Supplementary Data includes:

Detailed Materials and Methods
Figure S1 to S16
References

Materials and Methods (detailed)

Cell cultures & Mice

Human embryonic kidney 293T cells, H4 cells, HeLa cells, *EGR1*$^{-/-}$ and *EGR1*$^{+/+}$ MEFs, *p19ARF*$^{-/-}$ and *p19ARF*$^{+/+}$ MEFs were cultured in DMEM containing 10% FBS, penicillin, and streptomycin at 37°C and 5% CO2. *p19ARF*$^{-/-}$ and *p19ARF*$^{+/+}$ MEFs, which were prepared from *p19ARF*-null and wild type 129 embryos, were gifts from Dr. C.J. Sherr. *p19ARF*$^{+/+}$ MEFs enter senescence at passage 7-9 and thus were used at earlier passage 3-6. For IGF-1 stimulation, cells were incubated in 0.5% CS for 24 hr and then 100 ng/ml of IGF-1 (PerpoTech Inc.) was added. *p19ARF*$^{-/-}$ 129sv/BL6 mice were kindly provided by the NCI Mouse Repository (Frederick, MD) with the permission of Dr. C.J. Sherr. Twelve of these mice and twelve control mice of the same strain (3 mice for each treatment) were irradiated with 5 Gy and sacrificed 0, 1, 3, or 7 h later. Total RNAs were prepared from 6 different organs (spleen, heart, kidney, liver, lung and thymus) and analyzed by quantitative real-time RT-PCR for *CPH, EGR1*, *p19ARF* and *PTEN* mRNAs. The same tissue samples were lysed in regular RIPA buffer with 20 mM N-ethylmaleimide (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 20 mM N-ethylmaleimide and complete protease inhibitor cocktail) for Western blot. Total RNAs from *EGR1*$^{-/-}$ mice were described before (Yu *et al*,2007). For later use, aliquot tissues were stored in liquid nitrogen and RNA samples were preserved at -80℃.

For genotyping analysis of *p19ARF*$^{-/-}$ and wild-type 129sv/BL6 mice,

Primers:

C018 : 5'-AGT AcA GcA GcG GGA GcA TGG-3'

C019 : 5'-TTG AGG AGG Acc GTG AAG ccG-3'

C020 : 5'-Acc AcA cTG cTc GAc ATT GGG-3'

Product Sizes:	Primer Combination	Product Size	
	C018 /C019	415 bp	Wild-type
	C020 /C019	250bp	Knockout

Generation of PTEN or EGR1 Retrovirus and Infection of Cells

To generate a high titer of viral stocks, pBabe-PuroL, pBabe-PuroL-PTEN (WT), pBabe-PuroL-PTEN (C124S), pBabe-PuroL-EGR1(WT), or pBabe-PuroL-EGR1(K272R) were transfected into the BOSC-23 packaging line (American Type Culture Collection) using Lipofectamine 2000 (20 μg of DNA per 100-mm dish). Approximately 60–72 h after the beginning of transfection, the supernatants were harvested, and cleared by filtering through 0.45 mm filter or by spinning in a 15-ml tube at 1500 RPM for 5 minutes, and used for infection or stored in aliquots at -80 °C for later use. Wild-type primary MEFs and *ARF*$^{-/-}$ MEFs in 12-well plates at 60% confluence were incubated for 18 h with 2 ml of viral supernatants in the presence of polybrene (8 μg/ml). The infected cells were then kept in complete medium for 48 h. And then transduced cells (colonies) were selected by puromycin (for *ARF*$^{-/-}$ MEFs, 2 μg/ml for 2 weeks; for wild-type primary MEFs, 2 μg/ml for 3-4 days). To quantitate proliferation, cells were seeded at 5000 cells per well in 96-well plates in six-replicates. Cell proliferation ratios were determined by CyQUANT Cell Proliferation Assay Kit (Invitrogen/Molecular Probes) and the relative proliferation rates were normalized with the cell number at 0.5 day.

Plasmids

The human *EGR1* cDNA was amplified by using Expand High Fidelity PCR Systems (Roche) with a pair of primers TGCT*GAATTC*GGATGGCCGCGGCCAAGGCCGAG (*EcoR*I) and

TTCG*CTCGAG*TTAGCAAATTTCAATTGTCCTGGG (*Xho*I) and subcloned into three different epitope tagging mammalian expression vectors pCMV-Tag2C (FLAG) (Stratagene, San Diego, CA), pCMV-HA and pCMV-MYC (BD Biosciences, Clontech) and a GST-fusion vector pGEX4T1, thus creating pCMV-Tag2C-hEGR1, pCMV-HA-hEGR1, pCMV-MYC-hEGR1 and pGEX4T1-hEGR1, each was confirmed by sequencing. Mutations were induced by directed mutagenesis using Quick-Change Site directed Mutagenesis Kit (Stratagene, San Diego, CA). Since the full-length EGR1 was poorly soluble in *E. coli*, we decided to generate three proteins: GST-EGR1N (aa 1-278), which contains the transactivation domain, GST-EGR1M (aa 275-421), which contains the repressor domain and the Zn fingers, and GST-EGR1C (aa 416-543), which contains the C-terminal regulatory stabilization region. The human *p14ARF* cDNA was amplified by using Advantage-GC Genomic polymerase Mix (Clontch, BD Biosciences) with a pair of primers AGCG*GAATTC*ATGGGCAGGGGGCGGTGCGTGGGT(*EcoR*I) and AGCG*CTCGAG*TCAGCCAGGTCCACGGGCAGACGG (*Xho*I) and subcloned into the epitope tagging mammalian expression vectors pCMV-Tag2B (FLAG) (Stratagene).

Antibodies and Reagents

Antibodies to EGR1 (C-19 and 588), mouse p19ARF (5-C3-1), p53 (DO-1) and Sumo-1 (FL-101) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to PTEN (26H9, 138G6), phospho-S473-Akt1 and Akt1 were from Cell Signaling Technology, antibody to human p14ARF (Ab-2) was from Calbiochem (San Diego, CA). Anti-β-actin, anti-Flag M2 monoclonal antibodies, and anti-Flag M2 agarose affinity gel were from Sigma (St. Louis, MO). Anti-PTEN (A2b1) was from Chemicon International (San Diego, CA). Anti-HA (mouse IgG) was from Roche Applied Science (Indianapolis, IN). Etoposide and LY294002 were from Sigma. Human p14ARF siRNA was from Santa Cruz Biotechnology.

GST fusion protein pull-down assays

Forty-eight hours after transfection with Flag-p14ARF or Flag-EGR1, 293T cells were collected and lysed in (modified) M-RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, and complete protease inhibitor cocktail). For interaction experiments, 1 mg of lysate was added 1~2 μg of bacterially expressed purified GST fusion-protein and 30 μl of Glutathione-Sepharose beads (GE Healthcare), incubated for overnight at 4°C. Then the bead bound proteins were washed 4-5 times with the same buffer with M-RIPA buffer.

