Supplementary Materials

Table 1: Antibodies and primers used in qRT-PCR

RT PCR Primers		
Gene	**Forward 5'-3'**	**Reverse 5'-3'**
FoxM1	GCAGGCTGCACTATCAACAA	TCGAAGGCTCCTCAACCTTA
FoxA2	CTTCAAGCACCTGCAGATTC	AGACCTGGATTTCACCGTGT
c-Myc	TAGTGGAAAACCAGCAGCCTCC	CCTCGTCGCAGTAGAAATACGG
Sox2	TGAATGCCTTCATGGTGTGGTC	CCGTCTCCGACAAAAGTTTCC
Oct4	GGGGTTCTATTTGGGAAGGTATTC	GGTTCGCTTTCTCTTTCGGG
Nanog	CCAGTCCCAAAGGCAAACAAC	TGGAGGCTGAGGTATTTCTGTCTC
FYN	GGTGTGAACTCTTCGTCTCATAC	CTTCTGTCCGTGCTTCATAGTC
Survivin	TCAAGGACCACCGCATCTCTA	TGAAGCAGAAGAAACACTGGGC
Aurora B	ATCTGCTCTTAGGGCCAAGGG	CACATTGTCTTCCTCCTCAGGG
ALB	GTTGCCAAGCTGCTGATAAAG	GGCACACTTGAGTCTCTGTT
AAT	TGGGTGCTGCTGATGAAATA	GTGATGATATCGTGGGTGAGTT
HNF4α	CCTACCTCAAAGCCATCATCTT	GTCGTTGATGTAGTCCTCCAAG
GAPDH	ACACCCACTCCTCCACCTTT	TTCCTCTTGTGCTCTTGCTG
CHIP primers		
Gene	**Forward 5'-3'**	**Reverse 5'-3'**
FoxA2 (-1294)	GGACAGAGACGCTCTTGAA	AAACAGGGCAGGAGGTG
FoxA2 (-3581)	CATATCTGCCTTATGTTGC	CACATGAAACCAACCAGTGC
FoxA2 (-4156)	CTGGTCTTTTGACCATCCAAGAAC	GGCCCATGCCTATAATCCCAGCTAC
FoxA2 (-4535)	GAGAATGTAATAATAAAGTAGTG	GGGGGAGGCAAGGTGCAACATT
Non-Specific	TTTTACGGGGCAACTACGGC	CAGTGGCATTAGCAGGTC
FoxM1 (-580)	CATTGTATCTTCAGGGCCTAGC	AGCGCGAAACCTGTCTTT
FoxM1 (-739)	CCCAGCCCACATTTGTTTATTT	CAATGGCAGACAAGGTTCTTTC
FoxM1 (-1029)	TTCCTGTCCTACCTCTCAAGAT	GGCGACAGAGGGAGACA
FoxM1 (-1830)	AGAGCAAGACCCTGTCTCTA	ACTACAAAGCAATGCTCAGAATG
FoxM1 (-2585)	GCAAACTCATCTGCCTGTATTTC	GGGTCCCAAACTTAAGGAATTCTA
FoxM1 (-2761)	CAGAAGACATCAAAGGAAATACTCTC	CAGCAGAATGAACTGACCTCTA
FoxM1 (-3420)	AGCTATGATCGTGCCACTG	GAGATCACGCTACTGCACTC
MSP Primers (Human)		
Gene	**Forward 5'-3'**	**Reverse 5'-3'**
FoxA2 (-356)	CGCGTTATATTATTAGTTTTTTACGT	CAATACCGAACTACCCCGAA
FoxA2 (-882)	GGTTTTTATAGGGATTTGTCGG	AAAAAAAACCACCCTCTAAAACG
FoxA2 (-1080)	AATTTTAGTTTTTTAATCGTCGGTC	CCTATTACAATTCAAACCCGAA
FoxA2 (-1974)	GAGTTTTTAGTATTCGGGGGATC	CATAAAAAAAAACATTAATAAACCCG
FoxA2 (-2656)	GTATTTTTATGGGTAGGCGTGTC	CTAACGAAATTCTAAAAACTCCGAT
Non-Specific	ATTTAATACGATTTTGTTGATTCGT	AAAAAAAACTAAATTTTCCCGC
MSP Primers (Mouse)		
FoxA2	TTAGAAAGAGGATTGAGGTAATTGAC	CAAATAACAACCAATTTACAAAACG
Non-Specific	TTCAGTCCAAAAGGATGCTG	GGATACAGTCCCAAACTCTTC
Antibody		
Protein	**Company**	**Catalogue number**
FoxM1	Santa Cruz	SC-500
FoxM1	Santa Cruz	SC-376471
FoxA2	Santa Cruz	SC-374376
Rb	Cell Signaling	CST9309
DNMT3b	Imgenex	52A1018
Actin	Sigma	AC-40
GAPDH	Cell Signaling	CST5174

Supplemental Methods:

Soft Agar Assay

For transformation and anchorage independent colony formation ability, soft agar assay was performed. Cells (2×10^4) were suspended in a medium containing 0.4% agarose and then poured onto 60mm culture dish coated with 0.8% agarose. The top agar surface was layered with complete medium every third day and cells were allowed to grow for 20 days. Colonies larger than 1mm in soft agar were stained with 0.1% crystal violet for counting and capturing images.

