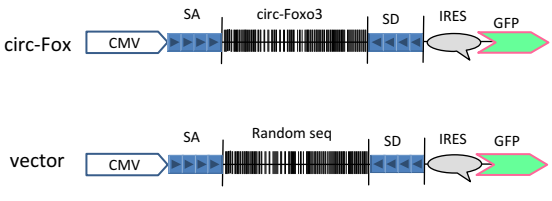
a



b

Primers used in this study

name	sequence
Cir.Foxo3-F	5’ gtggggaacttcactggtgctaag
Cir.Foxo3-R2	5’ ggg ttg atga tcc acc aag agc tctt
Foxo3-PCR-F	5’ gcaagagctcttggtggatcatcaa
Foxo3-PCR-R	5’ tgggg ct gcc agg ccac ttgg agag
Foxo3-F	5’ cttggacctggacatgttcaatgg
ectopic-Foxo3-R	5’ tttc ggatcc ttc agcc tgg cac c

c

Biotinylation probe
detecting circ-Foxo3.
B: biotinylation

5’ B aaaTGTGCCGGATGGAGTTCTGCTTTGCCCACTTC

Cy5 labelled probe
detecting circ-Foxo3.
Cy: Cy5 labelling

5’ Cy aaaTGTGCCGGATGGAGTTCTGCTTTGCCCACTTC

d

mouse
human

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Sbjct  118   AACTCCATCCGGCACAACCTGTCACTGCATAGTCGATTCATGCGGGTCCAGAATGAGGGA  177
Query  61    ACGGGCAAGAGCTCTTGGTGGATCATCAACCCCGATGGGGGAAAGAGCGGGAAGGCCCCC  120
Sbjct  178   ACTGGCAAGAGCTCTTGGTGGATCATCAACCCTGATGGGGGGAAGAGCGGAAAAGCCCCC  237
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Sbjct  238   CGGCGGCGGGCTGTCTCCATGGACAATAGCAACAAGTATACCAAGAGCCGTGGCCGCGCA  297
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Sbjct  298   GCCAAGAAGAAGGCAGCCCTGCAGACAGCCCCCGAATCAGCTGACGACAGTCCCTCCCAG  357
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Sbjct  358   CTCTCCAAGTGGCCTGGCAGCCCCACGTCACGCAGCAGTGATGAGCTGGATGCGTGGACG  417
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Sbjct  1498  CAGAGCTGGGTGCCAGGCTGAAGGATCACTGAGG-AAGGGGAAGTGGGCAAAGCAG  1552
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Fig S1. Structure and primers used in the study. (a) Structures of the circ-Foxo3 expression construct and the control vector. (b) Primers used in this study. (c) Probe sequences for binding assay. (d) The sequences of human circ-Foxo3 and mouse circ-Foxo3 are highly conserved, suggesting conservation in physiological functions.