Three methods for analysis of SUMO-modified proteins

a) Analysis of exogenous SUMO1-EGR1 by directly lysing in NEM-RIPA buffer

In general, 293T cells were co-transfected with three genes SUMO1, Ubc9 and EGR1 (including p14ARF gene in some experiments). 48-72 h after transfection, cells were collected and washed with NEM-PBS buffer (20mM N-ethylmaleimide in PBS), and then the cell pellets were directly lysed in NEM-RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 20mM N-ethylmaleimide and complete protease inhibitor cocktail).

b) Analysis of endogenous SUMO1-EGR1 by immunoprecipitation

The method was described (Babic et al., 2006) with several modifications. For analysis of endogenous SUMO1-EGR1 protein, HeLa cells were grown in 10cm plates. For IGF-1 stimulation, cells were incubated in 0.5% CS for 24 hr and then 100 ng/ml of IGF-1 (PerpoTech Inc.) was added. For γ-irradiation, cells were exposed to 5 Gy, then collected in NEM-PBS and the cell pellets lysed by adding 200 μl of SUMO lysis buffer (62.5mM Tris pH 6.8, 2%SDS) and boiling for 10 min. The samples were centrifuged for 20 min at full speed in an eppendorf microcentrifuge. The supernatant was transferred to a new tube and either stored at -80°C until required for further analysis or used for direct protein determination. This lysate was diluted

1/20 with NEM-RIPA buffer. Immunoprecipitation (IP) with 5 μl of anti-SUMO1 (FL-101) antibody was used and immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-EGR1(C-19) antibody.

c) ***Analysis of His-tagged SUMO1 conjugates by using Ni^{2+}-NTA beads***

This method was as described (Xirodimas et al., 2001) with several modifications. Briefly, *p19ARF*$^{-/-}$ MEFs were transfected with MEF Nucleofector Kit 1 (from Amaxa Biosystems) according to the manufacture's protocol. Where indicated 1 μg of each of Flag- or Myc- hEGR1 (WT or Mutants), Flag-UBC9 and His_6-SUMO1 expressing plasmids were used. In some experiments, other plasmids such as Flag-p14ARF or p14RNAi were added. 48 h after transfection cells were collected and 25% of cells were used for M-RIPA buffer lysis and Western blot. The remainder was lysed in 3 ml of His-lysis buffer. 60 μl of Ni^{2+}-NTA-agarose beads (Qiagen) were then added and lysates were rotated at 4°C overnight. The beads were successively washed for 5 min in each step at room temperature with 750 μl of each of the following buffers: Washing buffer1, Washing buffer2, Washing buffer3, Washing buffer4. After the last wash His6-tagged sumoylateded products were eluted by incubating the beads in 75 μl of Elution buffer for 20 min at RT. The eluates were analysed by Western blot. His-lysis buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0, 5 mM imidazole and 10 mM β-mercaptoethanol). Washing buffer 1 (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0 plus 10 mM β-mercaptoethanol). Washing buffer 2 (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl,pH 8.0, 10 mM β-mercaptoethanol). Washing buffer 3 (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol (buffer A) plus 0.2% Triton X-100). Washing buffer 4 (buffer A plus 0.1% Triton X-100).Elution buffer (200 mM imidazole, 0.15 M Tris/HCl pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, 5%SDS).

Cell staining and confocal microscope

For 293T cells, the uncoated glass bottom culture dishes (MatTek Corporation, Ashlan, MA) were pretreated and coated by using 0.01% Poly-L-lysine solution (Molecular weight range 70-150 kDa (Sigma) according to the product procedure. 293T cells were seeded into the above coated 35 mm dishes at a density of 3×10^5 cells, 1 day before transfection in order to achieve 75-95% confluence. And HeLa cells were directly seeded into the uncoated 35mm dishes at a density of 1.5×10^5 cells. After 24 hr transfection, or treatment of IGF-1 and LY294002, cells were washed in PBS once, fixed with 4% freshly prepared formaldehyde in PBS for 8-10 min, and then washed 3 times with PBS. Cells were permeabilized with 0.1% Saponin/PBS for 15 min, blocked in 2.5% NGS (normal goat serum) in 0.1% Saponin/PBS for 30 min-24 hrs, incubated in the primary antibody (EGR1, 588, dilution 1:50; p14ARF dilution 1:400) diluted in blocking solution for 1 hr, washed three times with blocking solution and then incubated in the second antibody (Alexa 568 anti rabbit, or Alexa 488 anti mouse, dilution 1:500) in blocking solution for 30 min. Washed twice with blocking solution, and once with PBS. DAPI was added for DNA staining. Pictures were taken with a Fluoview 1000 Olympus Laser Point Scanning Confocal Microscope.

Luciferase Assays

3-5 $\times10^3$ H4 cells and 5-8 $\times10^3$ 293T cells were inoculated 1 day before, and transfection was performed with the Lipofectamine 2000 (Invitrogen). For transfection of *p19ARF*$^{-/-}$ and *p19*ARF$^{+/+}$ (passage 4~5) MEFs were electroporated with MEF Nucleofector Kit 1 (from Amaxa Biosystems) according to the manufacture's protocol. Luciferase assay were performed as described previously (Yu *et al*, 2007). Briefly, typically 90 ng of total plasmid DNA, 40 ng of reporter construct, 40 ng of EGR1 expressing vector, and 10 ng of pRL-SV40 were mixed with appropriate volume of Lipofectamine 2000. Cells were lysed 24 hr after transfection and luciferase activities were assessed using Dual-Luciferase Reporter Assay System (Promega,

Madison, WI). Each value represents the mean fold change of three independent experiments with three triplicates each.

In vitro phosphorylation, tryptic peptide mapping

For *in vitro* kinase assays, GST-EGR1M fusion proteins were incubated with Akt1 (Upstate, Lake Placid, NY), for 30 min at 30°C in 20 mM Hepes pH 7.4, 0.05 mM ATP, 10 mM MgCl2, 1 mM DTT, 2 mM sodium orthovanadate, 5 μCi of [γ-32P]ATP. Assays without radiolabeled ATP had 1 mM cold ATP for Mass Spectrometry. The reactions were terminated by adding SDS sample buffer and heating to 95°C for 5 min followed by SDS-PAGE and autoradiography. Tryptic peptide mapping was performed with the protocol of Luo *et al* (1990).

Mass spectrometry

Identification of phosphorylated residues by LC-MS/MS was performed on *in vitro* phosphorylated GST-EGR1M and digested with highest grade trypsin. The digest was injected into a high-pressure liquid chromatography instrument (LC Packings Inc.), which first separates the peptides on a reverse-phase column, from which they elute directly into a quadrupole time-of-flight mass spectrometer (Q-TOF API-US) equipped with a nanoelectrospray ionization source (Waters-Micromass, UK).

RNA purification, cDNA synthesis and real time PCR

Approximately 20-30mg of tissues preserved in RNAlater (Ambion, Inc., Austin, TX) were immersed in the Trizol reagent (Invitrogen, Carlsbad, CA) and homogenized using MagNA Lyser (Roche Diagnostics, Indianapolis, IN). After chloroform extraction, the RNA fraction was mixed with appropriate volume of 70% ethanol and loaded onto Qiagen column. Total RNA was purified using RNeasy column and DNase I

digestion was performed on the column as recommended by the manufacturer (Qiagen, GmbH, Germany). For cDNA Synthesis, 1.0 μg of total RNA was primed with oligo dT, and reverse transcription was performed with the QuantiTech Reverse Transcription Kit (Qiagen, GmbH, Germany). This kit contains a Genomic Wipeout Buffer which reduced the effects of residual genomic DNA, as monitored by real-time PCR. Real time PCR was performed using an Mx3000p instrument (Stratagene Inc, La Jolla, CA) by following the procedures as described in RT Profiler PCR Array User manual (SuperArray Bioscience Corp. Frederick, MD). In brief, 10~25 ng of the cDNA was used in a 25 μl reaction, on the SuperArray RT^2 ProfilerPCR array plates. RTProfiler Master Mix was used, and PCR product amplification was monitored by SYBR green fluorescence and normalized to the Rox dye standard. A standard curve was generated with cyclophilin A (CPH) primers for each cDNA preparation, and was linear over 4 orders of magnitude. This curve was used to determine relative differences in cDNA from changes in C_T values. CPH gene is also set up for internal reference. Results (at least 3 replicates for each genes analysis) with each cDNA sample were normalized to CPH gene.