Clonogenic assay

To check the effect of the FoxA2 on FoxM1 expressing cell line proliferation, clonogenic assay was performed with Huh7 cells transfected with Control, T7-FoxM1, CMV-FoxA2 and co-transfected with T7-FoxM1 and FoxA2 were seeded at the density of $1X10^4$ cell in 24 well plate. Cells were feed with fresh medium every third day and were allowed to grow for 6 days. At the end of the assay, pictures of the cells were captured under bright field microscope after staining with crystal violet solution (0.1% crystal violet in 10% ethanol). Stained colonies were counted.

Supplemental Figure Legends:

Figure S1: Expression of FoxM1 and FoxA2 in normal liver: (A) Upper panel shows the expression pattern of FoxM1 and FoxA2 in non-tumor human liver. (B) lower panel shows the expression in mouse normal liver. Image scale bar is 50µm in upper panel and 100µm in lower panel.

Figure S2: FoxM1 regulates the expression of FoxA2 in human HCC cells: (A) Equal amounts (100µg) of SNU449, Huh7 and HepG2 cell extracts were resolved by SDS-PAGE and western blot was performed with antibodies against FoxM1 and Actin. (B) Expressions of FoxM1 transgene in Huh7 and HepG2 are shown (C) Huh7 and HepG2 cells were transfected with empty vector (control) or vector expressing T7-FoxM1b. Forty-eight hours post transfection, cells were harvested for protein and RNA assays. Total protein (70 µg) of whole cell extracts were subjected to western blotting for the relative expression of FoxM1 and FoxA2. The western blot panel for FoxM1 expression was exposed for less than a second, whereas the other panels were exposed for greater than 10 seconds. Huh7 cells (D) and SNU449 cells (E) were transfected with control-siRNA or FoxM1-siRNAs. Seventy-two hours after siRNA transfection, cells were harvested for RNA and protein assays. Whole cell (100 µg) extracts were resolved through SDS-PAGE and Western blots were performed for the respective proteins. Blots were exposed for greater than 10 sec. (F) Single cell clones of Huh7 cells stably expressing pINDUCER-shFoxM1 and pINDUCER-RFP as control were treated with 300ng of doxycycline for four days, at the end of treatment cells were harvested and subjected to western blotting with antibodies against FoxM1, FoxA2 and Actin. The number above each band in western blots represents Image J quantification relative to control set at 1. Result is the representative of three independent experiments.

Figure S3: Predicted FoxM1 binding site in the FoxA2 promoter and assessment of FoxM1 interaction along with Rb and DNMT3b: (A) Schematic shows the predicted FoxM1 binding sites (Red) (MacVector) and the CpG methylation islands (sky blue) (Methprimer tool http://www.urogene.org) on the human FoxA2 promoters. (B) Equal amounts (1mg) of Huh7 cell extracts were used for immunoprecipitation assay with anti-FoxM1 and anti-IgG. Immunoprecipitates were resolved by SDS-PAGE and co-immunoprecipitation of Rb and DNMT3b were confirmed by using respective antibodies as shown in the figure. SNU449 cells were subjected to crosslinking for chromatin-IP (ChIP). The chromatin preparations were immunoprecipitated with FoxM1-ab (C) or Rb-ab (D) or DNMT3b (E) or IgG as control. Enrichments of the FoxA2 promoter fragments were assayed by quantitative RT-PCR, and the relative enrichments with FoxM1-ab, Rb-ab and DNMT3b-ab over that with IgG, after normalization against a non-specific site, are shown. (F) Huh7 cell transfected with either control vector or T7-FoxM1 expression vector, 48h post transfection cells were harvested and isolated genomic DNA was subjected to bisulphite conversion followed by identification of CpG island on FoxA2 promoter. (G) Huh7 cells transfected with control siRNA or FoxM1 siRNA. Extracts were subjected to western blot to confirm the extent of FoxM1 knock down. Western blots were analyzed using Image J software followed by intensity calculation against control band set at 1. Statistical calculations were done using GraphPad Prism online tool for t-test and p values stated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and error bar represents ±SE. Result is the representative of three independent experiments.

