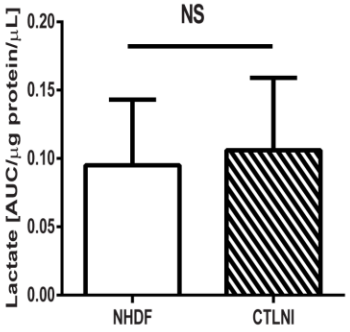
Supplementary Figures

Supplementary Figure 1. ASS1 expression is downregulated in hypoxic and acidic states

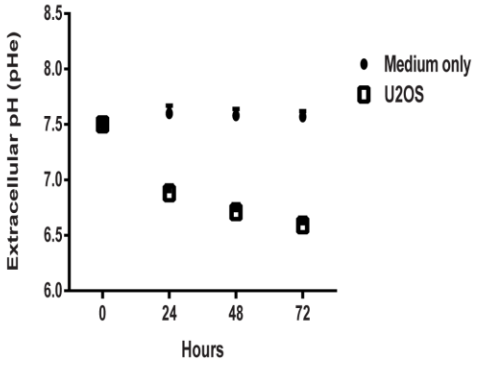
(**A**) Intracellular lactate measurements using GC/MS of fibroblasts taken by biopsies from a CTLN I patient as compared to normal human dermal fibroblasts (NHDF). The experiment was repeated twice, in triplicates. (**B**) Extracellular pH (pHe) measurements of control U2OS osteosarcoma cells infected with empty vector during a 72 hours of incubation in $-CO_2/-HCO_3^-$ conditions. As a control, we incubated under the same conditions a cell free medium. (**C**) A western blot quantification correlating with Figure **1F** showing ASS1 protein levels extracted from osteosarcoma cancer cells (pHe $\leq$ 6.6), following incubation with or without bicarbonate in the medium and CO_2 in the incubator. ($-CO_2/-HCO_3^-$ [pHe $\leq$ 6.6] or $+CO_2/+HCO_3^-$ [pHe 7.4] conditions). ASS1 levels are normalized to the expression levels of GAPDH. (**D**) **Left**: Representative normalized mRNA expression levels of osteosarcoma cells following 24 hours incubation under either pHe 7.4 or pHe 6.3 using 25mM PIPES. ASS1 levels are normalized to HPRT. **Right:** A quantification of a western blot correlating with Figure **1G** showing ASS1 protein levels extracted from osteosarcoma cells following 24 hours incubation under either pHe 7.4 or pHe 6.3 conditions using 25mM PIPES. ASS1 levels are normalized to the expression levels of α−tubulin.

We used student's t-test (2-tail) for statistical analysis. Unless otherwise specified, each graph represents the average of $n \geq 3$ experiments ± S.D. *, $P \leq 0.05$.

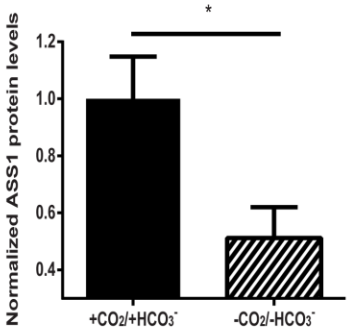
A.


NS
Lactate [AUC/μg protein/μL]
0.20
0.15
0.10
0.05
0.00
NHDF
CTLNI

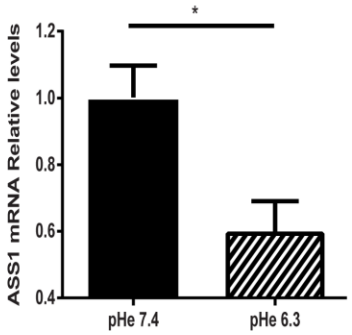
B.