Mouse *CPH* primers:

CACCGTGTTCTTCGACATC

ATTCTGTGAAAGGAGGAACC

Human *CPH* primers:

GACCCAACACAAATGGTTC

AGTCAGCAATGGTGATCTTC

Mouse *PTEN* primers:

CAAAGCAAACAAAGACAAGG

TGCTGATCTTCATCAAAAGG

Human *PTEN* primers:

AGCCAACCGATACTTTTCTC

TGCTGATCTTCATCAAAAGG

Mouse *p19ARF* primers:

CACTCCAAGAGAGGGTTTTC

GAATCTGCACCGTAGTTGAG

Mouse *p14ARF* primers:

GGTCCCAGTCTGCAGTTAAG

TCATGACCTGGTCTTCTAGG

Mouse Tumor Histological Analyses

The animal handling procedures were as described (Kamijo *et al*, 1999). Briefly, all mice were observed daily and were sacrificed when they were moribund or emaciated, and tumors from non-autolyzed tissues were histologically examined in detail following an IACUC approved protocol.

a) Paraffin-embedded Sample Preparation (20 to 26 hours)

1) Sacrifice animal
2) Harvest tissue
3) Dissect whole organs (allowing one small part for Western blot and immunoprecipitation)
4) Immediately place the sample in Z-Fix solution for 18 to 24 hours
5) Wash with PBS 2 times for 5 minutes each
6) Transfer to 70% ETOH for storage at room temperature
7) Deliver to the histology facility for processing.

b) Hematoxylin and Eosin Staining (H&E)

Solutions needed for H & E

1) Acid alcohol 1% - 1ml hydrochloric acid in 99 ml 70% ethyl alcohol
2) Saturated lithium carbonate - 1.54gm lithium carbonate in 100ml distilled water
3) Harris hematoxylin from Surgipath
4) Eosin Y from Surgipath

Procedure

1) Deparaffininize slides to water
2) Stain for three to six minutes in hematoxylin
3) wash in running tap water for three minutes
4) differentiate in 1% acid alcohol (5 to 10 dips)
5) Wash briefly in tap water
6) Place in saturated lithium carbonate solution for one minute
7) wash in tap water for five minutes
8) place in 70% alcohol for two minutes
9) counterstain in eosin solution for one minute
10) dehydrate and clear through two changes each of 95% alcohol, 100% alcohol and Xylene allow slides to sit for two minutes in each solution
11) coverslip with a resinous mounting media

Results: Nuclei---blue; Cytoplasm---pink to red; most other tissue structures---pink to red

b) Paraffin Section Immunohistochemistry Protocol

1) Deparaffinize slides and wash in water
2) Block with 3% hydrogen peroxide/PBS 30 minutes
3) Rinse with 3 changes of PBS
4) Block with 5% normal serum for 10 minutes
5) Apply diluted primary antibody PTEN (138G6 from Cell Signaling Technology 1:50 or A2B1 from Chemicon International 1:100) and incubate for 2 h-overnight
6) Wash with 3 changes of PBS
7) Apply secondary antibody (Alexa 488 anti mouse or rabbit, dilution 1:500) and incubate for 30 min
8) Wash with 3 changes of PBS
9) Wash slides with PBS and coverslip using Vectashield DAPI.

Whole Mount Staining

In briefly, the entire inguinal mammary gland (fourth at right side) was excised and fully spread onto glass sides, and fixed In Carnoy fixative overnight. Tissue preparations were immersed in 70% ethnol for 15min, and changed slowly to distilled water, and stained in carmine alum overnight. The tissue was then dehydrated through serial ethanol solutions and cleared in xylene.

Supplementary Figures S1-16

Figure S1. For Figure 1B, a similar analysis of qRT-PCR for *p19ARF* mRNA in four tissues: kidney, heart, liver and lung from wild-type 129sv/BL6 mice that were γ-irradiated with 5 Gy and sacrificed 0 (Non-irradiated), 1, 3, 7 h later.

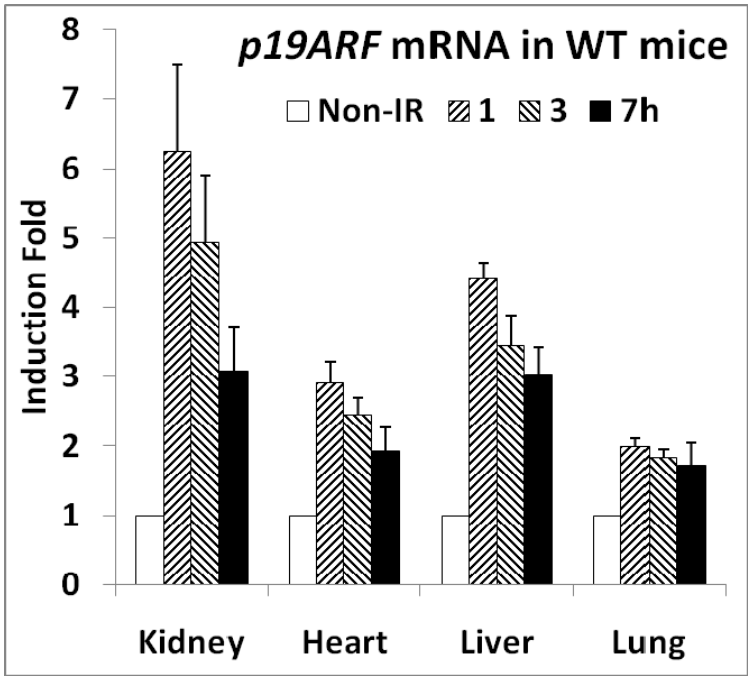

Figure S2. Structure of the PTEN gene promoter. The highest-affinity binding sites (Yu j *et al*, 2004; Yu j *et al*, 2007 and Hamilton *et al*, 1998) for EGR1 in the *PTEN* regulatory regions are marked EBS1 and EBS2. The numbers indicate the position relative to the start of transcription.

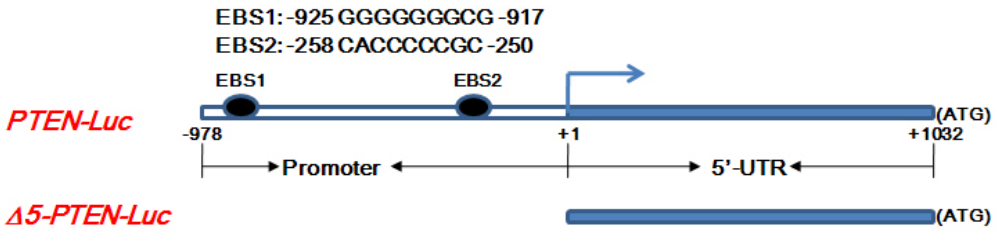

Figure S3. SUMOplot prediction of human EGR1 protein. SUMO consensus sequence ΨKXE/D (where Ψ is a large hydrophobic amino acid, K is the target lysine, X is any amino acid, D or E is an acidic residue).Sumoylation sites of human EGR1 protein were predicted by the program of Abgent SUMOplot™ (**http://www.abgent.com/tool/sumoplot**). K5 is the highest score in all possible EGR1 sumoylation sites in the above table; however it is not a SUMO site. In contrast, K272 which is one of the lowest score was proved to be a true SUMO site by the experiments here.

