Supplemental Information

Aspartate β-hydroxylase modulates cellular senescence via glycogen synthase kinase 3β in hepatocellular carcinoma

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Supplementary Material

Cell Lines

The FOCUS cell line was derived from a hepatitis B virus (HBV)-related poorly differentiated human HCC tumor as described previously.(1) Huh7, HepG2 (as the other human HCC cell lines) and HEK293 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM of L-glutamine and cultivated in a humidified incubator at 37°C with 5% CO_2.

Lentivirus Production and Infection for knockdown of ASPH

HEK293 cell line was used to produce lentivirus by transfection with TransIT®-LT1 Reagent (Mirus Bio LLC, Madison, WI, USA). Lentivirus plasmids, pLKO.1-shRNA-luciferase and pLKO.1-shRNA-ASPH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fifteen μg of lentivirus plasmid, 8.5 μg of psPAX2 (contains GAG, POL) and 5 μg of pMD2.G (contains VSVg) were used to produce virus in 10-cm dishes. Culture medium containing virus was collected 48 hours after transfection, filtrated through 0.45 μm filter to remove cell debris or cells, and then added to the target HCC cells.

Stable cell line with knockout of ASPH

We established stable HepG2 cell line with knockout of ASPH using the CRISPR/Cas9 system. The sgRNA expression oligos targeted human ASPH (ATGGAGGACACAAGAATGGG; TAAACAGAGACAAAGCATGG; and CCTAGTACAAAATACGTGACGTAGAA, respectively) were introduced into the pRSGC1-U6-sg-CMV-Cas9-2A-Puro vector (Cellecta, Mountain View, CA, USA). A mixture of 0.5 彡g of pRSGC1-U6-sg-CMV-Cas9-2A-Puro plasmid DNA containing each sgRNA sequence and 0.5 彡g of HIV Packaging Mix plasmids (GeneCopoeia, Rockville,

MD, USA) were transfected into about 80% confluent HEK293 cells in 6-well plates. Supernatants containing viral particles were collected after 2 days of transfection and diluted by the corresponding medium added with polybrene at the final concentration of 8 μg/ml. HepG2 cells were infected with the supernatants overnight. The next day cells were split into 10 cm dishes. One day later these cells were selected in EMEM medium containing 1.2 μg/ml puromycin.

Transient Transfection

Wild type (WT)-GSK3β (#16260) and constitutive active (CA)-GSK3β (#16261) plasmids were purchased from Addgene (Cambridge, MA, USA). WT-ASPH (#EX-Z8758-Lv105) plasmid was purchased from GeneCopoeia. The structure of WT-ASPH construct was inserted into the pcDNA3 vector with 5' HA tagged vector. Empty vector (EV), WT-GSK3β, CA-GSK3β and HA-tagged ASPH plasmids were transfected using TransIT®-LT1 Reagent (Mirus Bio LLC) according to the protocol provided by the manufacturer. All assays were performed after 48-hour's transfection.

Western Blot

Cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. Blots were incubated with each primary antibody overnight at 4°C. Appropriate secondary antibodies were added for 1 hour, and protein expression was visualized with enhanced chemiluminescence by ECL Western blotting system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Waltham, MA, USA). All experiments were performed in triplicate. Densitometric analysis was performed by Image J software (NIH).

Antibodies for Western Blot

Western blot analysis was performed using primary antibodies against ASPH (rabbit polyclonal), GSK3β (Cell Signaling Technology Inc, Danvers, MA, #9315), phospho-GSK3β (Ser9) (Cell Signaling, #9336), Glycogen Synthase (GS) (Cell Signaling, #3893), phospho-GS (Ser641) (Cell Signaling, #3891), p16 (Abcam, Cambridge, MA, ab51243), p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-1311), p15 (Santa Cruz, sc-612), p21 (Santa Cruz, sc-6246), cyclin D1 (Santa Cruz, sc-718), PCNA (Santa Cruz, sc-7907), AKT (Cell Signaling, #9272), phospho-AKT (Ser473) (Cell Signaling, #9271), p38 (Cell Signaling, #9212), phospho-p38 (Thr180/Tyr182) (Cell Signaling, #9211), ERK (Cell Signaling, #9102), phosphor-ERK (Thr202/ Tyr204) (Cell Signaling, #4370), JNK (Cell Signaling, #9252), phospho-JNK (Thr183/Tyr185) (Cell Signaling, #9251), S6K (Cell Signaling, #9202), phospho-S6K (Thr389) (Cell Signaling, #9205), Caspase-3 (Cell Signaling, #9662), α-tubulin (Sigma-Aldrich, T9026), Actin (Santa Cruz, sc-1616) and HA-probe (Santa Cruz, sc-7392).