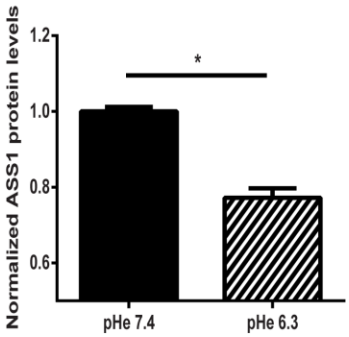

Extracellular pH (pHe)
8.5
8.0
7.5
7.0
6.5
6.0
0
24
48
72
Hours
Medium only
U2OS

C.

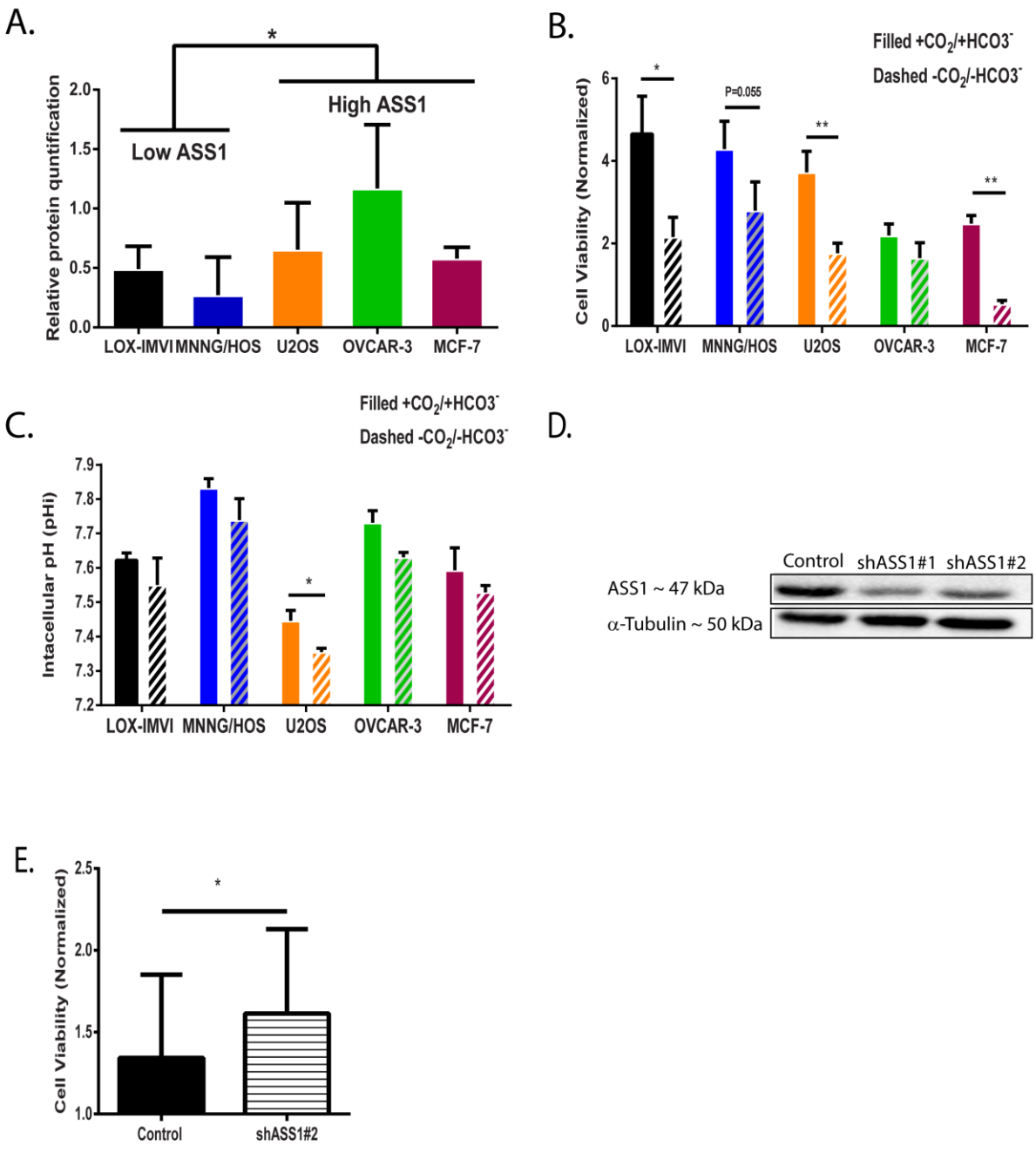

*
Normalized ASS1 protein levels
1.2
1.0
0.8
0.6
0.4
+CO2/+HCO3-
-CO2/-HCO3-

D.