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  1 MAAAKAEMQL MSPLQISDPF GSFPHSPTMD NYPKLEEMML LSNGAPQFLG
 51 AAGAPEGSGS NSSSSSSGGG GGGGGGSNSS SSSSTFNPQA DTGEQPYEHL
101 TAESFPDISL NNEKVLVETS YPSQTTRLPP ITYTGRFSLE PAPNSGNTLW
151 PEPLFSLVSG LVSMTNPPAS SSSAPSPAAS SASASQSPPL SCAVPSNDSS
201 PIYSAAPTFP TPNTDIFPEP QSQAFPGSAG TALQYPPPAY PAAKGGFQVP
251 MIPDYLFPQQ QGDLGLGTPD QKPFQGLESR TQQPSLTPLS TIKAFATQSG
301 SQDLKALNTS YQSQLIKPSR MRKYPNRPSK TPPHERPYAC PVESCDRRFS
351 RSDELTRHIR IHTGQKPFQC RICMRNFSRS DHLTTHIRTH TGEKPFACDI
401 CGRKFARSDE RKRHTKIHLR QKDKKADKSV VASSATSSLS SYPSPVATSY
451 PSPVTTSYPS PATTSYPSPV PTSFSSPGSS TYPSPVHSGF PSPSVATTYS
501 SVPPAFPAQV SSFPSSAVTN SFSASTGLSD MTATFSPRTI EIC
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Motifs with high probability

Motifs with low probability

Overlapping Motifs

No.	Pos.	Group	Score
1	K5	MAA AKAE MQLMS	0.79
2	K244	PAYPA AKGG FQVPM	0.62
3	K34	TMDNY PKLE EMMLL	0.61
4	K293	TPLST IKAF ATQSG	0.59

No.	Pos.	Group	Score
5	K323	KPSRM RKYP NRPSK	0.34
6	K366	RIHTG QKPF QCRIC	0.15
7	K394	RTHTG EKPF ACDIC	0.15
8	K272	LGTPD QKPF QGLES	0.15

Figure S4. For Figure 3A, EGR1 band with two different exposures (1 min and 5 min) showed that at least the amount of the 80-kD band in K272R-EGR1 is equal to the amount of the 80-kD band in WT-, or K34R-, or K317R- or k366R-EGR1, therefore the change in sumo form is not due to change in total amount.

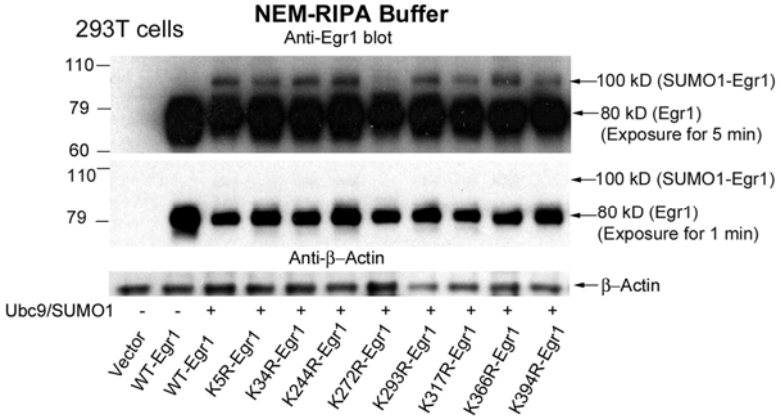


Figure S5. For Figure 4A, EGR1 band with two different exposures (1 min and 5 min) showed that the amounts of 80-kD bands are almost equal, showing that change in sumo form is not due to change in total amount.

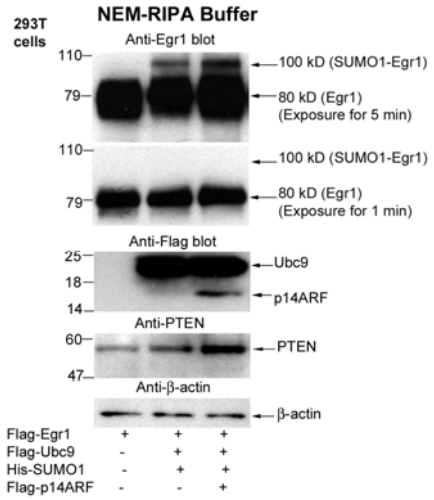


Figure S6. Immunoblots for EGR1, EGR1 in SUMO immunoprecipitates, ARF, PTEN, and β-actin (as a loading control) from *ARF*$^{-/-}$ and *ARF*$^{+/+}$ mouse embryonic fibroblasts given 5 Gy of γ-irradiation for the indicated times. In wild-type mouse embryonic fibroblasts (passage 6) given 5 Gy of γ-irradiation, the 100-kDa sumoylated EGR1 appeared with a time-course that closely matched that of ARF induction (lanes 5-8). In *ARF*$^{-/-}$ embryonic fibroblasts, this upper band of EGR1 was absent and PTEN levels remained low (lanes 1-4) thereby increasing Akt1 phosphorylation. Thus, it seems that EGR1 sumoylation in cells depends on the presence of ARF and that induction of PTEN depends on this modification of EGR1.

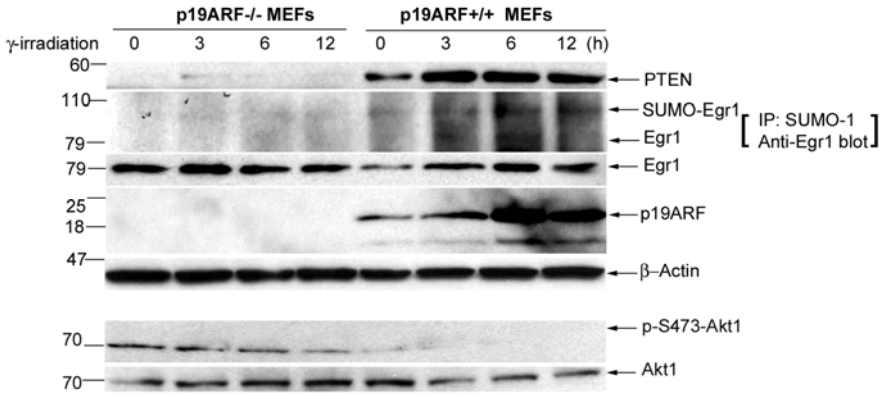


Figure S7. For Figure 5C, EGR1 and p14ARF location as a function of time after addition of IGF-1. All individual stains and merged are shown below. Under no treatment of LY294002, upon addition of IGF-1, EGR1 is largely cytoplasmic at t=0, but almost exclusively nuclear at 3, 6, 12h; and EGR1 was mostly located in the nucleus, but also located in the cytoplasm at 12-24h. In contrast, under the treatment with LY294002, it appeared that EGR1 located in both nucleus and cytoplasm all the time.

