyses. **f**, Western blot analysis. KO + cDNA, *ERas* knockout cells expressing the *ERas* transgene. **g**, Cell expansion after 16 d of culture. Double asterisk, $P < 0.01$ versus wild type; dagger, $P < 0.05$ versus KO1; double dagger, $P < 0.01$ versus KO2 ($n = 4$). **h**, Teratoma formation. One million cells were subcutaneously injected into nude mice. Teratomas were dissected and weighed after 28 d. Double asterisk, $P < 0.01$ versus wild-type; double dagger, $P < 0.01$ versus KO.

(Supplementary Fig. S2d). These data show that ERas constitutively binds and activates PI(3)K.

We next examined whether impaired growth and tumorigenicity in *ERas*-null ES cells could be rescued by forced expression of active PI(3)K[26]. Expression of the transgene was detected in two clones (one from each *ERas*-null clone) by northern blot analysis (data not shown). In both clones, the phosphorylation of Akt was higher than in wild-type cells (Fig. 4b) and impaired growth reverted to wild-type levels (Fig. 4c). Teratoma formation was also rescued and was even faster than in wild-type ES cells (Fig. 4d). In addition, we found that growth-promoting activity of ERas was blocked by the specific PI(3)K inhibitor LY294002, but not by the MAPK kinase inhibitor PD098059 (Supplementary Fig. S2e). These results show that the PI(3)K cascade is important in the growth-promoting activity of ERas.

Our study has established a previously unknown pathway that activates PI(3)K in ES cells. The role of PI(3)K in the cell-cycle control of ES cells has been shown by studies in which LY294002 markedly increased the proportion of ES cells in G0/G1 phase[27]. By contrast, we observed only small changes in the cell cycle in *ERas*-null cells as compared with wild-type cells (G1, 24.6 ± 0.3%; S, 60.5 ± 1.5%; G2, 14.8 ± 1.3% versus G1, 22.8 ± 0.7%; S, 59.8 ± 1.3%; G2, 17.4 ± 0.8%), suggesting that PI(3)K might also have a growth-promoting effect that is independent of cell-cycle control. We have also shown that Akt is a downstream effector of the ERas/PI(3)K pathway, although other factors are also likely to be involved. The identification of ERas will facilitate our understanding of signalling pathways that maintain the tumour-like propagation of ES cells. □

