Supplementary Methods

Cell line, cell culture, drug treatment and animals

Human embryonic lung diploid fibroblast 2BS cells (obtained from the National Institute of Biological Products, Beijing, China) were isolated from female fetal lung fibroblast tissue (Tang et al, 1994). Young 2BS cells are defined as having completed < 30 PD, while replicative senescent 2BS cells are defined as having completed > 55PD. 2BS, HeLa, MCF-7, and the packaging cell line Phoenix and 293T were grown in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO BRL, USA) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. The IMR90 strain of fetal lung fibroblasts transduced with a tamoxifen-regulated form of activated H-Ras (Tarutani et al, 2003) was a gift from Dr. Masashi Narita (Cancer Research UK, Cambridge Research Institute). IMR90 cells for tamoxifen treatment are grown in phenol red-free Eagle's minimal essential medium (HyClone, Logan, UT) supplemented with 10% dextran-charcoal-stripped fetal bovine serum (HyClone). DZNep (National Cancer Institute, Bethesda, MD, USA) was used at the concentration of 5µM. For SNP experiment, two B-lymphocytic cell lines with the ID number GM10851 (carrying C/C) and GM10860 (carrying T/T) were purchased from Coriell Cell Repository (CCR, USA). These cells were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum at 37°C in 5% CO₂. BALB/c mice were maintained in a certified animal facility in accordance with the guidelines set forth by the Peking University Animal Ethics Committee.

Plasmids, viruses and infections

Retroviral vector WZL-H-Ras V12, mir30-shSUZ12, mir30-shEZH2, mir30-shp16INK4a and the empty vectors were kindly provided by Dr. Masashi Narita. Human FOXA1 was subcloned into the pQCXIN retroviral vector and pLenti6/V5-DEST lentiviral vector respectively. FLAG-tagged FOXA2 was subcloned into pQCXIN. Retroviral construct of pBabe-BRAF V600E was a gift from Dr. Mooi. Lentiviral shRNA constructs for RNAi based FOXA1 knockdown were
hosted in lentiviral vector pLL3.7 with the following targeting sequences #1: GCGTACTACCAAGGTGTGTAT; #2: GTATTCCAGACCCGTCCTAAA. The full-length FOXA1 or N-term, DBD, C-Term and ΔC truncated mutants for luciferase reporter assay were generated by PCR and cloned into pcDNA3.1. The pSIR-tgp16INK4a-T and -C constructs hosted in a self-inactivating retroviral vector (pSIR) were assembled as illustrated in the Figure 7D (top panel). Retroviral and lentiviral-mediated gene transfers were performed using the Phoenix packaging cells and 293T as described previously (Beausejour et al, 2003; Narita et al, 2003).

**Western blotting analysis and antibodies**

Western blotting assay was performed as described previously (Cao et al, 2011; Zhou et al, 2009). The following antibodies were used in this study: anti-FOXA1 (Abcam, ab23738 and ab5089); anti-SUZ12 (Millipore, 07-379) and anti-EZH2 (BD Biosciences, 612667); anti-pRb (Cell Signaling Technology Inc); anti-Flag (Sigma, M2); anti-BRAF (Epitomics, 1647-1); anti-p16INK4a (C-20, sc-468), anti-H-Ras (F235, sc-29), anti-p27kip1 (C-19, sc-528), anti-GAPDH (0411, sc-47724) and anti-β-actin (I-19, sc-1616) were all purchased from Santa Cruz Biotechnology.

**Co-immunoprecipitation Assays**

Cells were collected and lysed on ice with lysis buffer containing 0.5% NP40. The lysates were pre-cleared by incubation with protein A Sepharose beads (Sigma). The protein complex was then precipitated by a specific antibody together with protein A Sepharose beads followed by extensive washing. The resulting materials were analyzed by western blotting.

**Baculovirus production and generation of FOXA1 protein**

The baculovirus construct was generated by insertion of the open reading frame of human FOXA1 into the pFastBac HT A vector (Invitrogen), which contained a C-terminal FLAG tag for further affinity purification. The virus was generated and amplified according to the manufacturer's protocol. To purify FOXA1 proteins, sf9
cells were infected at a multiplicity of infection of 10 with virus expressing FLAG-FOXA1. Cells were harvested after 3 days and lysed by sonication, and the lysate was incubated for 4 h with M2 agarose beads (Sigma). Washes were performed with BC500 buffer containing 50 mm Tris, 2 mm EDTA, 500 mm KCl, 10% glycerol, and protease inhibitors. Proteins were eluted with FLAG peptide at a 0.2 mg/ml concentration. FOXA1 protein was generated by co-infecting sf9 cells with virus expressing FLAG-tagged FOXA1 (Zhang et al, 2011).