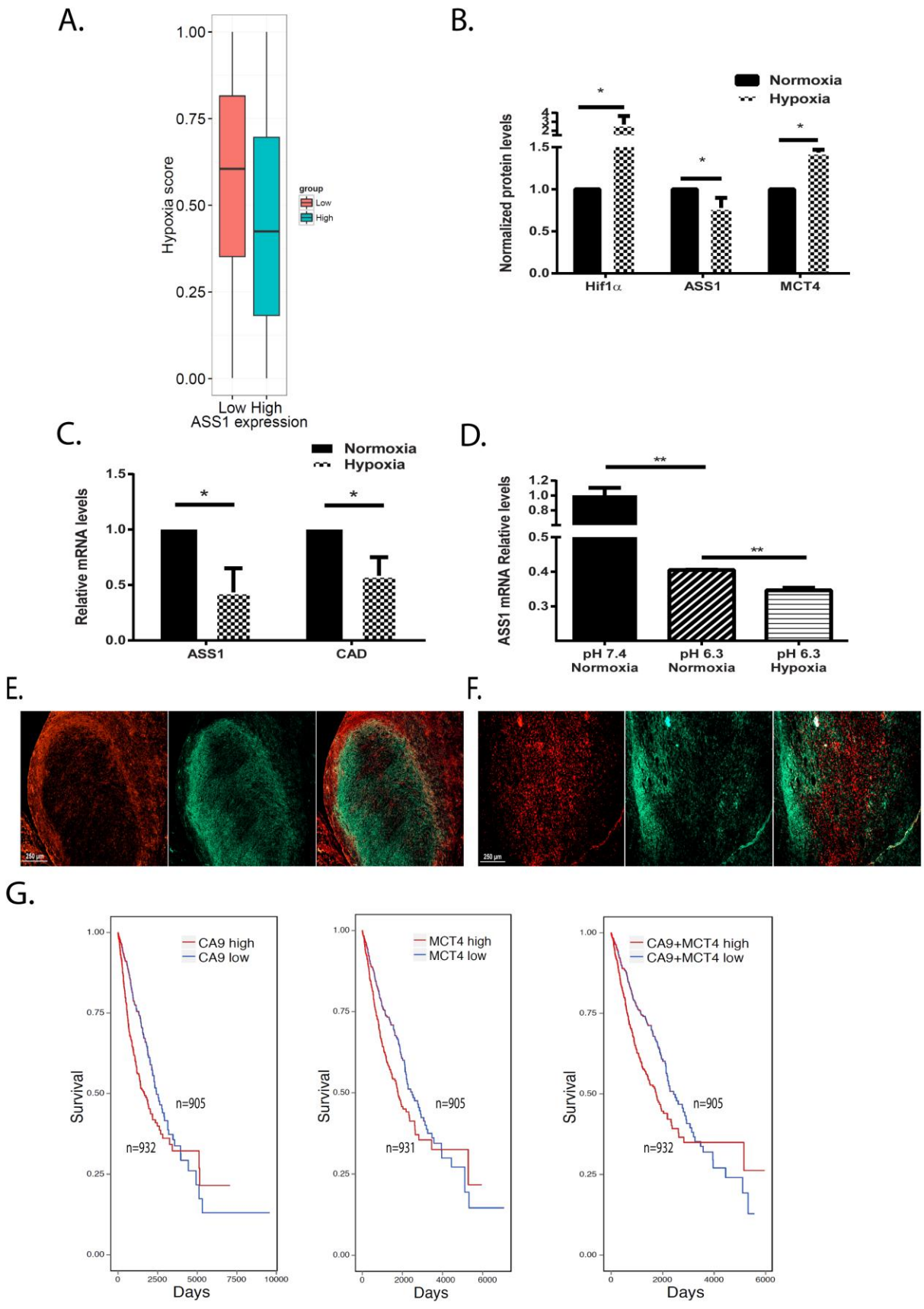

*
ASS1 mRNA Relative levels
1.2
1.0
0.8
0.6
0.4
pHe 7.4
pHe 6.3


*
Normalized ASS1 protein levels
1.2
1.0
0.8
0.6
pHe 7.4
pHe 6.3

Supplementary Figure 2. ASS1 downregulation contributes to cancer cells' survival in acidic conditions. (**A**) A western blot quantification showing ASS1 protein levels extracted from five different human cancers. ASS1 levels are normalized to the expression levels of GAPDH. The five different human cancers are divided into two groups based on ASS1 expression as either "High ASS1" or "Low ASS1" (**B**) Viability assay of five different cancer cell lines following incubation of 72 hours under either $+CO_2/+HCO_3^-$ (control) or $-CO_2/-HCO_3^-$ (buffer free) conditions. Viability is quantified relative to day 0. (**C**) Averaged pHi measurements (live confocal imaging) of five different cancer cell lines following incubation of 72 hours under either $+CO_2/+HCO_3^-$ (control) or $-CO_2/-HCO_3^-$ (buffer free) conditions correlating with (**B**). (**D**) A representative western blot image showing ASS1 levels in infected U2OS with empty vector *vs.