Co-Immunoprecipitation

HEK293 cells were transiently co-transfected with HA-tagged ASPH and either WT-GSK3β or EV plasmids **(for Figure 5A and 5B),** as well as with WT-GSK3β and either HA-tagged ASPH or EV plasmids **(for Figure 5C, 5D, and Supplementary Figure 6B)**. HEK293 cells were also transiently transfected with WT-ASPH to test the interaction of the overexpressed ASPH and endogenous GSK3β **(for Supplementary Figure 7)**. Cells were treated with 20ng/ml of insulin for 1 hour to enhance ASPH expression level as described.(2) Immunoaffinity purification was performed using either HA-probe (Santa Cruz, sc-7392) or anti-GSK3β (Cell Signaling, #9315) coupled to Protein A/G PLUS-Agarose (Santa Cruz, sc-2003). For negative control in immunoaffinity purification, normal mouse IgG (Santa Cruz, sc-2025) and normal rabbit IgG (Santa Cruz, sc-2027) were used. Western blot analysis was carried out using the following antibodies: HA-probe (Santa Cruz, sc-7392), anti-ASPH (rabbit polyclonal), anti-GSK3β (Cell

Signaling, #9315), anti-AKT (Cell Signaling, #9272), anti-p38 (Cell Signaling, #9212), anti-ERK (Cell Signaling, #9102), anti-JNK (Cell Signaling, #9252) and anti-S6K (Cell Signaling, #9202).

Quantitative real-time RT-PCR

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Grand Island, NY), and reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturers' instruction. Quantitative real-time PCR (qRT-PCR) was carried out on a Mastercycler ep realplex instrument and software (Eppendorf AG, Hamburg, Germany), using SYBR Green PCR reagents. Relative quantification was performed using ΔΔCt method. The expression of the target gene was normalized relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control. Dissociation curves were generated to evaluate PCR product specificity and purity. The designed PCR primers were as follows: GAPDH forward primer 5'-CTTAGCACCCCCTGGCCAAG-3' and GAPDH reverse primer 5'-GATGTTCTGGAGAGCCCCG-3'; IL6 forward primer 5'-CCTGAACCTTCCAAAGATGGC-3' and IL6 reverse primer 5'-TTCACCAGGCAAGTCTCCTCA-3'; IL1a forward primer 5'-AGATGCCTGAGATACCCAAAACC-3' and IL1a reverse primer 5'-CCAAGCACACCCAGTAGTCT-3'; IL1b forward primer 5'-ATGATGGCTTATTACAGTGGCAA-3' and IL1b reverse primer 5'-GTCGGAGATTCGTAGCTGGA-3'; IL8 forward primer 5'-ACTGAGAGTGATTGAGAGTGGAC-3' and IL8 reverse primer 5'-AACCCTCTGCACCCAGTTTTC-3'; and CXCL1 forward primer 5'-ACTCAAGAATGGGCGGAAAG-3' and CXCL1 reverse primer 5'-CCCTTCTGGTCAGTTGGATTT-3'.

Immunofluorescence

Cells were cultured on 4-well chamber slides and fixed with 4% paraformaldehyde and permeabilized. Cells were incubated with monoclonal rabbit anti-phospho-Histone H2AX (Ser139) (diluted 1:400; Cell Signaling, #9718) at 4 ℃ overnight. After incubation with Alexa Fluor anti-rabbit IgG (Cell Signaling) as the secondary antibody for 1 hour, the slides were counterstained by VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). The Zeiss LSM510 Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) was used to visualize the immunofluorescence staining. Camptothecin (CPT) treatment (10 μM for 1 hour) was performed to induce γH2AX (phospho-Ser139) (3) before staining.

Analysis of intracellular ROS

To assess the generation of intracellular ROS levels, cells were incubated with 20 μM DCF-DA (Abcam, ab113851) for 40 min at 37 ℃. The peak excitation wavelength for oxidized DCF was 485 nm and for emission was 535 nm. Treatment with H_2O_2 (100 μM or 400 μM, for 12 hours) at 37 °C was performed to induce intracellular ROS levels (4) before analysis.