**Quantitative RT–PCR (RT-qPCR)**

Total cellular RNAs were isolated with the RNeasy kits (Qiagen) and used for first strand cDNA synthesis with the Reverse Transcription System (Promega, A3500). Quantitation of all gene transcripts was done by quantitative PCR using Power SYBR Green PCR Master Mix and an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) with the expression of GAPDH as the internal control. The quantitative results were presented as mean±sd for triplicated experiments.

Primers used for RT-qPCR are listed below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAAGGTGAAAGGTCGGAGTC</td>
<td>GAAGATGGTGATGGGATTTTC</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>GAAGGTCCCTCAGACATCCCC</td>
<td>CCCTGTAGGACCTTCGGTGAC</td>
</tr>
<tr>
<td>FOXA1</td>
<td>CATCACCATGGCCATCCA</td>
<td>GGTCCATGATCCACTGGTAGATC</td>
</tr>
<tr>
<td>p27kip1</td>
<td>CATTGGTGGAACCAAGAC</td>
<td>TGCAGTGTCGCTCCCTTATTC</td>
</tr>
<tr>
<td>PCNA</td>
<td>AGCACAAAACCAGAGGAAG</td>
<td>CGTGCAAATTCCACAGAAGG</td>
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<tr>
<td>p14ARF</td>
<td>CCCCCCTGCTGATGCTACTG</td>
<td>ACCCTGGTCTTCTAGGAACGG</td>
</tr>
<tr>
<td>p15INK4b</td>
<td>CTTCCATGGATGCAACAAG</td>
<td>GCAATGGGAAGAAGCAAG</td>
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<tr>
<td>p18INK4c</td>
<td>GGACCACCTAGTCCCTTTC</td>
<td>TTTAGGGTCCCTGTCACAG</td>
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<td>p19INK4d</td>
<td>CCAAGGGCAGAGCATTTAAG</td>
<td>AAGCAACGTGCAACTTCAG</td>
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<td>p53</td>
<td>ACCCATCCACTACAACATCAT</td>
<td>CACAAACACGCACCTCACA</td>
</tr>
<tr>
<td>PTEN</td>
<td>TCACCAACTGAAGTGGCTAAAGA</td>
<td>CTCCATTCCTCCATAACCG</td>
</tr>
<tr>
<td>EZH2</td>
<td>GGAGCACATTTAAAATGTCCTTC</td>
<td>TGCACAGCAATAGTGCCTTT</td>
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</tbody>
</table>

Primers for determination of Forkhead family genes:

<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXA2</td>
<td>CCGCCCTACTCGTACATCTC</td>
<td>CGGTAGAAGGGAAGAGGTC</td>
</tr>
<tr>
<td>FOXA3</td>
<td>TCAACGACTGCTTCTGCAAG</td>
<td>CAAAACATGTCCCTCGAGCT</td>
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<tr>
<td>FOXB1</td>
<td>AGTTTCATCATGGACCCGCTTC</td>
<td>AGCAGTGGTGAGGAGAG</td>
</tr>
<tr>
<td>FOXB2</td>
<td>CGCTGAGCGACATCTACAAG</td>
<td>AGCAGTGGTGAGGAGAG</td>
</tr>
</tbody>
</table>

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed according to the protocol described previously (Shang et al, 2000). The precipitated DNA was quantified by realtime PCR with the
results presented as mean+sd for triplicate experiments. The antibodies used in ChIP were as following: anti-histone H1 (AE-4, sc-8030) and anti-sp1 (IC6, sc-420) were purchased from Santa Cruz Biotechnology; anti-histone H3 (abcam, ab1791); anti-EZH2 (17-662), anti-H3K27Me3 (07-449), anti-EED (17-663) were purchased from Millipore. The primers used in ChIP-qPCR analysis of INK4 loci as shown in Figure 3A, was reported previously (Bracken et al, 2007). The other primers for ChIP-qPCR analysis were listed below:

Primers for analysis of FOXA1 promoter:

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
</table>
| a  | AGACCTTTGTGGGATAA
tGACC | AGGAGGGAGGAAGC|AAAGAG |
| b  | AGCGACCAAAAAAGGAAGA
ga | CTCACGCTACTCCCTGAAG |
| c  | TCTTCC
cAACGC| AAAAAACACACGCACCAC|
| d  | CCAGCCTCTCTCCCATCTCA
g | GAAAGTCGACTGCGCTGCA|

Primers for analysis of H3 and H1 occupancy at p16\(^{INK4a}\) promoter:

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
</table>
| -300bp | ACCCGCATTC
cAAATTTGGCAG | AAAAAGAGAAA|
| -1kb  | CTCACCGGGATAA
tCAAGG | AAGCCTTAGAAGACGTC|
| +1kb  | ATCGTGAGC
gGTAAGGTAAG | ATCACA|

Primers for analysis of FOXA1 occupancy on the putative enhancers:

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
</table>
| 1  | TGGCCC
gTTTGAG | ATCCTGACT|
| 2  | AAGGGCAG
gAAGA | CAGGTGTGAA|
| 3  | GCCCTGATA
cAACACTTTC | GGGAGGT|
| 4  | CACAGAAT
cGCCAAGGA | AACGCAG|
| 5  | GAGGAA
tGGCAGGATTG | TGGG|
| 6  | GCCATCTG
tGTTGAG | TCTCC|
| 7  | ATTACTGAT
gGGGGT | CCGT|
| 8  | AATATCCGA
tGGGCAAGGA | TGGAGG|
| 9  | AGAGCAT
tGCTCAGAG | AAGG|
| 10 | TCATGTTG
tGTAAGGATG | GCCAGG|
| 11 | ACAGGGTGG
tAGGCTG | CCTG|
| 12 | ACCTAAACAC
tCAGATC | ATAACCCG|
| 13 | TACATCCGC
tAGGAAAAG | AGGCTGAT|
| 14 | TGGAAGGT
tGGAGAATG | GAAGG|
| 15 | CAGTTCT
tGCAAGT | CGAG|
| 16 | TTCCTGTGTG
tGGGGATTAG | TTTT|

Luciferase activity assay

The luciferase reporters were all constructed in the pGL3 basic vector. The reporters were transfected in 24-well plates using lipofectamine 2000 (Invitrogen). 48 h post-transfection, the cells were harvested and luciferase activity was measured with a dual luciferase kit (Promega) according to the manufacturer’s protocol. The quantitative results were presented as mean±sd for triplicated experiments.

EMSA

Biotinylated probes and cold competitors were synthesized commercially. The assay was performed using gel shift assay systems from Promega with purified FOXA1 protein or nuclear lysate from 2BS cells following manufacturers’ standard protocol. The sequences of probes synthesized for EMSA were listed as following:

Wild-type p16\textsuperscript{INK4a} promoter:
TCCTAACTGCAATCCGCGCTTTCGTCTCAGAAGT
TGGCTTCTT

Mutant p16\textsuperscript{INK4a} promoter:
TCCTAACTGCAATCCGCGCTTTCGTCTCAGAAGTATGGCTTCTT

SNP probe with T:
CAGCTCACCTCCAGCTTTTAGTTCATGACAGTATGGCTTCC

SNP probe with C:
CAGCTCACCTCCAGCTTTAGTTTTCCCATGACAGT
AAGTCTATTACCCTCC

**RNAi**

Chemically synthesized double-stranded siRNA was used against the transcript of FOXA1 (Carroll et al, 2005). Cells were transfected with 50 nm small interfering RNA oligonucleotides for 72 h using Lipofectamine RNAiMAX (Invitrogen). The siRNA sequences were as following: siFOXA1, GGACUUCAAGGCAUACGAA; and siNS, UUCUCCGAACGUGUCACGU.

**Micrococcal nuclease (MNase) mapping assay**

Collected cell pellets were washed with PBS buffer and then resuspended in hypertonic buffer A (300 mM sucrose, 2 mM Mg acetate, 3 mM CaCl₂, 10 mM Tris [pH 8.0], 0.1% Triton X-100, and 0.5 mM DTT) followed by a 5-min incubation on ice, and homogenization with a 2 ml dounce homogenizer for 20 times. Nuclei were collected by centrifuging at 4°C for 5 min at 720g. The pellets were then washed twice in buffer A and then resuspended in buffer D (25% glycerol, 5 mM Mg acetate, 50 mM Tris [pH 8.0], 0.1 mM EDTA, 5 mM DTT). The resultant chromatin was collected, washed and then resuspended in buffer MN (60 mM KCl, 15 mM NaCl, 15 mM Tris [pH 7.4], 0.5 mM DTT, 0.25 M sucrose, 1.0 mM CaCl₂). Diluted MNase (USB) in buffer MN, was then added with 0, 0.1, 1, 10, and 100 units used per reaction followed by incubated at room temperature for 30 min. The reactions were stopped with the addition of EDTA and SDS to the final concentrations of 12.5 mM and 0.5%, respectively. DNA was then subjected to 0.8% agarose gel for visualization and the mono-nucleosome sized fragments were recovered by QIAquick columns (Qiagen) and subjected to quantitative PCR. The quantitative results were presented as mean±sd for triplicated experiments. Primers used in this experiment are listed below:

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GCCCCACCGAGAATCGAA</td>
<td>GGTTTCTGACTTAGTGAACCC</td>
</tr>
<tr>
<td>2</td>
<td>GCAAAACCTATTTCCTTCTAGTTGTA</td>
<td>GAAAATCAAGGGTTGAGGGG</td>
</tr>
<tr>
<td>3</td>
<td>GAAAGTATGGCTTCTTCTTTAATC</td>
<td>TTTCTAGTCGTACAGGTGATTTCG</td>
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</table>
DNase I accessibility assay

Nuclei for DNase I accessibility assay were prepared by incubating cells on ice in lysis buffer (10 nM Tris at pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.3% NP40) for 5 min. The nuclei were then pelleted and washed in the lysis buffer without detergent. Two micrograms of DNA were then partially digested for 3 min at 37°C in a total volume of 200 μL with 2 U of DNase I. The reaction was stopped by addition of 50μL of 0.5 M EDTA. The resultant DNA was then purified using the DNeasy genomic DNA preparation kit (Qiagen) and analyzed by realtime PCR. The relative DNase I accessibility at a given region was measured as the ratio of the amount of digested DNA to the undigested control. The quantitative results were presented as mean±sd for triplicated experiments. Primers used in this experiment are listed below:

<table>
<thead>
<tr>
<th>Forward primer</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1 CACCGAGAATCGAAATCACC</td>
<td>CCGACTCTCCAAAAGGAATC</td>
</tr>
<tr>
<td>2 AGTATGGCTTCTTCTTCTTCTAATCATAAGA</td>
<td>GAAAAATCAAGGGTTGAGGGG</td>
</tr>
<tr>
<td>3 GCGATACAACCTCTTCCTACTCACTGC</td>
<td>TCCCTATGACAACAAACCAC</td>
</tr>
<tr>
<td>4 GGCTCCTCCCCACCTG</td>
<td>CAGAGTGAAACGCACCTCAACAC</td>
</tr>
<tr>
<td>5 CACCGAGCGAGAAGCAG</td>
<td>GGCAAGGGTTGGGAGGAG</td>
</tr>
<tr>
<td>6 GAGTGCTCGAGGAGGTTG</td>
<td>CTGCTCAGGGTCTG</td>
</tr>
<tr>
<td>7 GACCGCGTGATCTTCTTCCAG</td>
<td>AACCTGGAATGGGCAGCTTCAG</td>
</tr>
<tr>
<td>8 AGCCCCCTCTCTCCTTCG</td>
<td>CTTGCGCTTTGAAAGATACC</td>
</tr>
</tbody>
</table>

DNase I accessibility assay for nuclei prepared by incubating cells on ice in lysis buffer (10 nM Tris at pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.3% NP40) for 5 min. The nuclei were then pelleted and washed in the lysis buffer without detergent. Two micrograms of DNA were then partially digested for 3 min at 37°C in a total volume of 200 μL with 2 U of DNase I. The reaction was stopped by addition of 50μL of 0.5 M EDTA. The resultant DNA was then purified using the DNeasy genomic DNA preparation kit (Qiagen) and analyzed by realtime PCR. The relative DNase I accessibility at a given region was measured as the ratio of the amount of digested DNA to the undigested control. The quantitative results were presented as mean±sd for triplicated experiments. Primers used in this experiment are listed below:
**Bioinformatics analysis**

FOXA1 binding motif was predicated through JASPAR database with default threshold (http://jaspar.genereg.net). Multiply sequence alignment and phylogenetic tree comparison of FOXA1 binding site at the potential enhancer element -150kb away from p16\(^{\text{INK4a}}\) TSS were performed using MulAlin and MEGA5 respectively with default settings (Corpet, 1988; Tamura et al, 2011). Expression levels of FOXA1 in human cancer were obtained from data sets collected in Oncomine portals (Buchholz et al, 2005; Chen et al, 2003; Kaiser et al, 2007; Pomeroy et al, 2002).