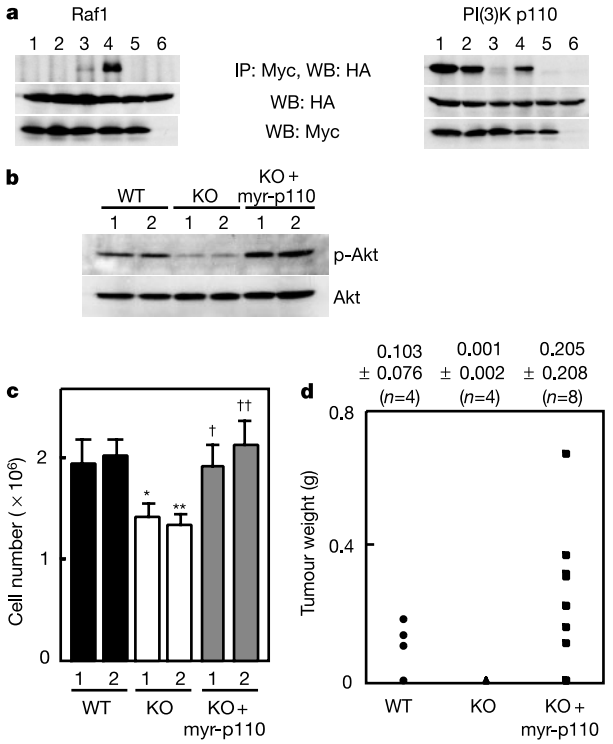

Figure 4 PI(3)K activation by ERas. **a**, Interaction with Raf1 and PI(3)K p110δ. MG1.19 cells were transfected with constructs expressing Myc–hERas (lane 1), Myc–mERas (lane 2), Myc–HRas (lane 3), Myc–HRasV12 (lane 4), Myc–HRasN17 (lane 5) or EGFP (lane 6), along with HA–Raf1 (left) or HA–PI(3)K (right). Cell lysates were analysed by western blot with antibodies against Myc (bottom) or HA (middle). Precipitants with agarose-conjugated anti-Myc antibodies were analysed with antibodies against HA (top). **b**, Akt phosphorylation. Wild-type ES cells (WT), *ERas*-null ES cells (KO) and *ERas*-null ES cells expressing active PI(3)K (KO + myr-P110) were analysed by western blot with antibodies against phosphorylated (Ser 473) or total Akt. **c**, Cell growth. Ten thousand of the cells in **b** were plated and counted after 6 d. Asterisk, $P < 0.05$ and double asterisk, $P < 0.01$ versus mock transfected; dagger, $P < 0.05$ versus KO1; double dagger, $P < 0.01$ versus KO2 ($n = 4$). **d**, Teratoma formation. One million of the cells in **b** were subcutaneously injected into nude mice. Teratomas were dissected and weighed after 16 d.

Methods

Isolation of full-length cDNA of mouse *ERas*

We obtained the 5′ portion of *ERas* cDNA using a 5′ RACE system (version 2.0, Invitrogen) with primers 45328-AS1 for reverse transcription and 45328-race11 for polymerase chain reaction (PCR). All primer sequences are given in the Supplementary Information. Amplified products were cloned into pCR2.1 (Invitrogen) and four independent clones were sequenced.

Analysis of *HRasp*

A DNA fragment containing human *HRasp* was amplified from genomic DNA of two adults by PCR with the primers hHRAS2-S and hHRAS2-AS. The amplified product was cloned into pCR2.1 to construct pCR2.1–hERas and sequenced.

Analysis of GTP/GDP association

We transfected pCAG-IP–EGFP, pCAG-IP–Myc–hERas, pCAG-IP–Myc–mERas, pCAG-IP–Myc–HRas, pCAG-IP–Myc–HRasV12 or pCAG-IP–Myc–HRasN17 into MG1.19 cells by using Lipofectamine 2000 (Invitrogen). The construction of expression vectors is described in Supplementary Information. After 24 h, GTP/GDP association was evaluated as described[28], except that the lysis buffer consisted of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4, 20 mM $MgCl_2$, 0.5% Triton X-100 and protease inhibitor cocktail (Nacalai Tesque). Signals were analysed with a BAS 5000 imaging plate scanner (Fujifilm). The ratio of GTP-bound form was calculated as GTP/(GTP + 1.5 × GDP).

Retroviral infection

MEFs were isolated and cultured as described[18]. We cultured NIH 3T3 mouse fibroblast cells in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cells of the ecotropic packaging line PLAT-E (8×10^6) were plated in a 100-mm dish, incubated for 24 h and then transfected using FuGENE6 (Roche) with a pMX-IP retroviral plasmid encoding ERas, ERas lacking the CAAX motif (ERas-ΔC) or HRasV12. After 24 h, the medium was replaced and incubation was continued for another 24 h. We then passed the virus-containing medium through a 0.45-μm filter (Schleicher & Schuell) and supplemented it with 4 μg ml^{-1} polybrene (Nacalai Tesque). We incubated MEFs (passage 1) or NIH 3T3 cells (~80% confluency) in this supernatant in a 100-mm dish for 24 h. The medium was then replaced and incubation was continued for another 24 h. Infected cell populations were selected with 2 μg ml^{-1} puromycin for 4 d. We did the transformation assay as described[29].

Episomal expression in MG1.19 ES cells

A sense strand cDNA of ERas, ERas-ΔC or HRasV12 or an antisense strand cDNA of ERas was inserted into a pCAG-IP vector containing the Polyoma replication origin. We transfected these plasmids with Lipofectamine 2000 (Invitrogen) into MG1.19 ES cells expressing Polyoma large antigen. Cells were selected with puromycin for 4 d.

Targeted disruption of mouse *ERas*

A targeting vector designed to replace the *ERas* coding region with β-geo was transferred into RF8 ES cells[13] by electroporation. Targeted clones were identified by PCR and confirmed by Southern blot (Supplementary Information).