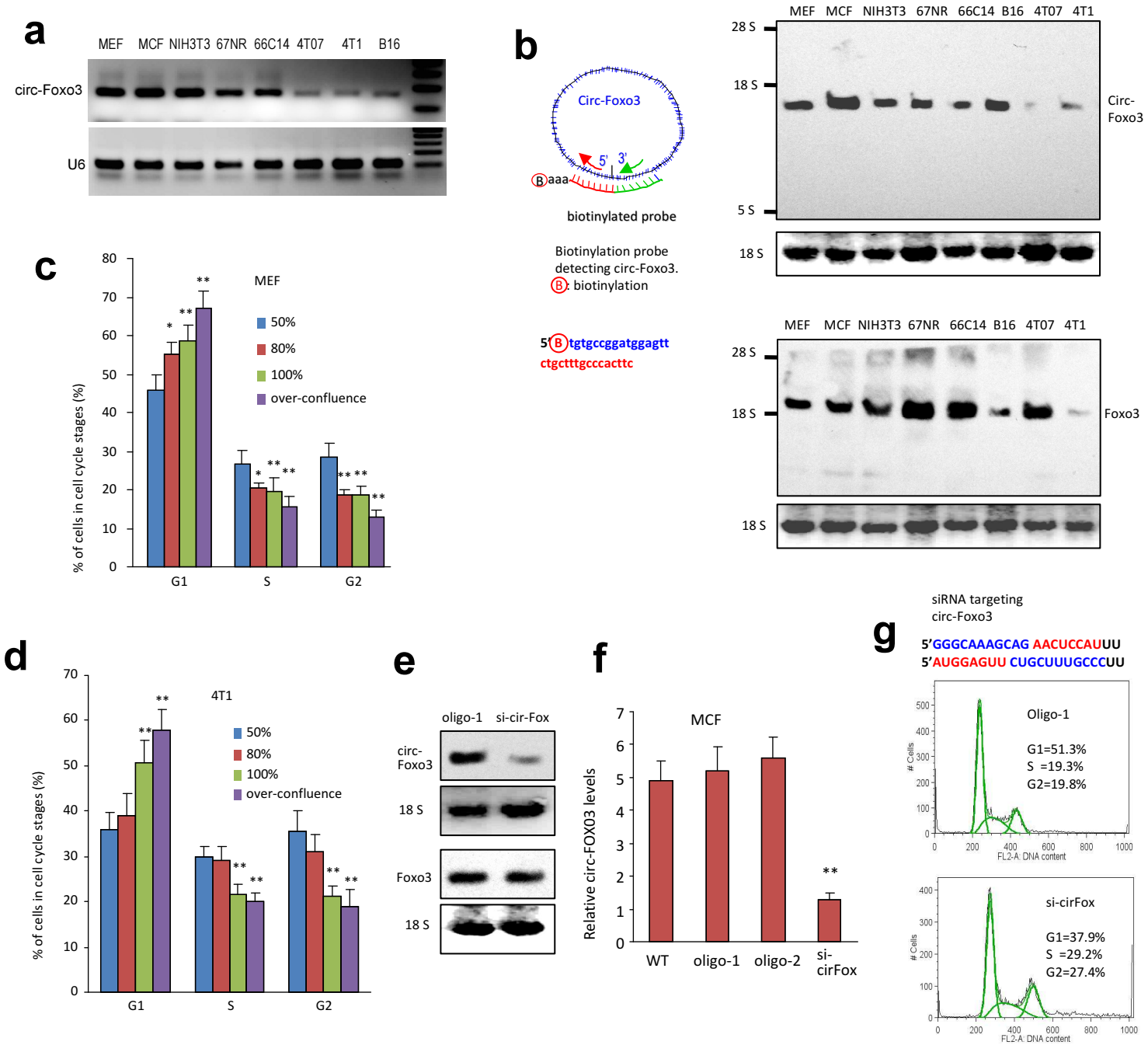

Fig S2. Expression of circ-Foxo3 affected cell cycle progression. (a) RT-PCR showed that expression of circular RNA Foxo3 in mouse cell lines, including mouse embryo fibroblast (MEF), mouse cardiac fibroblast (MCF), NIH3T3, 67NR, 66C14, 4T07, 4T1 and B16 cell lines. (b) Left, structure of oligo probe binding to circ-Foxo3. Right, Northern blot showing expression of circ-Foxo3 and Foxo3 mRNA in a number of cancer and non-cancer cell lines. (c-d) $1x10^5$ cells of MEF (c) and 4T1 (d) were seeded onto 6-well dishes in 10% FBS/DMEM medium until 50%, 80%, 100% or over-confluence, followed by determination of cell cycle distribution. Increased cell densities had more cells detected in the G1 phase, but less cells detected in G2 and S phases. (e) Northern blot showing expression of circ-Foxo3 and Foxo3 in circ-Foxo3 siRNA-transfected NIH3T3 cells. (f) Real-time PCR showed that MCF cells transfected with circ-Foxo3 siRNA expressed lower levels of circ-Foxo3 compared with cells transfected without (WT) or with two oligos with random sequences. Asterisks indicate significant differences. **, $p<0.01$. Error bars, SD ($n=4$). (g) Upper, sequence of circ-Foxo3 siRNA. Lower, circ-Foxo3 siRNA- and control oligo-transfected NIH3T3 cells were cultured in DMEM with 10 % FBS for 2 days, and processed to cell cycle assays measured by flow cytometry. Typical pictures showed silencing circ-Foxo3 decreased the number of G1 phase cells, and increased the number of S and G2 phase cells.