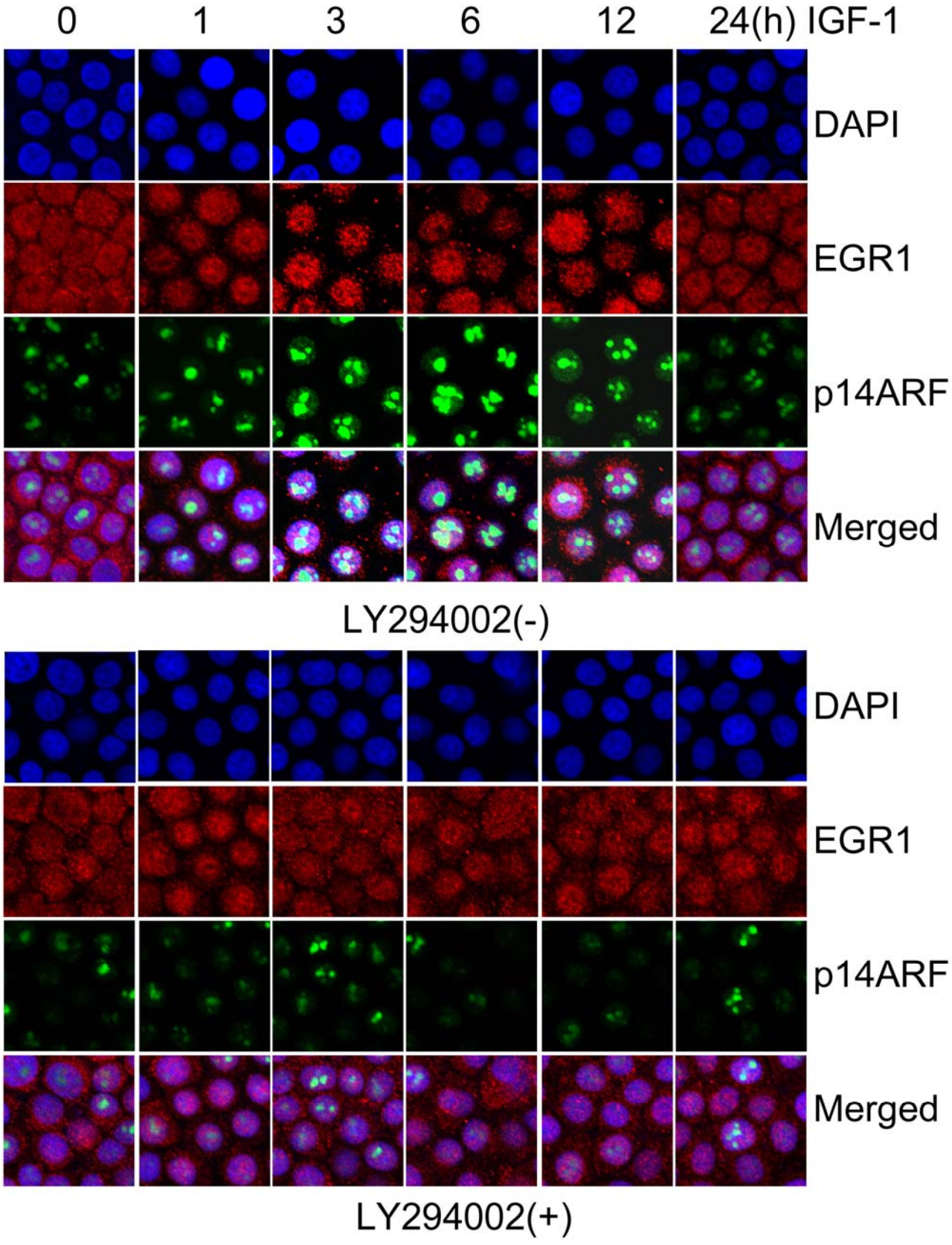
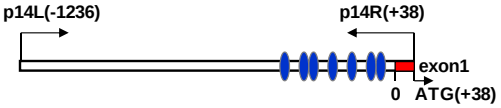


Figure S8. Structure of the *p14ARF* gene promoter and its regulation by EGR1.

(A) The highest-affinity binding sites for EGR1 in the *p14ARF* regulatory regions are marked ebs1-7. The numbers indicate the position relative to the start of transcription. The *p14ARF* regulatory fragment was amplified with primers p14L and p14R from genomic DNA and cloned into pGL3-Basic.



ebs1, -238 CGCCCGCGC –230; ebs2, -210 GTGGGGGTG–202; ebs3, -204 GTGGGGGTG –196;
ebs4, -139 CGCCCGCGC –131; ebs5, -43 GTGGGGGTG –35; ebs6, -32 GTGGGGGTG –24;
ebs7, -26 GTGGGGGTG –18;

PRIMER: p14L, GGTaccgcgc tcaggacccg gctcctccgc attctccggc tgc
p14R, aagcttCTCC GCCCCGCAGG CGCGCACCCG CCTTCCCTGA GCG

(B) pGL3-Basic and pGL3-p14ARF were transiently transfected into 293T cells with or without EGR1, or WT1 (Wilms Tumour protein 1) and WT1/EGR1 (EGR1 dominant-negative construct, Drummond *et al*, 1992; Rauscher, 1993; Yu *et al*, 2004) constructs. The activities of p14ARF promoter were assayed 24h after transfection with the Dual-Luciferase Reporter Assay System as relative luciferase activity (fold change). The results showed EGR1 strongly induced p14ARF promoter-luciferase activity, while WT1 strongly decreased luciferase as did the EGR1 dominant-negative construct WT1/EGR1, suggesting that EGR1 is the agent responsible for up-regulating p14ARF promoter and that preventing activated EGR1 from binding blocks this effect.

Note: Wilms Tumour protein 1, a transcription factor which binds to the DNA sequence as EGR1, and frequently activates or suppresses genes with reverse effects to EGR1, and may therefore compete with EGR1.

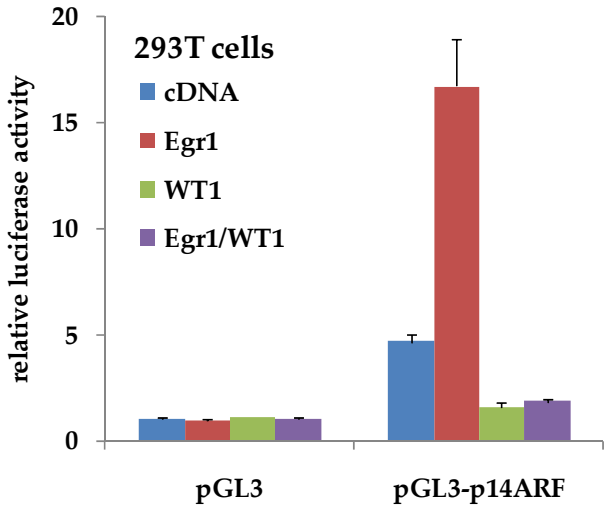


Figure S9. Akt1 phosphorylated the central M portion (aa 274–421) of EGR1. Autoradiogram of the ^{32}P-labeled GST-EGR1 proteins phosphorylated by Akt1 kinase.

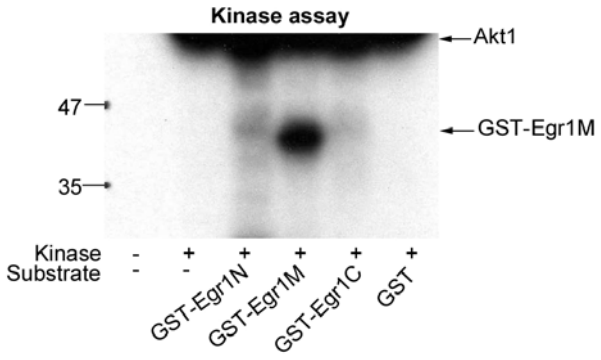


Figure S10. Identification of T309 as a phosphorylation site for Akt in EGR1 by LC-MS/MS. MS/MS Spectrum of the m/z 893.462+ ion from GST-EGR1M Akt1-phosphorylated. MS/MS spectrum of the precursor ion that corresponds to the tryptic phosphopeptide containing phospho-T309. The y-series ion defining the phosphorylated T is in red.