* two different clones of *shASS1*. (**E**) Viability measurements of control U2OS osteosarcoma cells infected with either an empty vector or *shASS1#2* and incubated under $-CO_2/-HCO_3^-$ conditions for 72 hours (viability is quantified relative to day 0). Unless otherwise specified, we used student's t-test (2-tail) for statistical analysis. For the multiple cell line comparisons in **A**, we used Two-way ANNOVA (with A-priori contrast: MNNG/HOS, LOX-IMVI vs. U2OS, MCF-7 and OVCAR-3). Unless otherwise specified, each graph represents the average of $n \geq 3$ experiments ± S.D. *, $P \leq 0.05$.


A.
*
High ASS1
Low ASS1
Relative protein quntification
2.0
1.5
1.0
0.5
0.0
LOX-IMVI
MNNG/HOS
U2OS
OVCAR-3
MCF-7
B.
Filled +CO2/+HCO3-
Dashed -CO2/-HCO3-
Cell Viability (Normalized)
*
P=0.055
**
**
6
4
2
0
LOX-IMVI
MNNG/HOS
U2OS
OVCAR-3
MCF-7
C.
Filled +CO2/+HCO3-
Dashed -CO2/-HCO3-
Intacellular pH (pHi)
7.9
7.8
7.7
7.6
7.5
7.4
7.3
7.2
*
LOX-IMVI
MNNG/HOS
U2OS
OVCAR-3
MCF-7
D.
Control
shASS1#1
shASS1#2
ASS1 ~ 47 kDa
α-Tubulin ~ 50 kDa
E.
Cell Viability (Normalized)
2.5
2.0
1.5
1.0
*
Control
shASS1#2

