Supplementary methods

Shoc2 *In Vitro* Ubiquitination Assay

^{35}S-labelled Shoc2 was prepared using a TNT quick Coupled transcription/ translation System (Promega) as recommended by manufacturer. For the ubiquitination assay 5 µl of synthesized ^{35}S-Shoc2 was combined with GST-HECT (2 µg), Mg-ATP (4µM), His-Ub (5µg), His6 UbE1 (100 nM), GST-UbcH5a (200 nM) in a buffer (50 mM Tris-HCl pH 7.5, 2.5 mM MgCl2, 0.2 mM DTT and 2 mM ATP) and incubated for 90 min at 37°C. Shoc2 was immunoprecipitated using anti-Shoc2 antibody (Proteintech) and eluted into 2X Laemmli sample buffer. Samples were resolved by SDS-PAGE and immunoblotted anti-ubiquitin antibody (Covance). His-Ub was detected using enhanced chemiluminescence detection, followed by Shoc2 detection using phosphor imaging system Typhoon FLA 9500 (GE).

Fluorescence Imaging of Living Cells:

Cells were plated 24 hours before the experiment onto 35-mm glass-bottom dishes (Mattek). All images were acquired using a Mariannas Imaging system consisting of a Zeiss inverted microscope equipped with a cooled CCD CoolSnap HQ (Roper, CA), dual filter wheels and a Xenon 175 W light source, all controlled by SlideBook 5.32 software (Intelligent Imaging Innovations, Denver, CO). The detection of tag YFP fluorescence was performed using a YFP channel. Images were acquired using 2x2 binning mode. Image analysis was performed using the SlideBook 5.32 software. The final arrangement of images was performed using adobe Photoshop (Adobe Systems, Mountain View, CA).

Supplementary Figures Legends

Supplementary Figure 1

(A) 293FT cells were co-transfected with HA-HECT (W/T), HA-HECT (C/A) and GST-Shoc2. GST-Shoc2 immunoprecipitates were analyzed by immune-blot using HA and GST antibodies.
(B) GST-HECT proteins were affinity purified, separated by SDS-PACE and visualized by SYPRO® Ruby Protein Gel Staining.
(C) MBP-Shoc2 proteins were affinity purified, separated by SDS-PACE, visualized by SYPRO® Ruby Protein Gel Staining (left panel; SR), and detected with anti-Shoc2 antibody (right panel; IB).

Supplementary Figure 2

(A) GST-Shoc2 was immunoprecipitated from 293FT cells in presence of SDS. Shoc2 ubiquitination was detected with anti-Ub antibody.
(B) GST-Shoc2 was immunoprecipitated from 293FT cells co-transfected with HA-Ub. Cells were treated with the proteasome inhibitor MG132. Shoc2 ubiquitination was detected with anti-HA antibody.
(C) ^{35}S-labeled, *in vitro* translated Shoc2 (^{35}S-Shoc2) was monitored by autoradiography.
(D) *in vitro* ubiquitination assays were performed with ^{35}S -labeled, *in vitro* translated Shoc2 (^{35}S-Shoc2). Shoc2 was immunoprecipitated using anti-Shoc2 antibody and separated by SDS-PACE. ^{35}S-Shoc2 ubiquitination was detected with anti-Ub antibody.

Supplementary Figure 3

Cos-NT, Cos-LV1 and Cos-SR cells were transiently transfected with HUWE1 siRNA. 48 hours post-transfection cells were treated with MG132. RAF-1 was precipitated using anti-RAF-1 antibody. RAF-1 ubiquitination was detected with anti-Ub antibody. The expression of HUWE1, RAF-1 and Shoc2 was analyzed using specific antibodies. IP-immunoprecipitation, IB-immuno-blot, NT-non-targeting siRNA, H-HUWE1 siRNA.

Supplementary Figure 4

T47D cells were transiently transfected with HUWE siRNA as indicated. 48 hours after transfection cells were starved for 16 hours, then treated with EGF and harvested for immunoblotting. The expression of Shoc2, HUWE1, RAF-1, pERK1/2, total ERK1/2 and GAPDH was analyzed using specific antibodies.