Statistical Analysis

Statistical analysis was carried out using Student's t tests. Results were shown as Mean ± S.D. *in vitro*, and Mean ± S.E.M. *in vivo*. A p value of <0.05 was considered as statistically significant.

Supplementary Figure 1. SASPs are up-regulated after inhibition of ASPH expression in human HCC cell lines. Relative IL6, IL1a, IL1b, IL8 and CXCL1 transcript levels in human HCC cell lines were determined by qRT-PCR. Levels of mRNA were normalized to GAPDH and presented relative to the control (shLuc or EV) cells. **(A)** FOCUS, **(B)** Huh7 and

(C) HepG2. Data are represented as Mean ± S.D. Similar results were observed in three independent experiments.

Supplementary Figure 2. Expression of γH2AX was induced after inhibition of ASPH in human HCC cell lines. Immunofluorescence was used to detect γH2AX (red) and nuclei (blue) in human HCC cell lines. Top panels showed non-treated control and bottom panels showed camptothecin (CPT) treated cells (10 μM for 1 hour). Right panels showed shLuc (or EV) control and left panels showed shASPH (or KO-ASPH) cells. **(A)** FOCUS, **(B)** Huh7 and **(C)** HepG2 (200×).

Supplementary Figure 3. Intracellular ROS levels are induced after inhibition of ASPH in human HCC cell lines. Intracellular ROS levels were analyzed and expressed relative to the control (shLuc or EV) cells. **(A)** FOCUS were treated with 0 μM or 100 μM H_2O_2 for 12 hours, **(B)** Huh7 were treated with 0 μM or 400 μM for 12 hours, and **(C)** HepG2 were treated with 0 μM or 400 μM for 12 hours. Results are represented as Mean ± S.D. of three independent experiments. $^{**}P < 0.01$ compared to the control cells (shLuc or EV).

Supplementary Figure 4. Treatment of human HCC cells with the β-hydroxylase inhibitor MO-I-1151 increased p15 and p21 protein expression levels. Protein expression of p15 and p21 were evaluated by Western blot analysis, after a 24-hour exposure in response to treatment with DMSO (control) or different concentrations of MO-I-1151 (from 1.25 to 5 μM) in FOCUS and Huh7 HCC cell lines.

Supplementary Figure 5. The transient transfection efficacy in human HCC cell lines. Representative photographs of morphology (top) and GFP (bottom, green) in **(A)** FOCUS and **(B)** Huh7 cell lines after 24-hour (left panel) and 48-hour transfection (right panel) (100×).

Supplementary Figure 6. ASPH did not influence the expressions of upstream kinases of GSK3β. (A) Protein expressions of the upstream kinases of GSK3β were evaluated in

human HCC cell lines (FOCUS and Huh7) with the control (shLuc) or knockdown of ASPH (shASPH) by Western blot analysis using antibodies against AKT, phospho-AKT, p38, phospho-p38, ERK, phospho-ERK, JNK, phospho-JNK, S6K and phospho-S6K. **(B)** Protein expression of the upstream kinases of GSK3β were evaluated in co-transfected HEK293 cells with WT-GSK3β and either HA-tagged ASPH or EV by Western blot analysis.

Supplementary Figure 7. ASPH binds to endogenous GSK3β in HEK293 cells. The following assays were performed after 48-hour transfection of WT- ASPH. Cells were treated with 20ng/ml of Insulin for 1 hour, before creating cell lysates. After immunoprecipitation using control IgG or anti-GSK3β antibody, immunoblotting was performed with antibody directed against ASPH. Antibody against α-tubulin was used as a loading control.