Figure S4: Regulation of the mouse FoxA2 promoter by FoxM1: (A) RNA assay showing knock down of DNMT3b in SNU449 cells. (B) DNA from SNU449 cells, 72h after transfection with control-siRNA or DNMT3b-siRNA were isolated and subjected to bisulphite conversion

followed by methylation specific pcr covering CpG island on FoxA2 promoter. (C) DNA from DNMT3b knock down SNU449 cells were isolated and subjected to quantitative real time PCR for DNMT3b and FoxA2 mRNAs. (D) Mouse FoxA2 promoter: Site of MSP (-1553bp to -1369bp upstream of transcription start site (TSS)), Non-Specific site (-4499bp to -4349bp). Schematic has been generated using Methprimer tool from http://www.urogene.org. (E) Genomic DNA was isolated from the FoxM1 +/+ and Fox Fl/Fl mice tumor were subjected to bisulphite conversion followed by identification of CpG island in the FoxA2 promoter by CYBR green qPCR. (F) Equal amount (1mg) of protein extract from the FoxM1 +/+ and Fox Fl/Fl mice tumor were used for immunoprecipitation assay with anti-FoxM1 and anti-IgG. Immunoprecipitates were resolved by SDS-PAGE and co-immunoprecipitation of Rb and DNMT3b were confirmed by using respective antibodies as shown in the figure. Western blots were analyzed using Image J software followed by intensity calculation against control band set at 1. Statistical calculations were done using GraphPad Prism online tool for t-test and p values stated as *p≤0.05, **p≤0.01, ***p≤0.001 and error bar represents ±SE. Result is the representative of three independent experiments.

Figure S5: Doxycycline does not affect the expression of FoxM1 and FoxA2 in control cells. Doxycycline inducible Huh7-pINDUCER-RFP and Huh7-pINDUCER-shFoxM1 cells were either mock treated or treated with 300ng/ml of Doxycycline for 4 days. RNAs corresponding to the indicated genes were assayed by quantitative real time PCR. Statistical calculations were done using GraphPad Prism online tool for t-test and p values stated as *p≤0.05and **p≤0.001 and ***p≤0.0001.

Figure S6: FoxA2 inhibits expression of FoxM1 and its downstream targets, whereas FoxM1 downregulation increases the hepatic differentiation genes. (A) SNU449 cells were

transfected with empty vector or FoxA2 expression plasmid. Two hundred cells were then seeded in sphere formation medium in low attachment petri dishes. Cells were allowed to grow for a week. Spheres were counted, and representative picture was captured under bright field microscope. (B) SNU449 cells were transfected with empty vector, and FoxA2, 48h post transfection, RNA was isolated, and cDNA was made. cDNA was subjected to qRT-PCR. Effects of FoxA2 on FoxM1, Survivin, AuroraB were assayed. (C) Huh7 cells were transfected with either empty vector of FoxA2 expression vector, 48h post transfection cells were harvested and mRNA assay was performed as depicted. (D) SNU449 cells transfected with either control siRNA or FoxM1 siRNA for 72 hours were used to assay the effect of FoxM1 knock down on hepatic differentiation gene such as ALB, AAT and HNF4α. Statistical calculations were done using GraphPad Prism online tool for t-test and p values stated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and error bar represents ±SE. Result is the representative of three independent experiments. Image scale bar is 500µm.

Figure S7: FoxA2 inhibit FoxM1-induced clonogenicity and anchorage independent growth of the Huh7 cells: (A) Huh7 cells were transfected individually with empty vector, Flag-FoxM1 or FoxA2 or a combination of Flag-FoxM1 and FoxA2, along with GFP expression plasmid as transfection control. The transfected cells were subjected to clonogenicity or soft agar assay. About $1x10^4$ cells were seeded in triplicate in 24 well plate and allowed to grow for one week. Cells were fixed and stained with crystal violate and pictures were taken under light microscope. (B) Quantification of the colonies is shown. (C) Huh7 cells ($2 x10^4$ cells) as described in panel (A) were seeded into soft agar in triplicate and allowed to grow for 20 days and the representative appearances are shown. (D) Graph shows the quantification of number of distinctly visible colonies with unaided eye. (E) Huh7 cells as described in panel (A) were lysed forty-eight hours

after transfection, and 70 ug of extracts were resolved on SDS-PAGE. Expressions of FoxM1 and FoxA2 were assayed by western blotting with anti-Flag, anti-FoxM1, anti-FoxA2 antibodies. For loading control, actin-ab and anti-GFP antibody were used. For the over-expressed Flag-FoxM1b, FoxA2 short exposure ($t<1$ sec) of the blots was taken. Western blots were analyzed using Image J software followed by intensity calculation against control band set at 1. Statistical calculations were done using GraphPad Prism online tool for t-test and p values stated as $*p\leq0.05$, $**p\leq0.001$ and $***p\leq0.0001$. Image scale bar is 500μm.