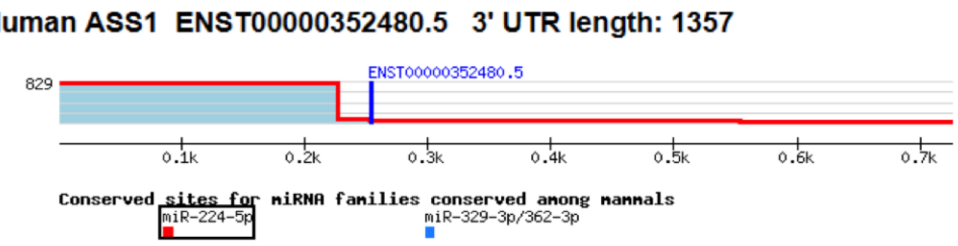
Supplementary Figure 3. ASS1 levels are downregulated together with CAD upon HIF1α activation. (**A**) Pan cancer analysis of the TCGA database demonstrating that tumors with low ASS1 expression (red) levels have a higher hypoxic score, independent of HIF1α target genes (Wilcoxon rank sum P<7.53e-4, see Methods). (**B**) Averaged western blot quantification correlating with Figure **3C**, representing 3 independent experiments of proteins extracted from osteosarcoma cancer cells following 24 hours incubation under either normoxic or hypoxic conditions (1% O2). Results are presented as fold change relative to normoxia. All proteins are normalized to the expression levels of α-Tubulin. (**C**) A representative Quantification of Real-Time PCR of cDNA produced from U2OS osteosarcoma cells showing a decrease in ASS1 and CAD mRNA expression levels following 24 hours incubation under either normoxic or hypoxic conditions (1% O2). mRNA expression levels are normalized to the mRNA levels under normoxic conditions. (**D**) Representative Quantification of Real-Time PCR of cDNA produced from U2OS osteosarcoma cells showing a decrease in ASS1 levels following incubation under pHe 6.3 buffer solution (25 mM PIPES) as compared with pHe 7.4 buffer solution (25mM PIPES) under normoxic conditions for 24 hours. Incubation under hypoxic conditions (1% O2) and pHe 6.3 buffer solution (25 mM PIPES) additively downregulates ASS1.(**E**) A representative immunofluorescence image of a mouse MC38 colon tumor section co-stained for ASS1 (red) and for MCT-4 (green). X6 magnification (**F**) A representative immunofluorescence image of a mouse Lewis Lung Carcinoma (LLC) tumor section co-stained for ASS1 (red) and MCT-4 (green). X6 magnification. (**G**) Kaplan-Meier plots showing that overexpression of CA9 (**left panel**), MCT4 (**middle panel**) or of both (**right panel**) in ASS1 upregulated tumors, does not associate with a significant difference in patients' survival (same number of patients as indicated in **3G**), as compared to patients with cancers in which ASS1 is downregulated shown in **3G** (the effect sizes are negligible: ΔAUC= -0.001, -1E-5, and 0.04, respectively). We used student's t-test (2-tail) for statistical analysis. Unless otherwise specified, each graph represents the average of $n \geq 3$ experiments ± S.D. *, $P \leq 0.05$.


A.
Hypoxia score
1.00
0.75
0.50
0.25
0.00
group
Low
High
Low High
ASS1 expression
B.
Normoxia
Hypoxia
Normalized protein levels
Hif1α
ASS1
MCT4
C.
Normoxia
Hypoxia
Relative mRNA levels
ASS1
CAD
D.
ASS1 mRNA Relative levels
pH 7.4 Normoxia
pH 6.3 Normoxia
pH 6.3 Hypoxia
E.
F.
G.
CA9 high
CA9 low
n=905
n=932
MCT4 high
MCT4 low
n=905
n=931
CA9+MCT4 high
CA9+MCT4 low
n=905
n=932
Survival
Days