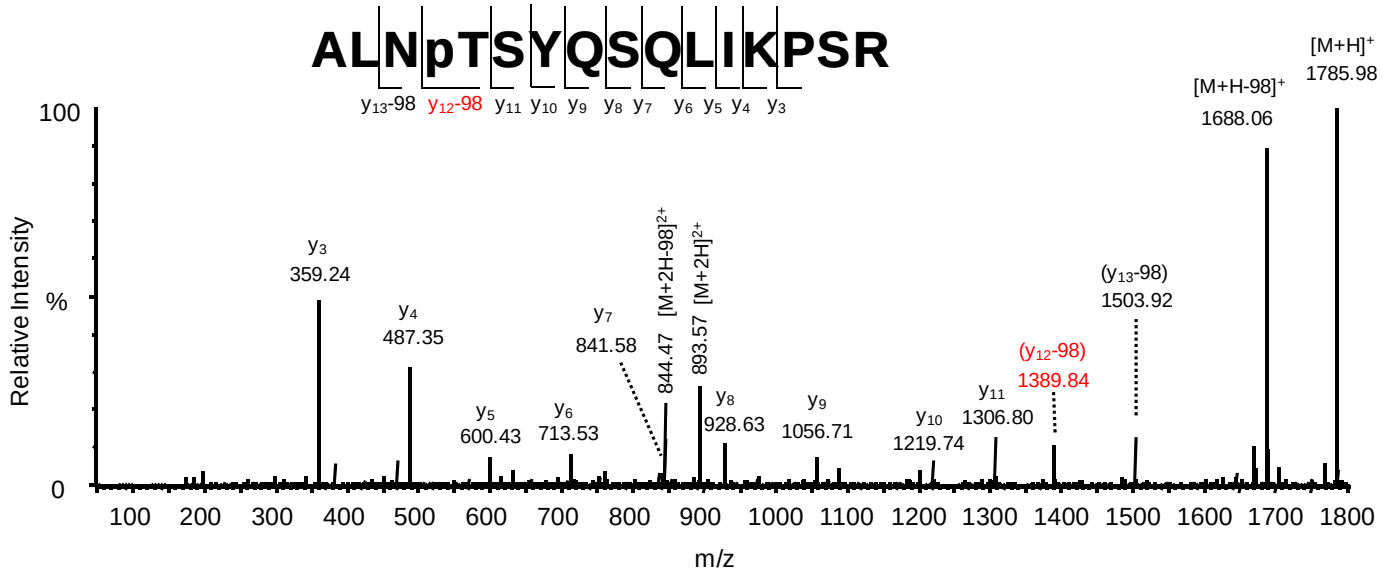


Figure S11. Verification that two Akt1-phospho-specific antibodies pT309 and pS350 of EGR1 reacted specifically. We used phosphopeptides corresponding to phospho-S350 (pS350) and pT309 for immunization of rabbits, followed by negative and positive affinity purification (service by QCB, San Diego, CA). Western blot of 150 ng of GST or GST-EGR1M proteins incubated without (lanes 1-5) or with (lanes 6-10) Akt1 kinase. The phospho-specific antisera were used at 1:2,000 and the blot developed by ECL. Of the resulting antisera, anti-pS350 was specific (**lower panel**): the antibody reacted with GST-Egr1M WT and GST-Egr1M T309A phosphorylated by Akt1, but did not react with GST-Egr1M S350A phosphorylated by Akt1, nor with any of these proteins without Akt1 or GST alone. The anti-pT309 also recognized the Akt1-phosphorylation of EGR1 at T309 (**upper panel**).

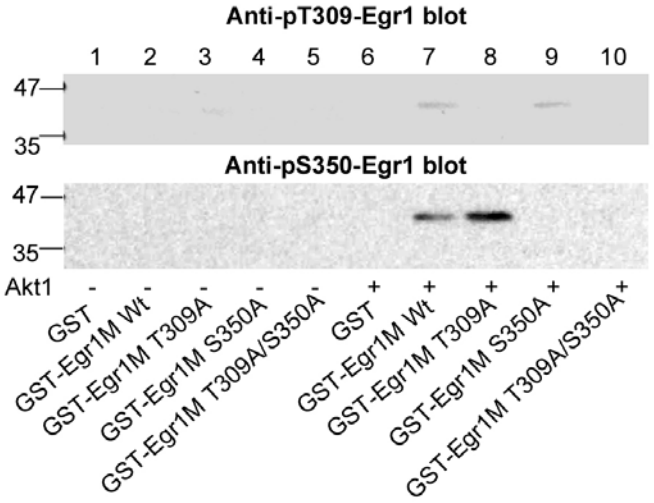


Figure S12. Defective *PTEN* induction by S350A or T309A or T309A/S350A mutated EGR1. *PTEN*-luc construct in the pGL3 vector was transfected into 293T cells together with empty pCMV-Tag2 vector (first column) or together with the wild-type or the indicated point-mutants of EGR1. Luciferase activity was measured 24 h later.

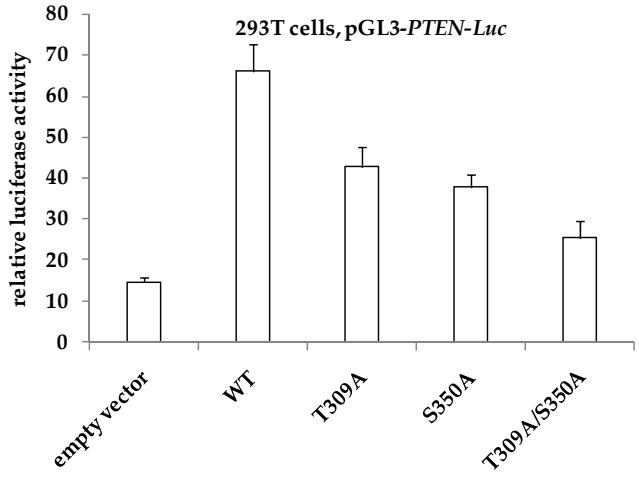
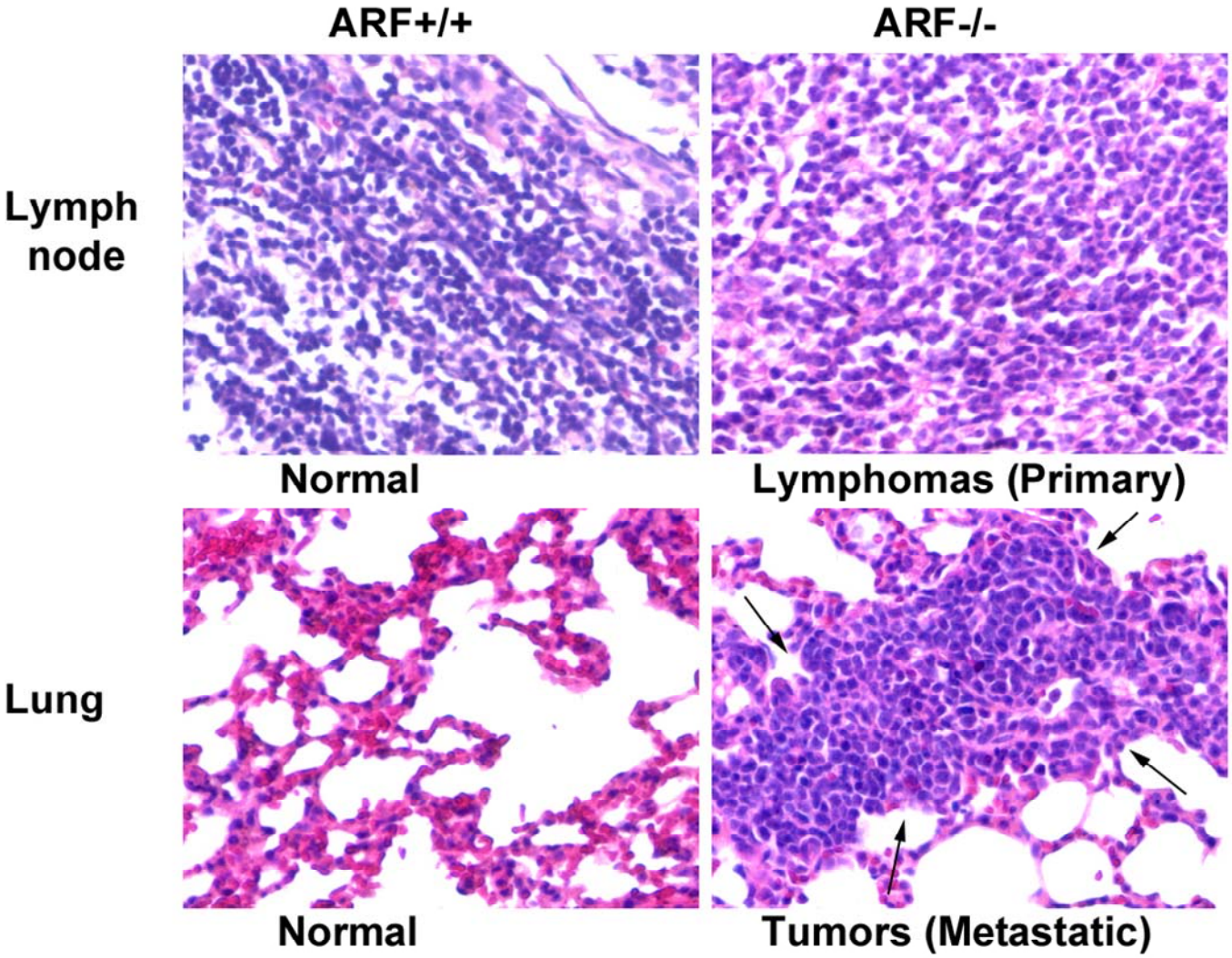