Figure S1

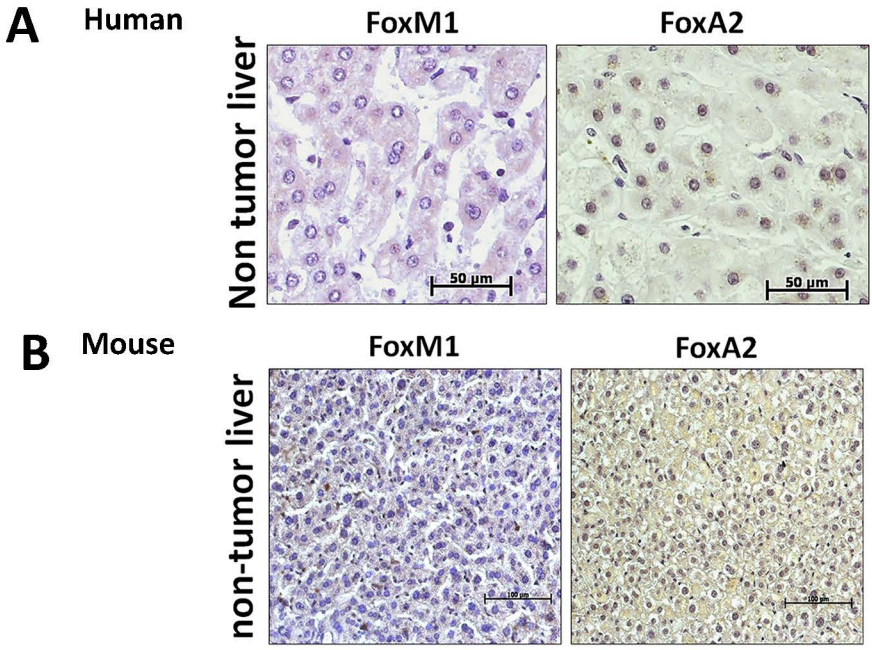

A
Human
FoxM1
FoxA2
Non tumor liver
50 µm
50 µm
B
Mouse
FoxM1
FoxA2
non-tumor liver