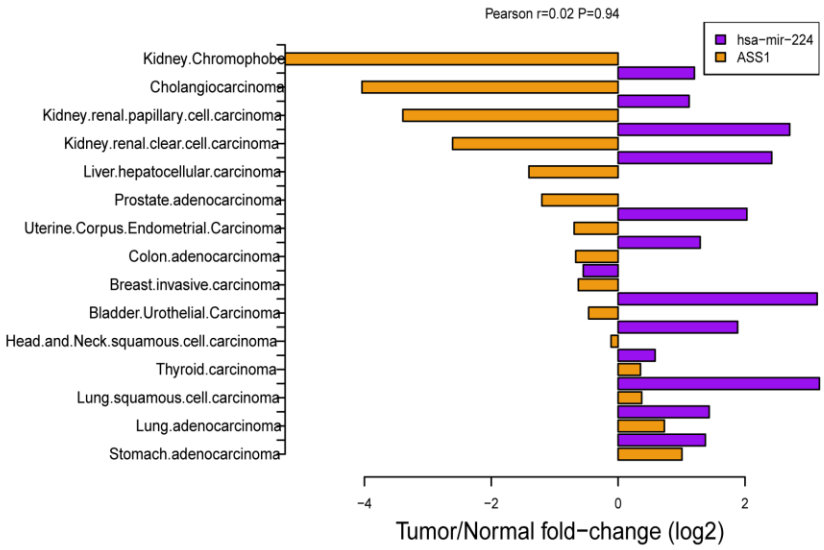
Supplementary Figure 4. miR-224-5p downregulates ASS1 in cancer.

(**A**) Target-scan analysis of the 3'UTR-ASS1 shows a conserved binding site in mammals for miR-224-5p. The image is taken from www.targetscan.org (**B**) TCGA analysis of RNA expression levels shows inverse correlation between ASS1 and miR224-5p expression levels. (**C**) Following 48 hours of hypoxia, miR-224-5p expression levels are upregulated in U2OS cells as compared to normoxic conditions. (**D**) Following hypoxia for 24 hours, ASS1 mRNA levels (**Left panel**) are downregulated whereas miR-224-5p expression levels (**Right panel**) are upregulated in murine B16-F10 melanoma as compared to normoxic conditions. (**E**) ASS1 mRNA levels are significantly downregulated in B16-F10 melanoma cells following transfection with miR-224-5p in normoxia. Addition of anti miR-224-5p rescued ASS1 levels. Averages done on four biological replicates generated in two independent experiments. Unless otherwise specified, we used student's t-test (2-tail) for statistical analysis and each graph represents the average of $n \geq 3$ experiments ± S.D. *, $P \leq 0.05$.

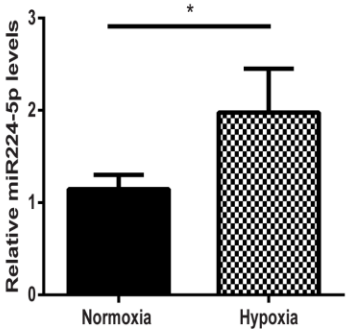
A.


Human ASS1 ENST00000352480.5 3' UTR length: 1357
829
ENST00000352480.5
0.1k
0.2k
0.3k
0.4k
0.5k
0.6k
0.7k
Conserved sites for miRNA families conserved among mammals
miR-224-5p
miR-329-3p/362-3p

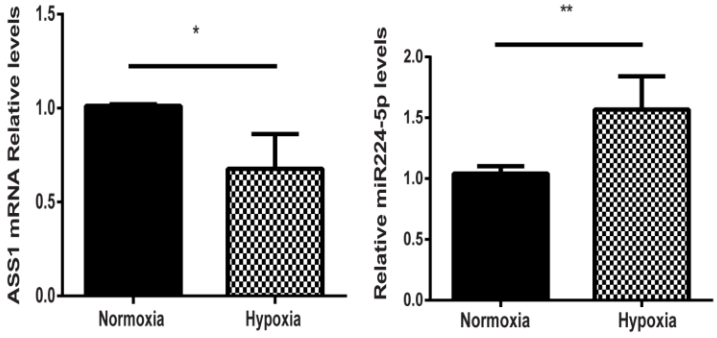
B.