Figure S13. Lymphoma histology. Upper panel, sections from an *ARF*$^{-/-}$ mouse lymphoma (primary tumours) show proliferating lymphoblastomas, compared with sections from an *ARF*$^{+/+}$ mouse normal lymph node. Middle panel, sections from the same *ARF*$^{-/-}$ mouse show lung metastases from lymphoma (arrows), compared with sections of normal lung tissues from the same *ARF*$^{+/+}$ mouse. Lower panel, inguinal mammary glands from the same *ARF*$^{-/-}$ mouse were stained with carmine. Scale bar: 200 μM.



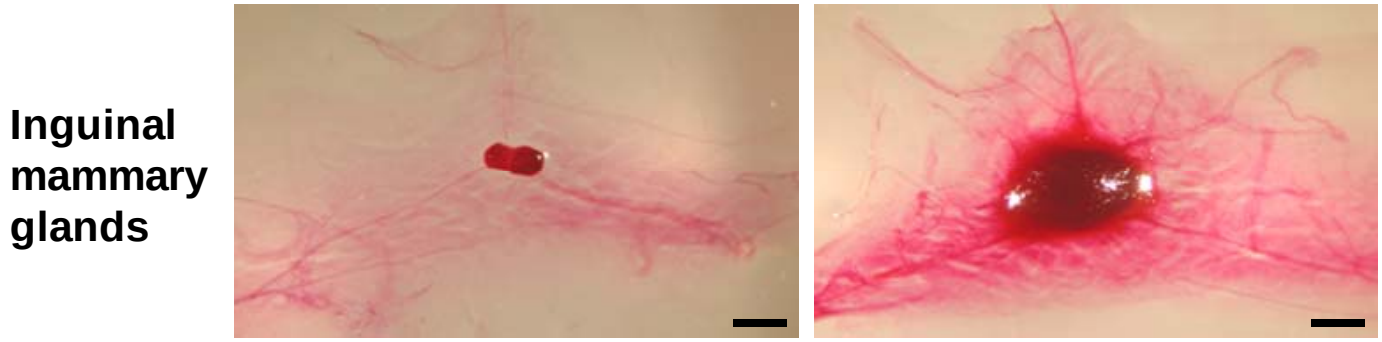


Figure S14. Muscle tumour histology. Upper panel, sections from an *ARF*$^{-/-}$ mouse primary muscle tumour. Lower panel, sections from the same *ARF*$^{-/-}$ mouse show lung metastases from muscle tumours.

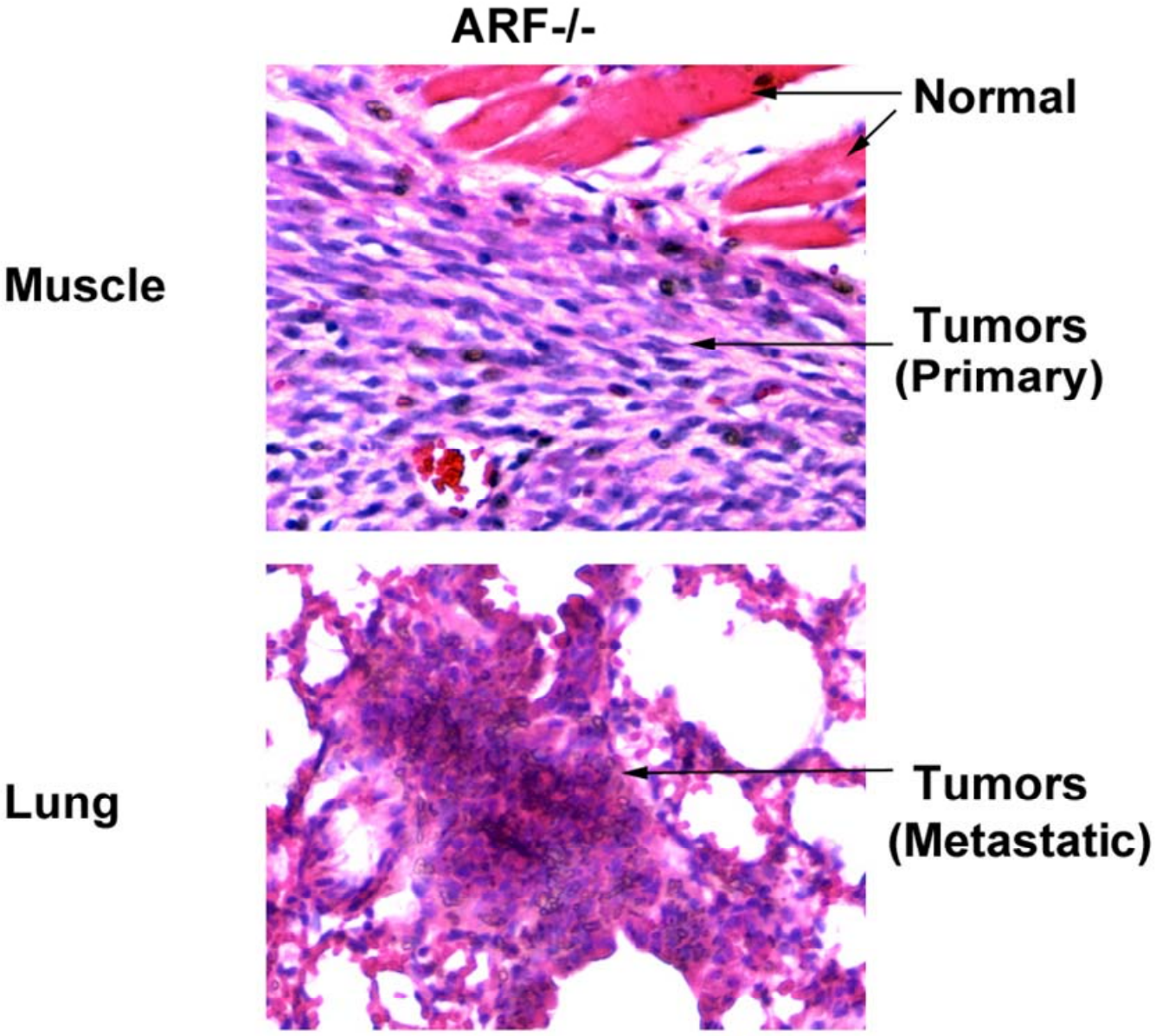


Figure S15. Immunohistochemical analysis of PTEN expression in normal lymph, and *ARF*$^{-/-}$ lymphoma reveals low expression of PTEN in lymphoma. Sections from the same *ARF*$^{+/+}$ and *ARF*$^{-/-}$ tissues as in **Figure S8** were immunostained with antibody PTEN (green) and DAPI (blue).