Figure S2

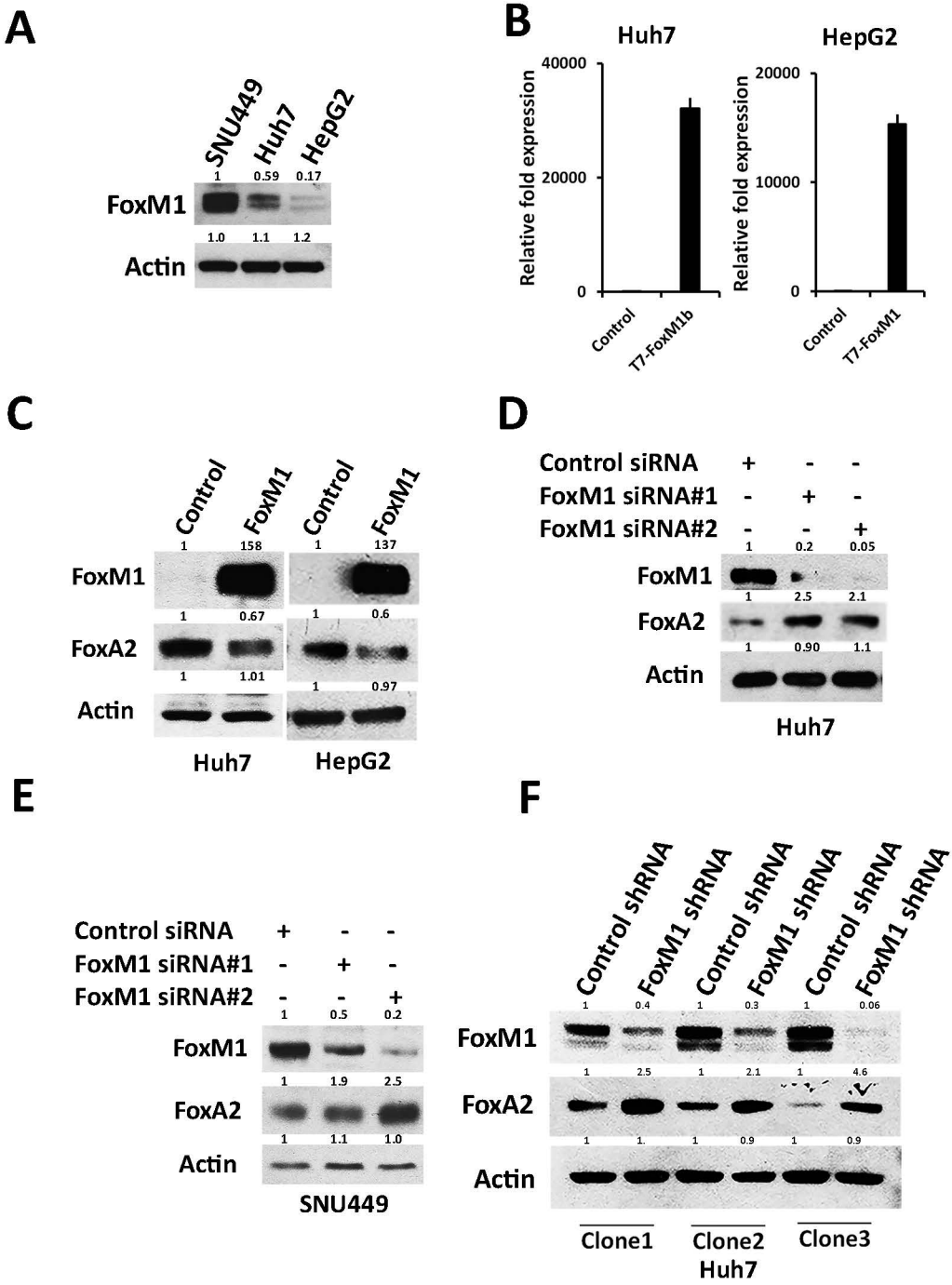

A
SNU449
Huh7
HepG2
1
0.59
0.17
FoxM1
1.0
1.1
1.2
Actin
B
Huh7
HepG2
Relative fold expression
40000
20000
0
20000
10000
0
Control
T7-FoxM1b
Control
T7-FoxM1
C
Control
FoxM1
Control
FoxM1
1
158
1
137
FoxM1
1
0.67
1
0.6
FoxA2
1
1.01
1
0.97
Actin
Huh7
HepG2
D
Control siRNA + - -
FoxM1 siRNA#1 - + -
FoxM1 siRNA#2 - - +
1
0.2
0.05
FoxM1
1
2.5
2.1
FoxA2
1
0.90
1.1
Actin
Huh7
E
Control siRNA + - -
FoxM1 siRNA#1 - + -
FoxM1 siRNA#2 - - +
1
0.5
0.2
FoxM1
1
1.9
2.5
FoxA2
1
1.1
1.0
Actin
SNU449
F
Control shRNA
FoxM1 shRNA
Control shRNA
FoxM1 shRNA
Control shRNA
FoxM1 shRNA
1
0.4
1
0.3
1
0.06
FoxM1
1
2.5
1
2.1
1
4.6
FoxA2
1
1.
1
0.9
1
0.9
Actin
Clone1
Clone2
Clone3
Huh7

Figure S3

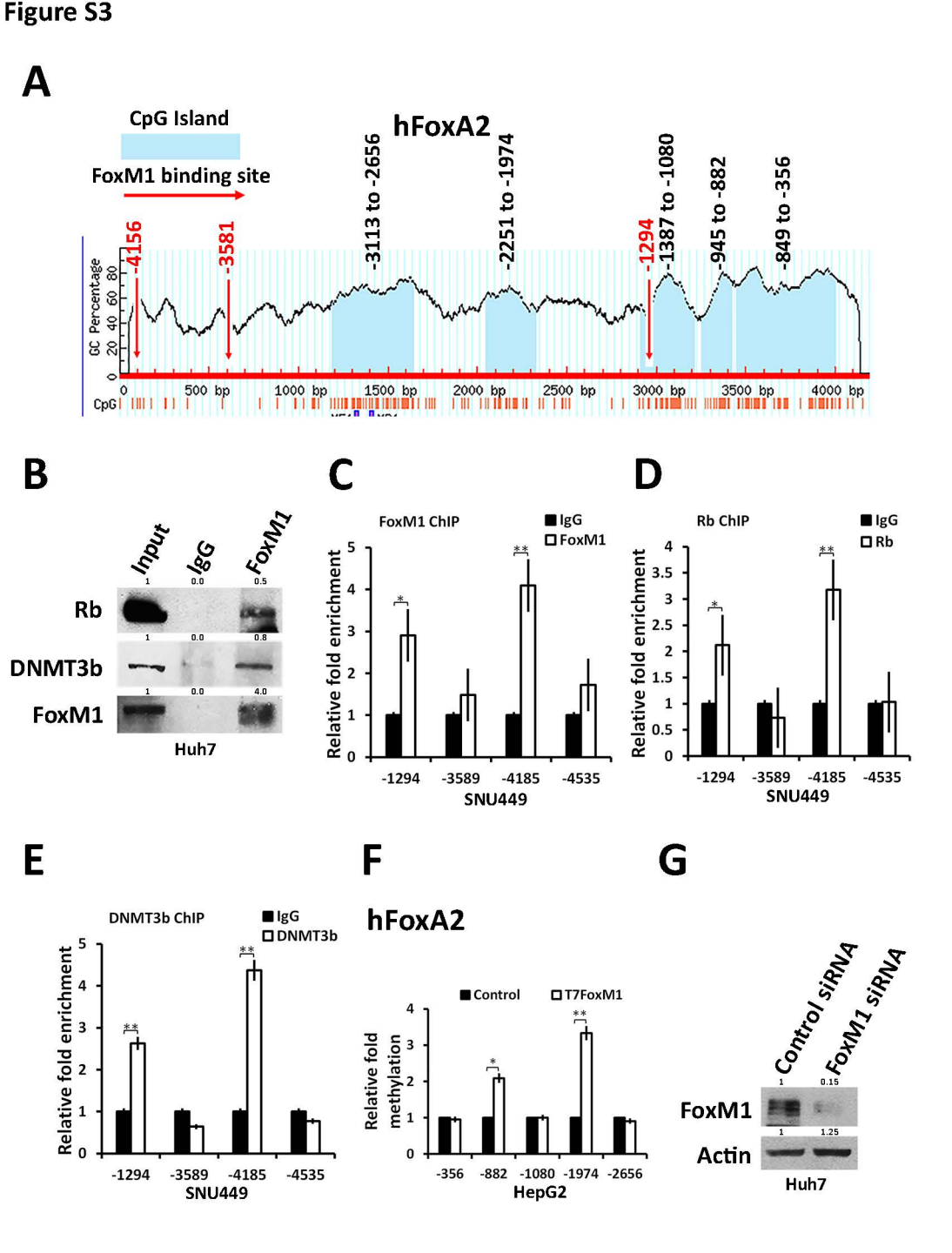
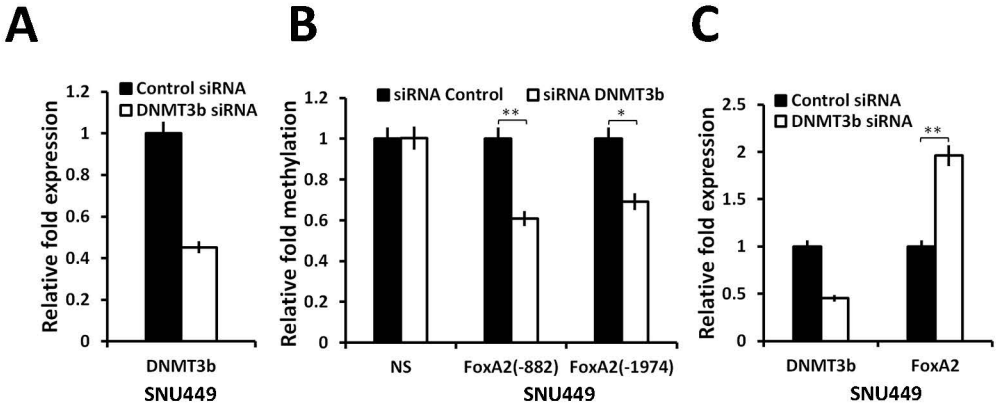
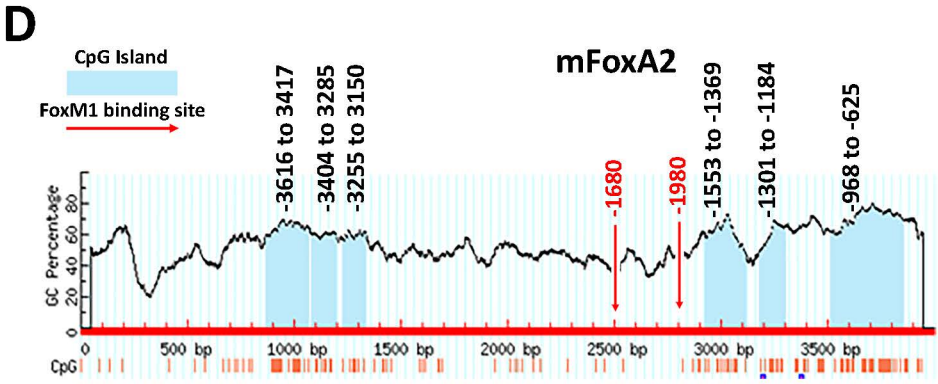
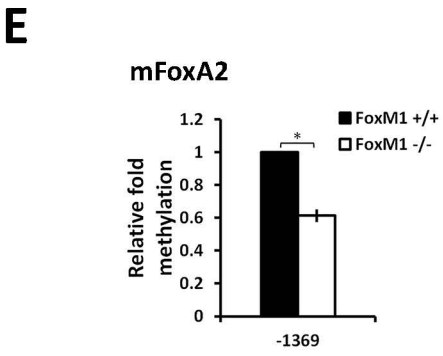