References

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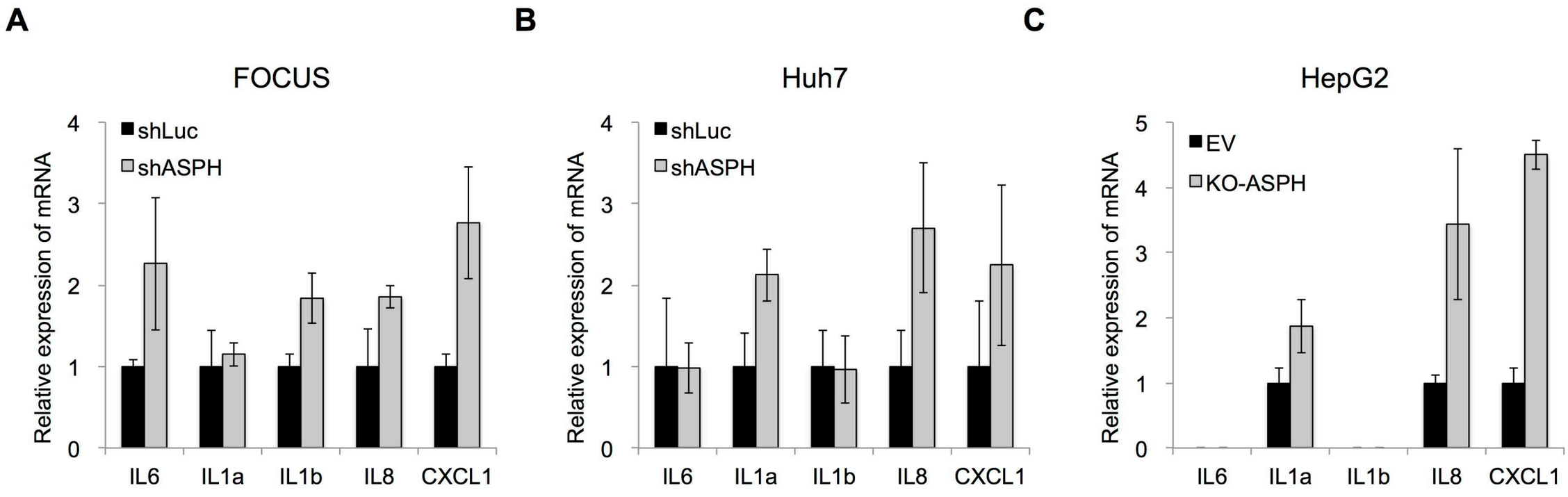
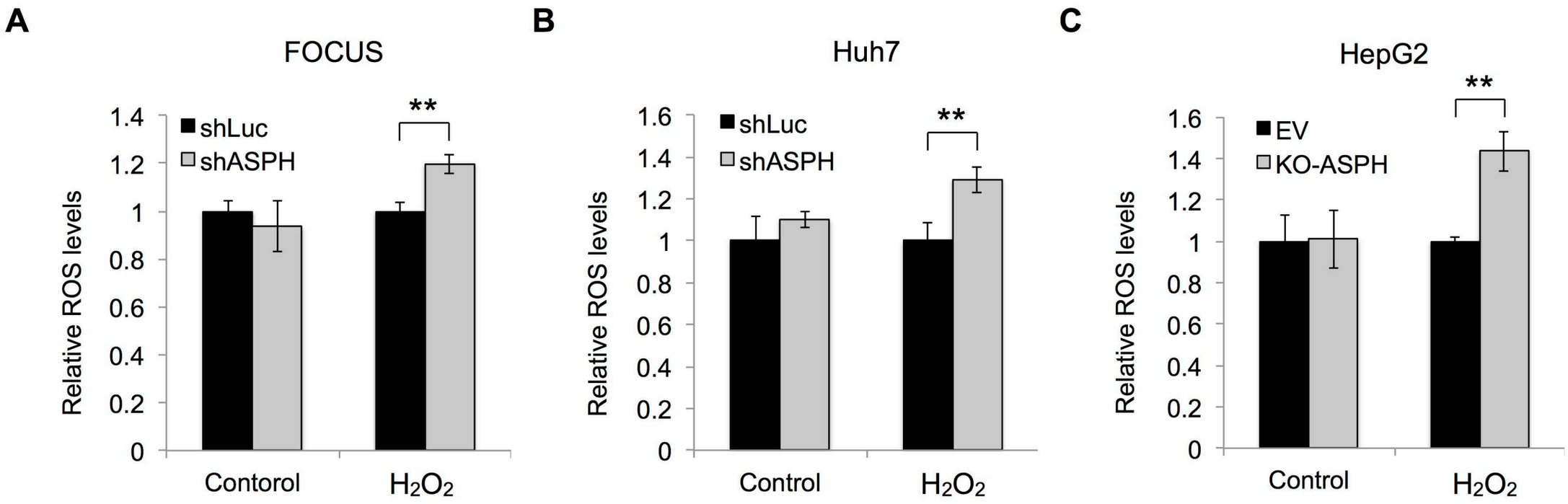
Supplementary Figure 1