Pearson r=0.02 P=0.94
hsa-mir-224
ASS1
Kidney.Chromophobe
Cholangiocarcinoma
Kidney.renal.papillary.cell.carcinoma
Kidney.renal.clear.cell.carcinoma
Liver.hepatocellular.carcinoma
Prostate.adenocarcinoma
Uterine.Corpus.Endometrial.Carcinoma
Colon.adenocarcinoma
Breast.invasive.carcinoma
Bladder.Urothelial.Carcinoma
Head.and.Neck.squamous.cell.carcinoma
Thyroid.carcinoma
Lung.squamous.cell.carcinoma
Lung.adenocarcinoma
Stomach.adenocarcinoma
−4
−2
0
2
Tumor/Normal fold−change (log2)

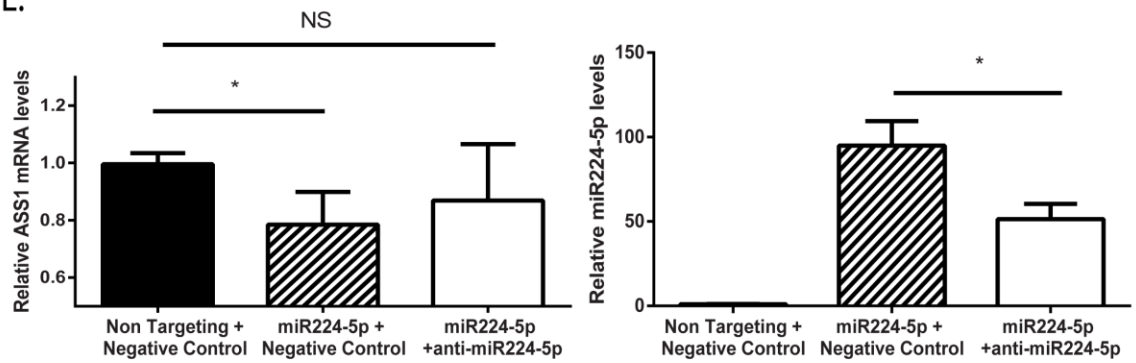
C.


Relative miR224-5p levels
3
2
1
0
*
Normoxia
Hypoxia

D.

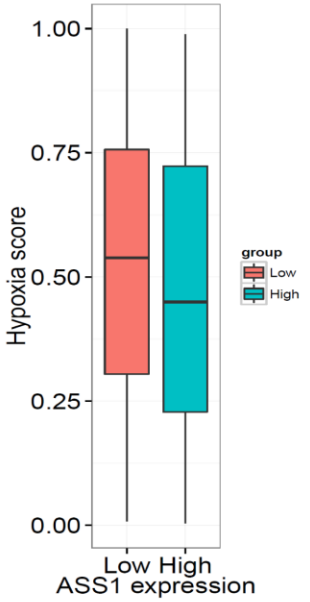

ASS1 mRNA Relative levels
1.5
1.0
0.5
0.0
*
Normoxia
Hypoxia
Relative miR224-5p levels
2.0
1.5
1.0
0.5
0.0
**
Normoxia
Hypoxia

E.

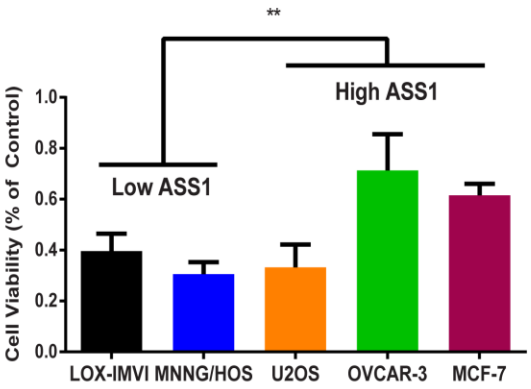

Relative ASS1 mRNA levels
1.2
1.0
0.8
0.6
NS
*
Non Targeting + Negative Control
miR224-5p + Negative Control
miR224-5p +anti-miR224-5p
Relative miR224-5p levels
150
100
50
0
*
Non Targeting + Negative Control
miR224-5p + Negative Control
miR224-5p +anti-miR224-5p