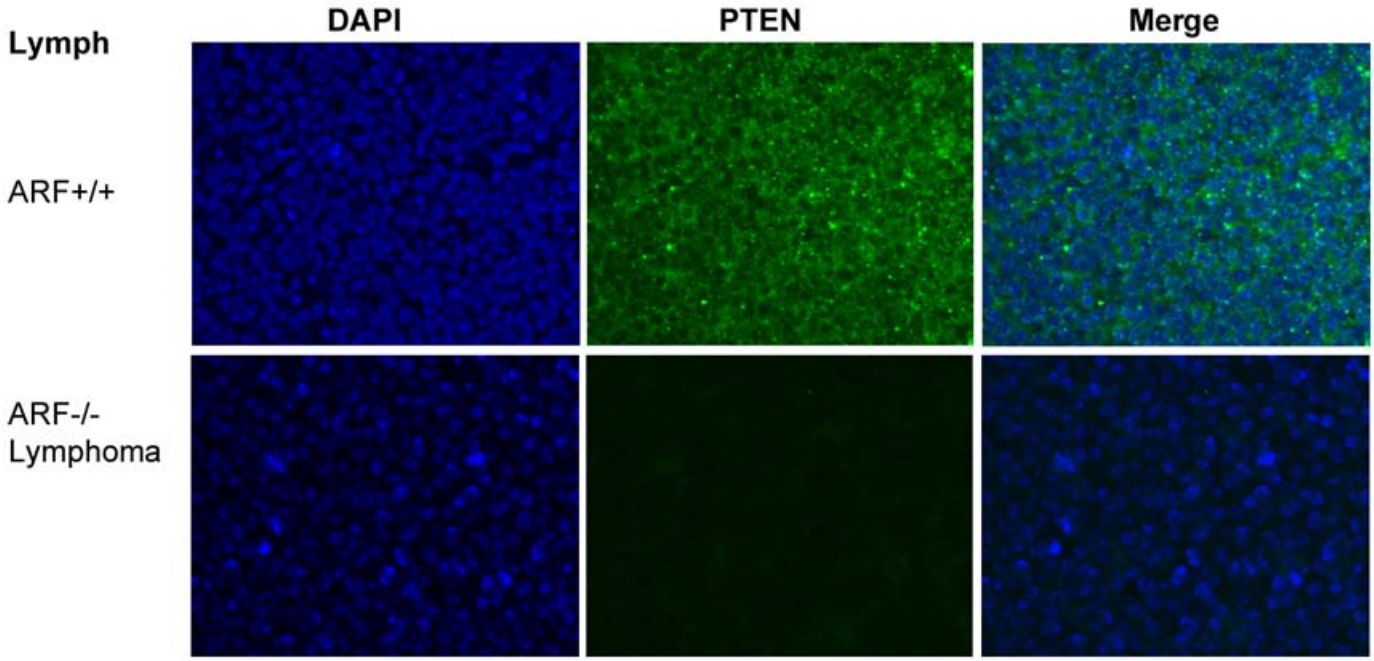
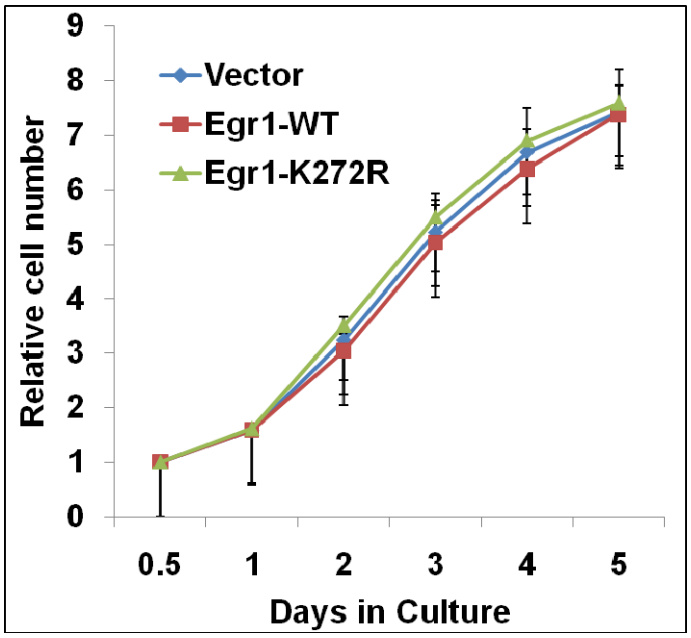


Figure S16. *ARF*$^{-/-}$ MEFs infected with pBabe-PuroL-EGR1(WT), pBabe-PuroL-EGR1(K272R), pBabe-PuroL vector were seeded on plates at 4~5×10^3 cells in 96-well plates and cell numbers were determined by CyQUANT fluorescence assay at 0.5, 1, 2, 3, 4, 5 days after seeding. The cell numbers were normalized against the value at 0.5 day (Mean values±SEM, from three independent experiments, *P<0.05, **P<0.01, ***P<0.001). Expression of either EGR1-WT or EGR1-K272R in *ARF*$^{-/-}$ MEFs did not significantly affect either cell proliferation or saturation density, suggesting that p19ARF is necessary for the effect of EGR1.



References

1. Drummond, I.A., Madden, S.L., Rohwer-Nutter, P., Bell, G.I., Sukhatme, V.P., and Rauscher, F.J.I. Repression of the insulin like growth factor II gene by the Wilms tumor suppressor WT1. Science *257*, 674–678 (1992).
2. Hamilton, T.B., Borel, F., and Romaniuk, P.J. Comparison of the DNA binding characteristics of the related zinc finger proteins WT1 and EGR1. Biochemistry *37*, 2051–2058 (1998).
3. Kamijo, T. et al., Tumor spectrum in ARF-deficient mice. *Cancer Res* 59 (9), 2217 (1999).
4. Kasper, L.H., Boussouar, F., Ney, P.A., Jackson, C.W., Rehg, J., van Deursen, J.M., and Brindle, P.K. A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. Nature 419, 738–743 (2002).
5. Luo, K., Hurley, T. R., and Sefton, B. M. *Oncogene* 5, 921-923 (1990).
6. Yu, J. et al., A network of p73, p53 and Egr1 is required for efficient apoptosis in tumor cells. *Cell Death Differ* 14 (3), 436-446 (2007).
7. Yu, J., de Belle, I., Liang, H., and Adamson, E. D., Coactivating factors p300 and CBP arr transcriptionally crossregulated by Egr1 in prostate cells, leading to divergent responses. *Mol Cell* 15 (1), 83-94 (2004).