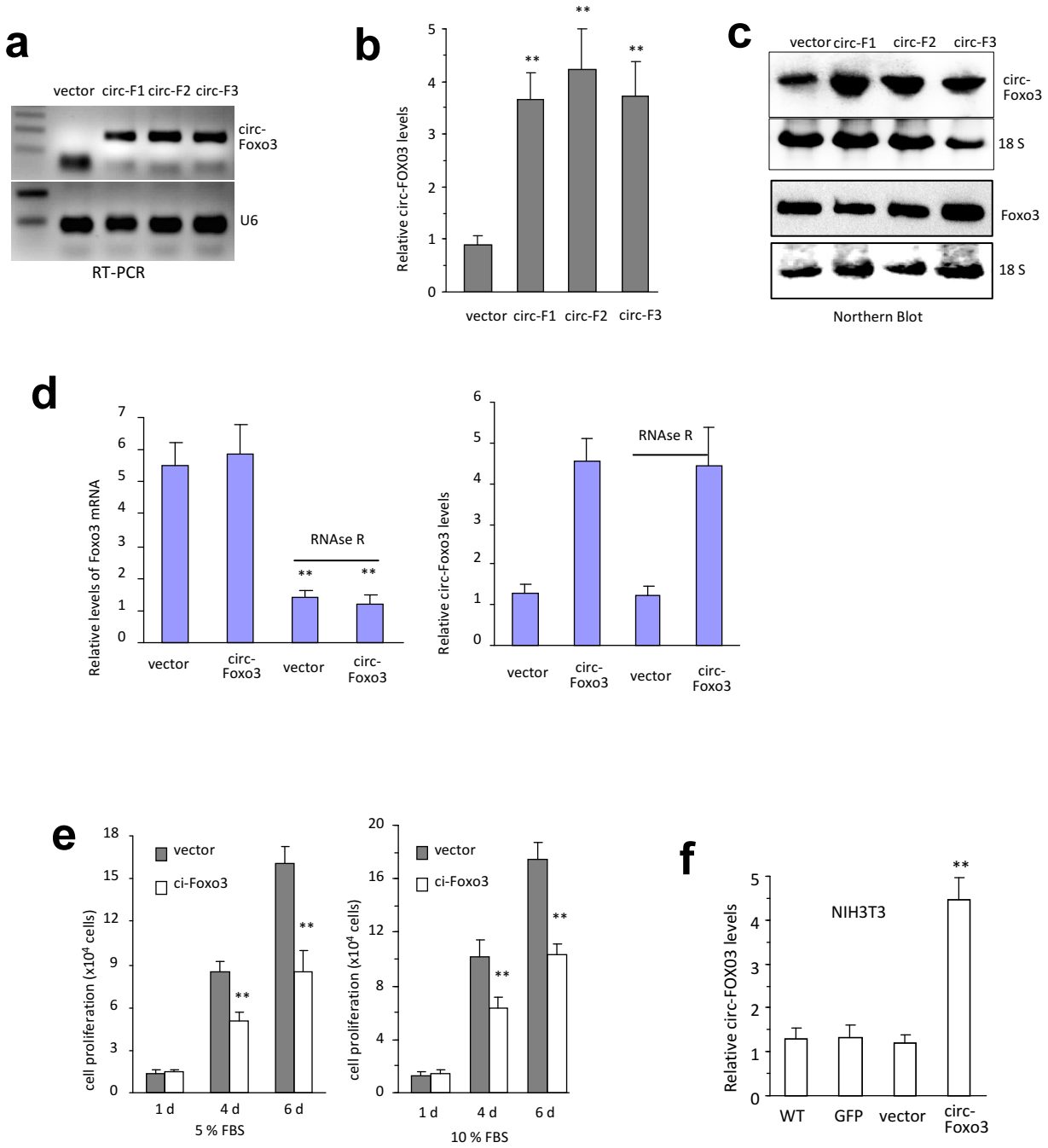

Fig S3. Circ-Foxo3 expression decreased cell proliferation. (a) RT-PCR showed that circ-Foxo3 construct transfected NIH3T3 cells expressed high levels of circ-Foxo3 compared with mock control cells. (b) Real-time PCR showed that three NIH3T3 cell lines transfected with circ-Foxo3 construct (circ-F1, circ-F2, and circ-F3) expressed higher levels of circ-Foxo3. *, $p<0.05$, **, $p<0.01$. Error bars, SD ($n=4$). (c) Northern blot showing expression of circ-Foxo3 in three circ-Foxo3-transfected cell lines. (d) Total RNA extracted from mock- or circ-Foxo3-transfected NIH3T3 cells was incubated with or without RNAse R followed by real-time PCR. While RNAse R treatment decreased Foxo3 linear mRNA levels, it did not affect circ-Foxo3 levels. **, $p<0.01$. Error bars, SD ($n=4$). (e) Circ-Foxo3- and mock-transfected NIH3T3 cells were cultured in DMEM containing 5% or 10% FBS for 6 days. Cell proliferation assays showed that circ-Foxo3 expressing cells grew slowly compared to the controls. **, $p<0.01$. Error bars, SD ($n=4$). (f) Real-time PCR showed that NIH3T3 cells transfected with circ-Foxo3 expressed higher levels of circ-Foxo3 compared with cells transfected without (WT) or with the vector or the GFP plasmid. **, $p<0.01$. Error bars, SD ($n=4$).