A
CpG Island
FoxM1 binding site
hFoxA2
-4156
-3581
-3113 to -2656
-2251 to -1974
-1294
-1387 to -1080
-945 to -882
-849 to -356
GC Percentage
0 500 bp 1000 bp 1500 bp 2000 bp 2500 bp 3000 bp 3500 bp 4000 bp
CpG
B
Input
IgG
FoxM1
Rb
DNMT3b
FoxM1
Huh7
C
FoxM1 ChIP
IgG
FoxM1
Relative fold enrichment
-1294 -3589 -4185 -4535
SNU449
D
Rb ChIP
IgG
Rb
Relative fold enrichment
-1294 -3589 -4185 -4535
SNU449
E
DNMT3b ChIP
IgG
DNMT3b
Relative fold enrichment
-1294 -3589 -4185 -4535
SNU449
F
hFoxA2
Control
T7FoxM1
Relative fold methylation
-356 -882 -1080 -1974 -2656
HepG2
G
Control siRNA
FoxM1 siRNA
FoxM1
Actin
Huh7

Figure S4


A
Control siRNA
DNMT3b siRNA
Relative fold expression
DNMT3b
SNU449
B
siRNA Control
siRNA DNMT3b
Relative fold methylation
NS
FoxA2(-882)
FoxA2(-1974)
SNU449
C
Control siRNA
DNMT3b siRNA
Relative fold expression
DNMT3b
FoxA2
SNU449


D
CpG Island
FoxM1 binding site
mFoxA2
-3616 to 3417
-3404 to 3285
-3255 to 3150
-1680
-1980
-1553 to -1369
-1301 to -1184
-968 to -625
GC Percentage
0 500 bp 1000 bp 1500 bp 2000 bp 2500 bp 3000 bp 3500 bp
CpG