ERas and PI(3)K expression in *ERas*-deficient ES cells

pCAG–mERas–IH or pCAG–myr-p110–IH was transferred into *ERas*-deficient ES cells by electroporation. To identify clones expressing ERas, we screened colonies resistant to 100 μg ml^{-1} hygromycin B by western blot analysis with a rabbit antiserum against ERas, which was generated against histidine-tagged recombinant ERas produced in *Escherichia coli*. Clones expressing active PI(3)K were screened by northern blot analysis using PI(3)K p110α cDNA as a probe.

Measurement of expansion in cell numbers

We plated ES cells at 1×10^4 cells per well in 24-well plates. After 4 d, the cells were counted with a Z2 Coulter Counter (Beckman Coulter) and replated into new wells at the same density. This procedure was repeated for four passages. We calculated the expansion of cell numbers after a total of 16 d as follows: expansion in cell number = (cell number after 1st passage/1×10^4) × (cell number after 2nd passage/1×10^4) × (cell number after 3rd passage/1×10^4) × (cell number after 4th passage/1×10^4).

Immunoprecipitation

We transfected pCAG-IP–EGFP, pCAG-IP–Myc–hERas, pCAG-IP–Myc–mERas, pCAG-IP–Myc–HRas, pCAG-IP–Myc–HRasV12 or pCAG-IP–Myc–HRasN17 into MG1.19 cells, along with pCAG-IP–HA–Raf1, pCAG-IP–HA–BRaf or pCAG-IP–HA–PI(3)K-p110δ. Cell lysates were collected and immunoprecipitation were done as described[30]. Precipitants and lysates were analysed by SDS–PAGE and western blot analysis. We used the following antibodies: agarose-conjugated monoclonal antibody against Myc (SC40

AC, Santa Cruz) for immunoprecipitation; a monoclonal antibody against haemagglutinin A (HA; 1867423, Roche), a polyclonal antibody against Myc (SC789, Santa Cruz), and antibodies against phosphylated (Ser 473) or total Akt (9270, New England Biolabs).

Statistical analysis

Results shown are the mean ± s.d. We analysed data by one-way analysis of variance (ANOVA). Individual statistical differences were determined by Scheffe's multiple range comparison test.

Accession numbers

The sequences of mouse and human ERas can be retrieved from DDBJ/GenBank/EMBL with accession numbers AB093573 and AB093575.

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Modulation of oestrogen receptor signalling by association with the activated dioxin receptor

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Environmental contaminants affect a wide variety of biological events in many species. Dioxins are typical environmental contaminants that exert adverse oestrogen-related effects[1]. Although their anti-oestrogenic actions[2,3] are well described, dioxins can also induce endometriosis[4–7] and oestrogen-dependent tumours[8,9], implying possible oestrogenic effects. However, the molecular mechanism underlying oestrogen-related actions of dioxins remains largely unknown. A heterodimer of the dioxin receptor (AhR) and Arnt, which are basic helix–loop–helix/PAS-family transcription factors, mediates most of the toxic effects of dioxins[10,11]. Here we show that the agonist-activated AhR/Arnt heterodimer directly associates with oestrogen receptors ER-α and ER-β. This association results in the recruitment of unliganded ER and the co-activator p300 to oestrogen-responsive gene promoters, leading to activation of transcription and oestrogenic effects. The function of liganded ER is attenuated. Oestrogenic actions of AhR agonists were detected in wild-type ovariectomized mouse uteri, but were absent in $AhR^{-/-}$ or ER-$\alpha^{-/-}$ ovariectomized mice. Our findings suggest a novel mechanism by which ER-mediated oestrogen signalling is modulated by a co-regulatory-like function of activated AhR/Arnt, giving rise to adverse oestrogen-related actions of dioxin-type environmental contaminants.

ERs, which are members of the nuclear receptor (NR) family[12,13], and AhR/Arnt are both ligand-dependent transcription factors. Ligand-activated AhR heterodimerizes with Arnt and activates the transcription of dioxin target genes such as *CYP1A1* (refs 10,11) through xenobiotic response elements (XREs). ERs bind to oestrogen response elements (EREs) and activate transcription in an oestrogen-dependent manner. This transcriptional activation