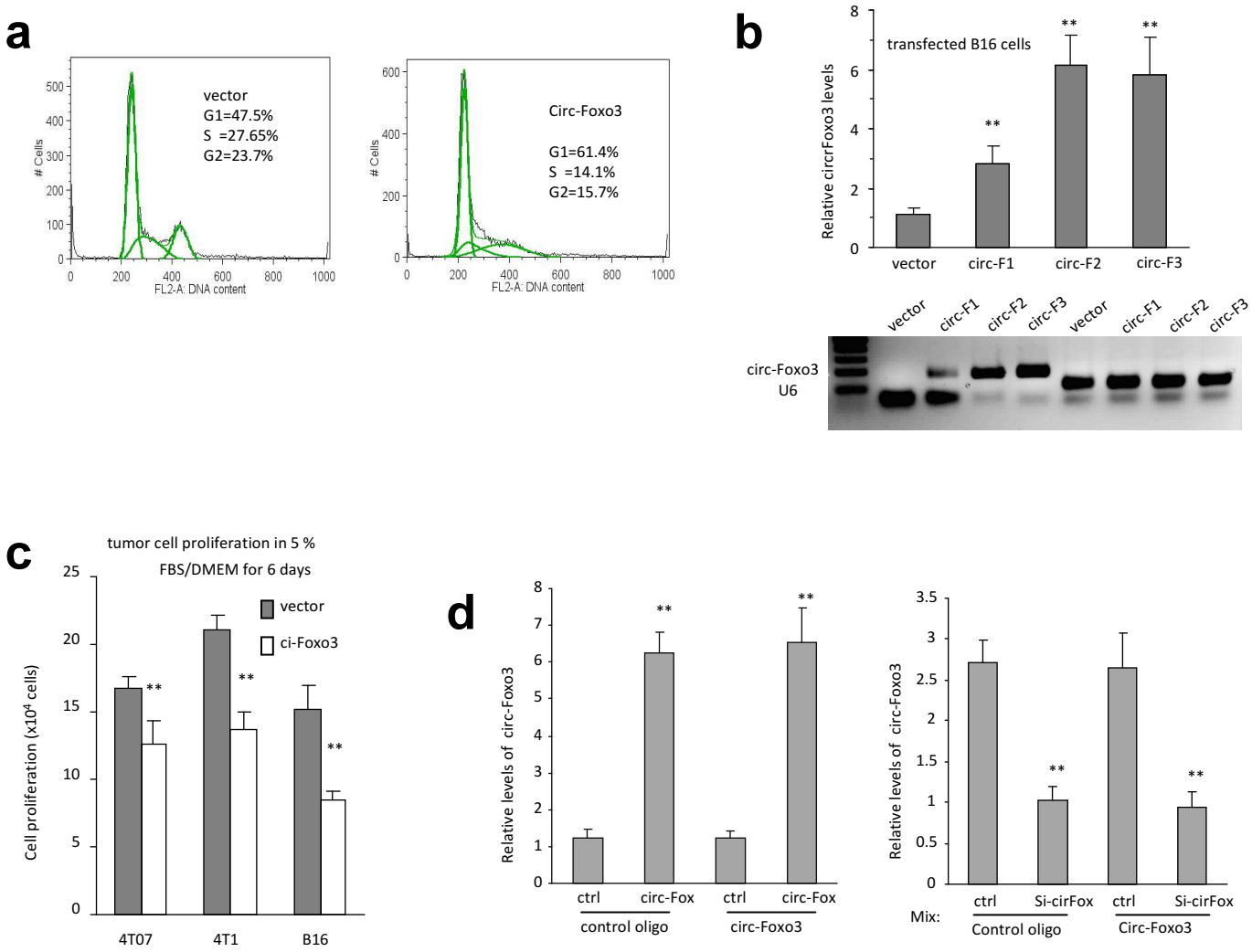

Fig S4. Circ-Foxo3 repressed cell cycle entry. (a) Circ-Foxo3- and mock-transfected NIH3T3 cells were cultured in DMEM with 10% FBS for 2 days, and processed to cell cycle assays measured by flow cytometry. Typical pictures showed that expression of circ-Foxo3 increased the number of cells in the G1 phase, and decreased the number of cells in the S and G2 phases. (b) RT-PCR showed that circ-Foxo3 construct transfected B16 cells expressed high levels of circ-Foxo3 compared with mock control cells. Left, real-time PCR showed that circ-Foxo3 construct transfected B16 cells expressed higher levels of circ-Foxo3. *, $p<0.05$, **, $p<0.01$. Error bars, SD ($n=4$). Right, PCR with primers for ectopic circ-Foxo3 showed expression of circ-Foxo3 in circ-Foxo3 construct and mock control transfected B16 cells. (c) Circ-Foxo3- and mock control-transfected tumor cell lines 4T07, 4T1 and B16 were cultured in DMEM containing 5% FBS for 6 days. Cell proliferation assays showed that circ-Foxo3 expressing cells grew slowly compared to control cells. **, $p<0.01$. Error bars, SD ($n=4$). (d) Left, Lysates prepared from NIH3T3 cells transfected with circ-Foxo3 or mock control, were hybridized with biotinylated DNA oligo probes against ectopic expression of circ-Foxo3 or control oligo, and then subject to RNA pull-down assays. Real-time PCR showed that circ-Foxo3-transfected NIH3T3 cells expressed high levels of circ-Foxo3. *, $p<0.05$, **, $p<0.01$. Error bars, SD ($n=4$). Right, Lysates prepared from NIH3T3 cells transfected with anti-circ-Foxo3 siRNA or control oligo, were hybridized with biotinylated DNA oligo probes against endogenous expression of circ-Foxo3 or control oligo, and then subject to RNA pull-down assays. Real-time PCR showed that anti-circ-Foxo3 siRNA-transfected NIH3T3 cells expressed decreased levels of circ-Foxo3 in the input. *, $p<0.05$, **, $p<0.01$. Error bars, SD ($n=4$).