Supplementary Figure 2

Supplementary Figure 3



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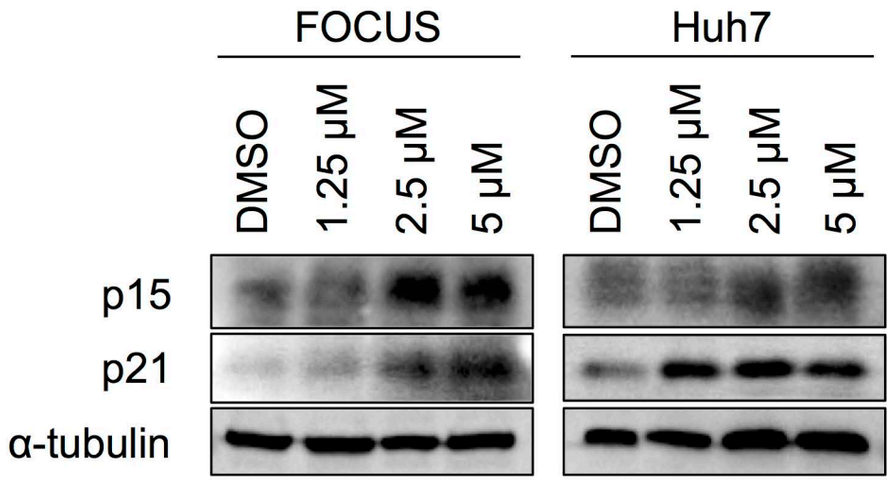