**Supplementary references**


by Lsh via recruitment of histone deacetylases in human diploid fibroblasts. *Nucleic acids research* **37**: 5183-5196
Supplementary Figure 1. (A) 2BS cells were transduced with retrovirus expressing FLAG-tagged FOXA1 or FOXA2, or a control vector. The resultant cell lysate was subjected to western blotting with indicated antibodies. (B) 2BS cells were infected with vector alone (V), or a combination of BRAF E600 (BRAF) and shRNA against FOXA1 (shF) or non-silencing control (shC). The cells were harvested for western blotting with indicated antibodies (left) and RT-qPCR analysis to determine the mRNA level of p16\textsuperscript{INK4a}.
Supplementary Figure 2. (A) Recombinant FOXA1 purified from *Spodoptera frugiperda* sf9 cells were analyzed by SDS-PAGE followed by coomassie blue staining (left panel). EMSA was performed using recombinant FOXA1 protein and biotin-labeled probes containing the predicated FOXA1 binding motif (right panel). Inclusion of bovine serum albumin (BSA) and increasing amounts of unlabeled wild-type probe (cold WT) or unlabeled mutant probe (cold MT) was indicated below. (B) Response of p16INK4a promoter to truncated FOXA1 mutants. Truncated FOXA1 was constructed as illustrated in the left panel. Western blotting analysis of FLAG-tagged FOXA1 truncations was performed with anti-FLAG antibody (middle panel). These constructs were co-transfected with p16INK4a promoter reporter in HeLa cells, and 48 hours after transfection, the relative luciferase activity was measured (right panel).
Supplementary Figure 3. (A) Profiling of the DNase I accessibility of p16\(^{\text{INK4a}}\) promoter in young and Ras-induced senescent IMR90 cells. DNase I accessibility assays were performed as described in Materials and Methods. qPCR primers used for determination of DNA accessibility were illustrated in the top panel. (B) Young 2BS cells were transduced with retrovirus expressing FOXA1 or its control vector. The resultant chromatin was extracted and subjected to ChIP analysis with antibody against linker histone H1 (upper panel). Middle-aged 2BS were transduced with two independent lentivirus expressing shRNA against FOXA1 (shF-1 and shF-2) or a non-silencing control. ~30 days post infection, the resultant chromatin was subjected to ChIP analysis with antibody against linker histone H1 (bottom panel).
Supplementary Figure 4. (A) Western blotting assay was performed in FOXA1- or control vector-infected 2BS cells with indicated antibodies. (B) Immunoprecipitation was performed in 2BS cells with antibodies against PRC2 component EZH2 (upper panel) or PRC1 component BMI1 (bottom panel). The precipitated complex was resolved on SDS-PAGE and blotted with indicated antibodies. The interaction between EZH2 and SUZ12 or BMI1 and CBX4 served as positive control.
Supplementary Figure 5. (A) Multiply sequence alignment of FOXA1 binding site at the potential enhancer element -150kb away from p16\textsuperscript{INK4a} TSS. Predicted FOXA1 binding motif was indicated by a box on the track of consensus nucleotides. (B) Phylogenetic tree based on the nucleotide sequences of the -150kb element as shown in (A).
Supplementary Figure 6. Illustration of the predicted FOXA1 binding site at the -150kb element from p16INK4a TSS. (A) The sequence logo and frequency matrix were retrieved from JASPAR database (http://jaspar.genereg.net). Predication of FOXA1 binding site on the -150kb element was performed using JASPAR analysis tools with the default relative profile score threshold. Aligned FOXA1 motif and rs10811661 DNA variant were shown below the sequence logo. (B) Sequencing results of rs10811661 surrounding region was shown and predicted FOXA1 binding motif was indicated. DNA variant was highlighted in GM10851 and GM10860 lymphocytes.
Supplementary Figure 7. FOXA1 down-regulation was associated with tumor progression. Differential mRNA expression of FOXA1 was analyzed with Oncomine database in (A) gastric adenocarcinoma, (B) desmoplastic medulloblastoma, (C) pancreatic ductal adenocarcinoma, (D) colon adenocarcinoma versus their normal tissue counterparts. The expression data are normalized and calculated as Log2 transformed median centered intensity. Data sets in a single panel were from the same study.