Supplementary Figure 5. Cancer cells with low expression of ASS1 invade more and have increased sensitivity to glutamine and bi-carbonate depletion. (**A**) Pan cancer analysis based on TCGA metastatic samples show that tumors with low ASS1 levels have a higher hypoxia-score, independent of HIF1α target genes (Wilcoxon rank sum P<0.019, see Methods). (**B**) A viability assay of five different cancer cell lines divided into two groups based on ASS1 expression as either "High ASS1" or "Low ASS1", following incubation of 48 hours with or without glutamine, under hypoxic ($1\%O_2$) conditions**.** Viability is normalized to control cells grown with glutamine. (**C**) Normalized mRNA expression levels of control breast cancer MCF-7 cells infected with empty vector *vs.* two clones of *shASS1* MCF-7 cells. (**D**) A representative western blot image showing MCF-7 breast cancer infected with either *shGFP* or two clones of *shASS1*. (**E**) Viability assays using an additional *shASS1*- clone#2**.** Similar to our results shown in Figure **5C-D**, MCF-7 breast cancer cells with ASS1 downregulation are less viable following incubation with either: 6-Diazo-5-oxo-L-norleucine (DON; 50 μM; **left panel**) or following incubation with S0859 (100 μM; **right panel**). Viability is normalized to non-treated cells (cells grown in the absence of inhibitors). The experiments have been performed under pHe 7.4**.** For the multiple cell line comparisons in **4B**, we used Two-way ANNOVA (with A-priori contrast: MNNG/HOS, LOX-IMVI vs. U2OS, MCF-7 and OVCAR-3). For all other comparisons, student's t-test was used (2-tail) for statistical analysis. Unless otherwise specified, each graph represents the average of $n \geq 3$ experiments ± S.D. *, $P \leq 0.05$; **, $P \leq 0.01$.

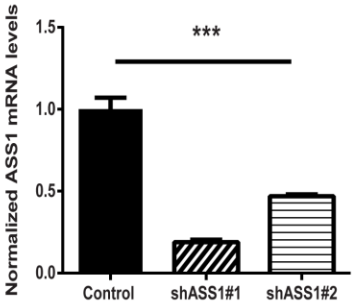
A.


1.00
0.75
0.50
0.25
0.00
Hypoxia score
group
Low
High
Low High
ASS1 expression

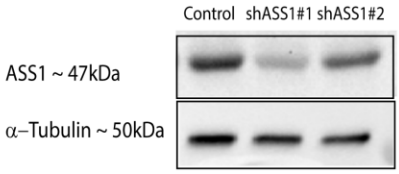
B.


**
High ASS1
Low ASS1
Cell Viability (% of Control)
1.0
0.8
0.6
0.4
0.2
0.0
LOX-IMVI
MNNG/HOS
U2OS
OVCAR-3
MCF-7

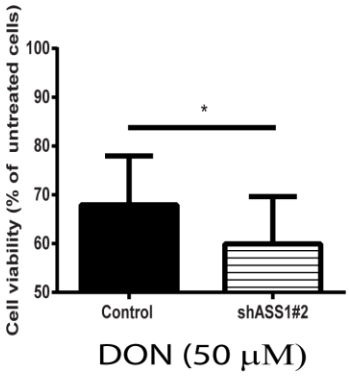
C.

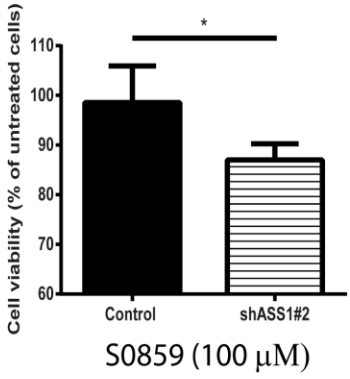

***
Normalized ASS1 mRNA levels
1.5
1.0
0.5
0.0
Control
shASS1#1
shASS1#2

D.


Control
shASS1#1
shASS1#2
ASS1 ~ 47kDa
α–Tubulin ~ 50kDa

E.


Cell viability (% of untreated cells)
100
90
80
70
60
50
*
Control
shASS1#2
DON (50 μM)


Cell viability (% of untreated cells)
110
100
90
80
70
60
*
Control
shASS1#2
S0859 (100 μM)