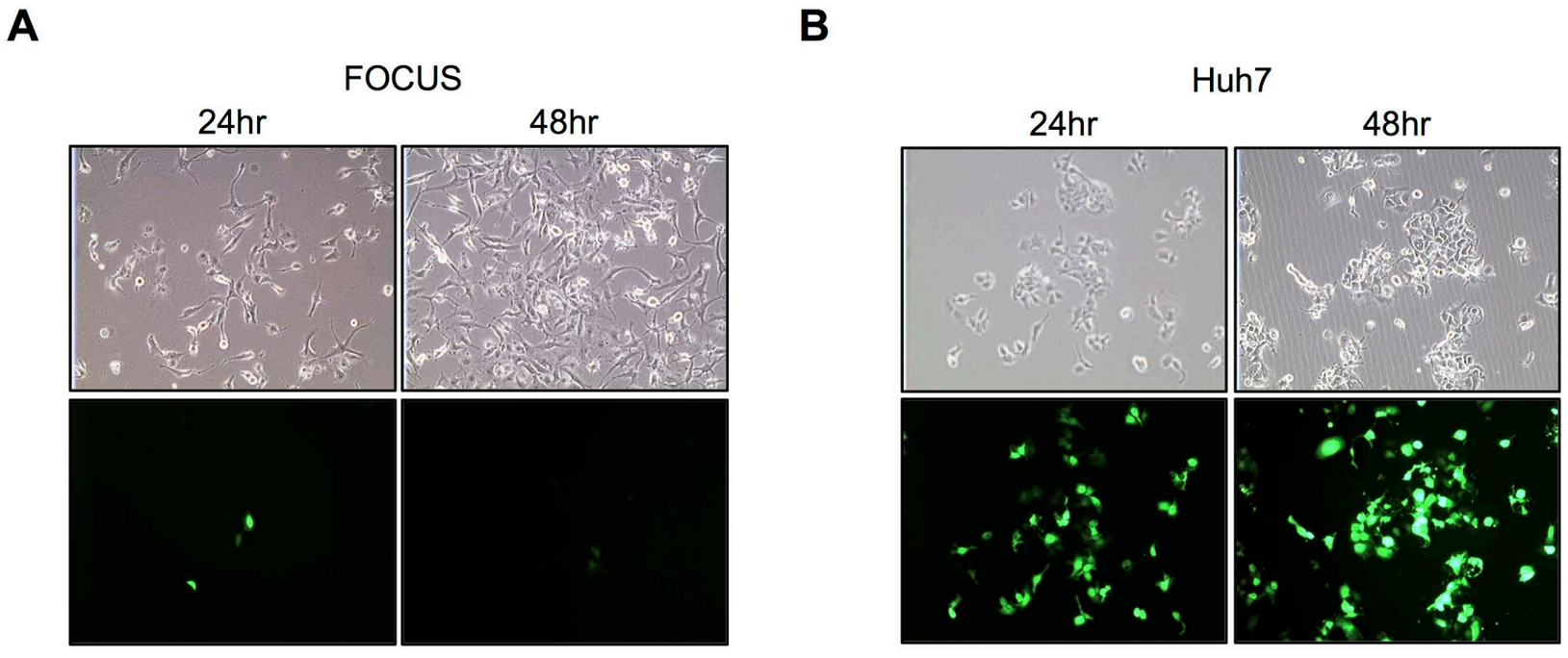
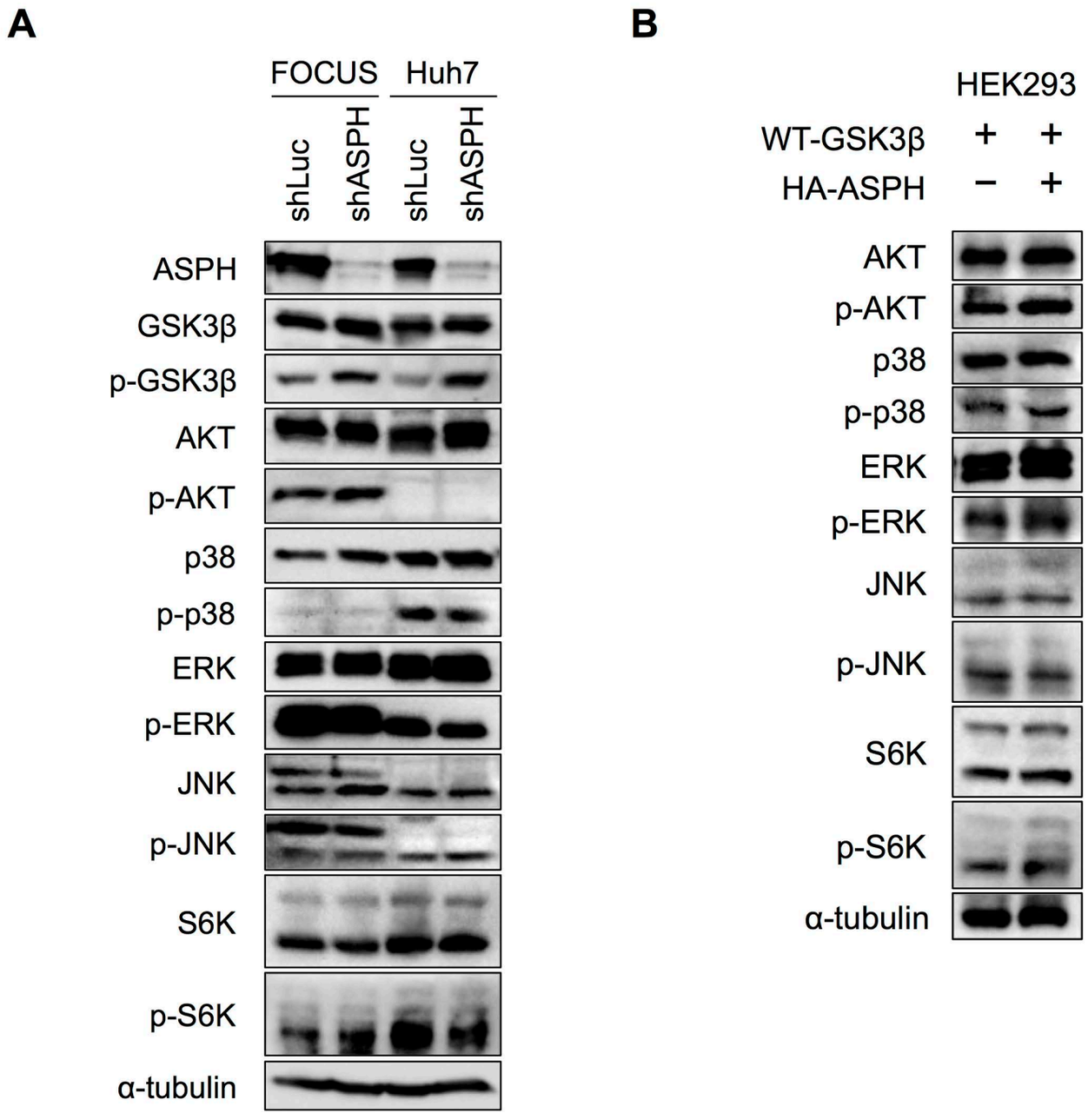


Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7