E
mFoxA2
FoxM1 +/+
FoxM1 -/-
Relative fold methylation
-1369

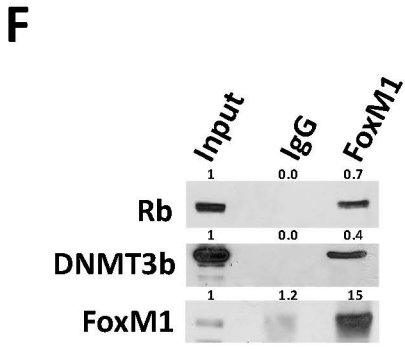

F
Input
IgG
FoxM1
Rb
1 0.0 0.7
DNMT3b
1 0.0 0.4
FoxM1
1 1.2 15

Figure S5

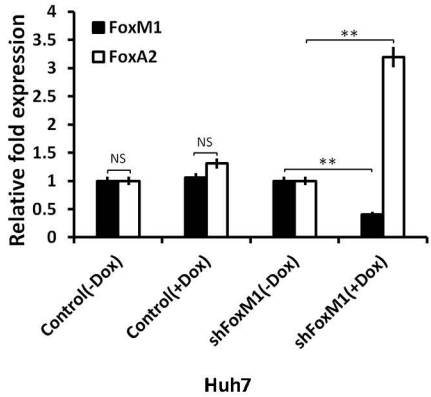

Relative fold expression
4
3.5
3
2.5
2
1.5
1
0.5
0
FoxM1
FoxA2
NS
NS
**
**
Control(-Dox)
Control(+Dox)
shFoxM1(-Dox)
shFoxM1(+Dox)
Huh7

Figure S6

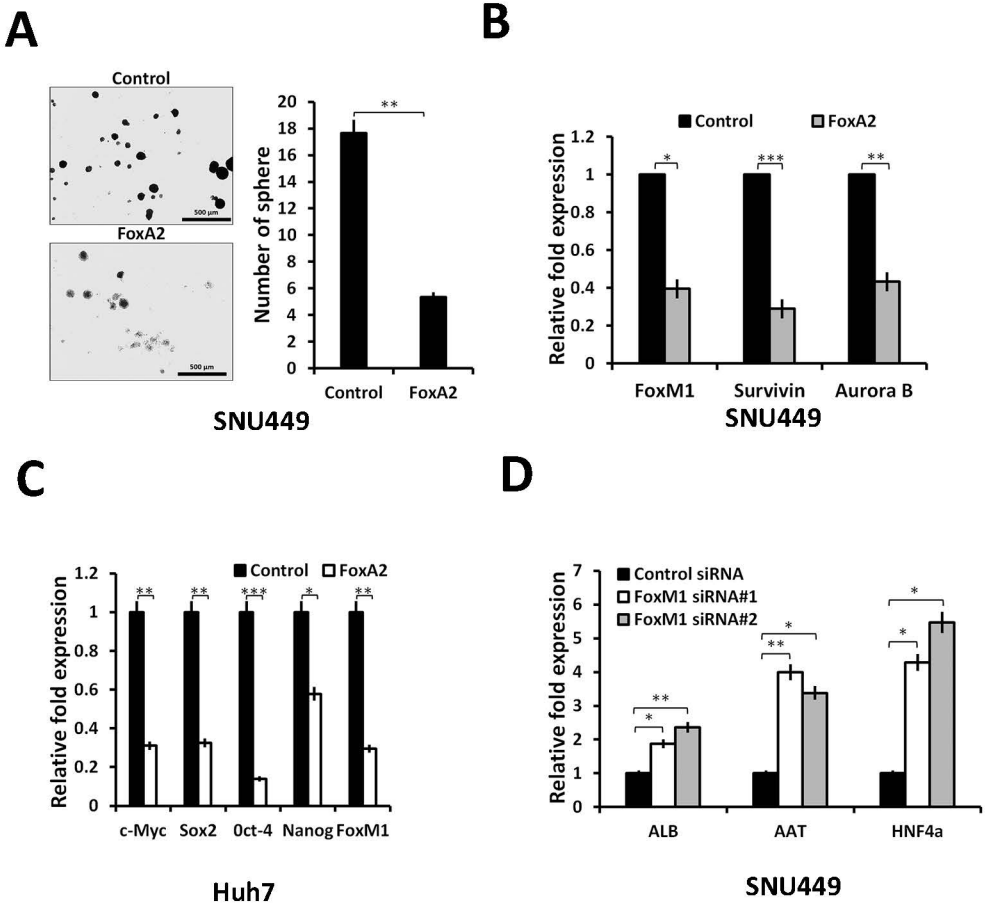
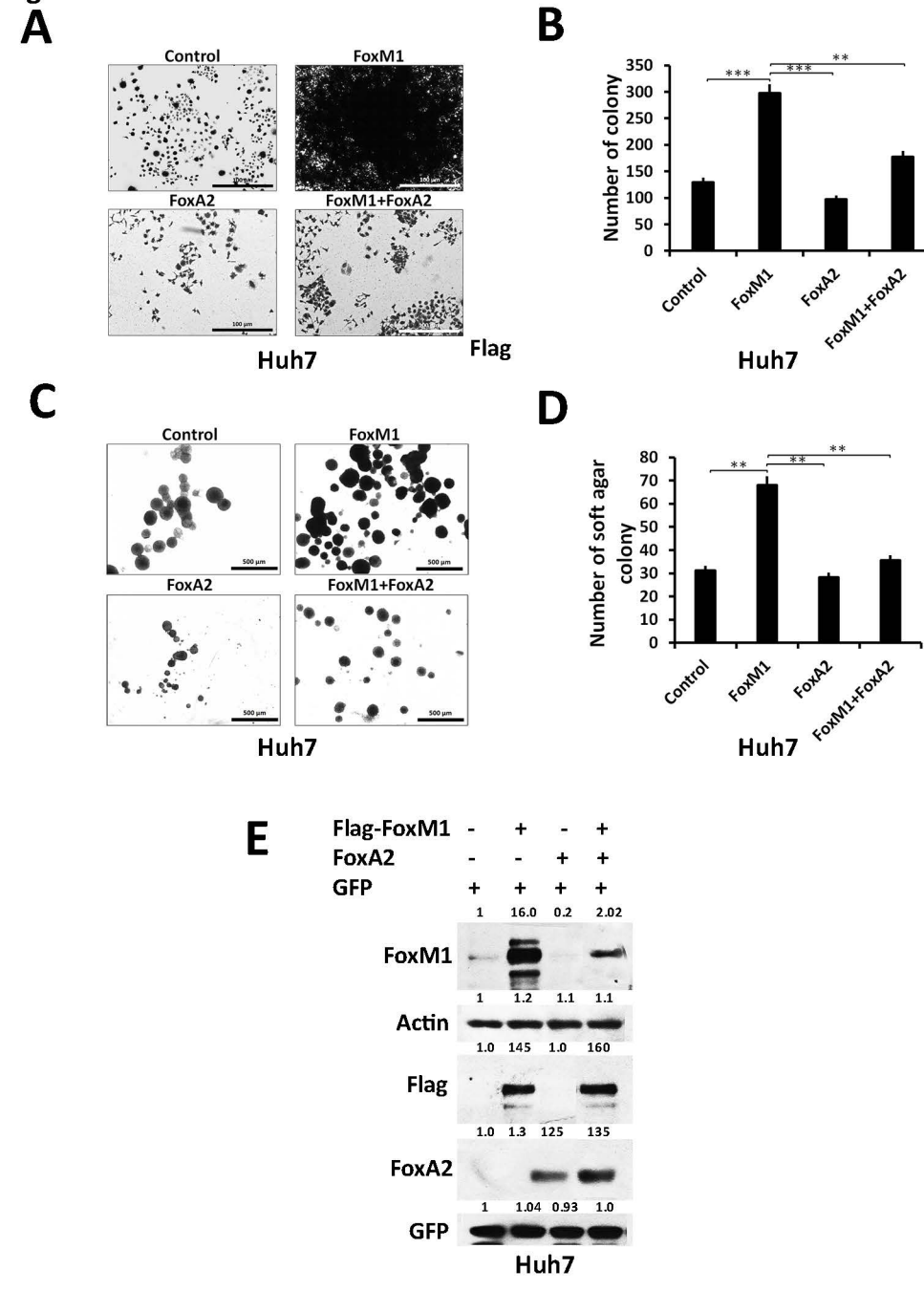

A
Control
FoxA2
500 μm
Number of sphere
20
18
16
14
12
10
8
6
4
2
0
**
Control
FoxA2
SNU449
B
Control
FoxA2
Relative fold expression
1.2
1
0.8
0.6
0.4
0.2
0
*
***
**
FoxM1
Survivin
Aurora B
SNU449
C
Control
FoxA2
Relative fold expression
1.2
1
0.8
0.6
0.4
0.2
0
**
**
***
*
**
c-Myc
Sox2
0ct-4
Nanog
FoxM1
Huh7
D
Control siRNA
FoxM1 siRNA#1
FoxM1 siRNA#2
Relative fold expression
7
6
5
4
3
2
1
0
*
**
*
**
*
*
ALB
AAT
HNF4a
SNU449

Figure S7


A
Control
FoxM1
FoxA2
FoxM1+FoxA2
Huh7
Flag
B
Number of colony
350
300
250
200
150
100
50
0
***
***
**
Control
FoxM1
FoxA2
FoxM1+FoxA2
Huh7
C
Control
FoxM1
FoxA2
FoxM1+FoxA2
Huh7
D
Number of soft agar colony
80
70
60
50
40
30
20
10
0
**
**
**
Control
FoxM1
FoxA2
FoxM1+FoxA2
Huh7
E
Flag-FoxM1 - + - +
FoxA2 - - + +
GFP + + + +
1 16.0 0.2 2.02
FoxM1
1 1.2 1.1 1.1
Actin
1.0 145 1.0 160
Flag
1.0 1.3 125 135
FoxA2
1 1.04 0.93 1.0
GFP
Huh7