Supplementary Figure 5

(A) A representative spectra that demonstrate ubiquitination at Lys 369 of Shoc2.
(B) 293FT cells were co-transfected with Shoc2(WT)-YFP, Shoc2(7KR)-YFP, HA-M-RAS or GST-RAF-1. Both of Shoc2(WT)-YFP and Shoc2(7KR)-YFP were detected in HA-M-RAS and GST-RAF-1 immunoprecipitates. Immunoprecipitates were analyzed by immunoblot using HA, RAF-1 and Shoc2 antibodies.
(C) Cellular distribution of the Shoc2(WT)-YFP and Shoc2(7KR)-YFP in HeLa cells constitutively expressing Shoc2, scale bar, 10 μm.

(D) HeLa cells constitutively expressing Shoc2-YFP or the Shoc2 (7KR)-YFP were treated with cycloheximide (30μM) for 4, 8, 15 and 24 hours. Protein levels were verified by western blot using anti-Shoc2, anti-Cyclin D1 and anti-GAPDH antibodies.

Supplementary Figure 6

(A) Cos-SY cells stably expressing either Shoc2(WT)-YFP or Shoc2(7KR)-YFP were transiently transfected with HUWE1 siRNA. 48 hours post-transfection cells were serum-starved for 16 hours and stimulated with EGF. The expression of indicated proteins was analyzed using specific antibodies. NT-non-targeting siRNA.

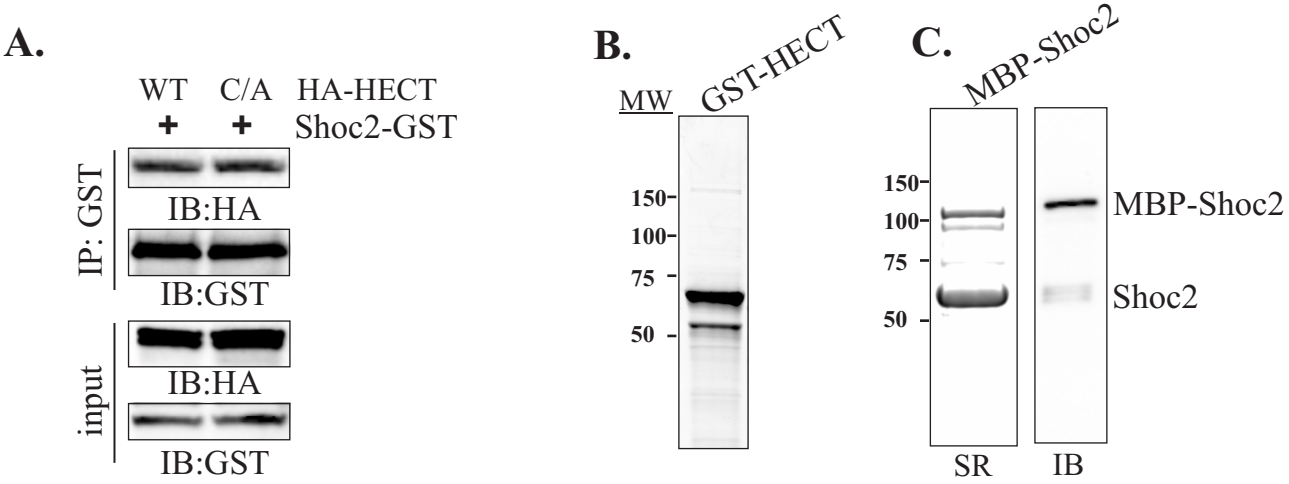
(B) Cos-SY cells stably expressing either Shoc2(WT)-YFP or Shoc2(7KR)-YFP were serum starved and treated with EGF. The cell lysates were probed for phosphorylated RAF-1(p338), phosphorylated MEK1/2, phosphorylated ERK1/2, RAF-1 and GAPDH. The experiment is representative of three independent experiments.

(C) Cos-SR cells were transfected with GST-RAF-1. 48 hours post-transfection cells were serum-starved for 16 hours and stimulated with EGF for 7 min. The expression of RAF-1, pERK1/2 and GAPDH was analyzed using specific antibodies

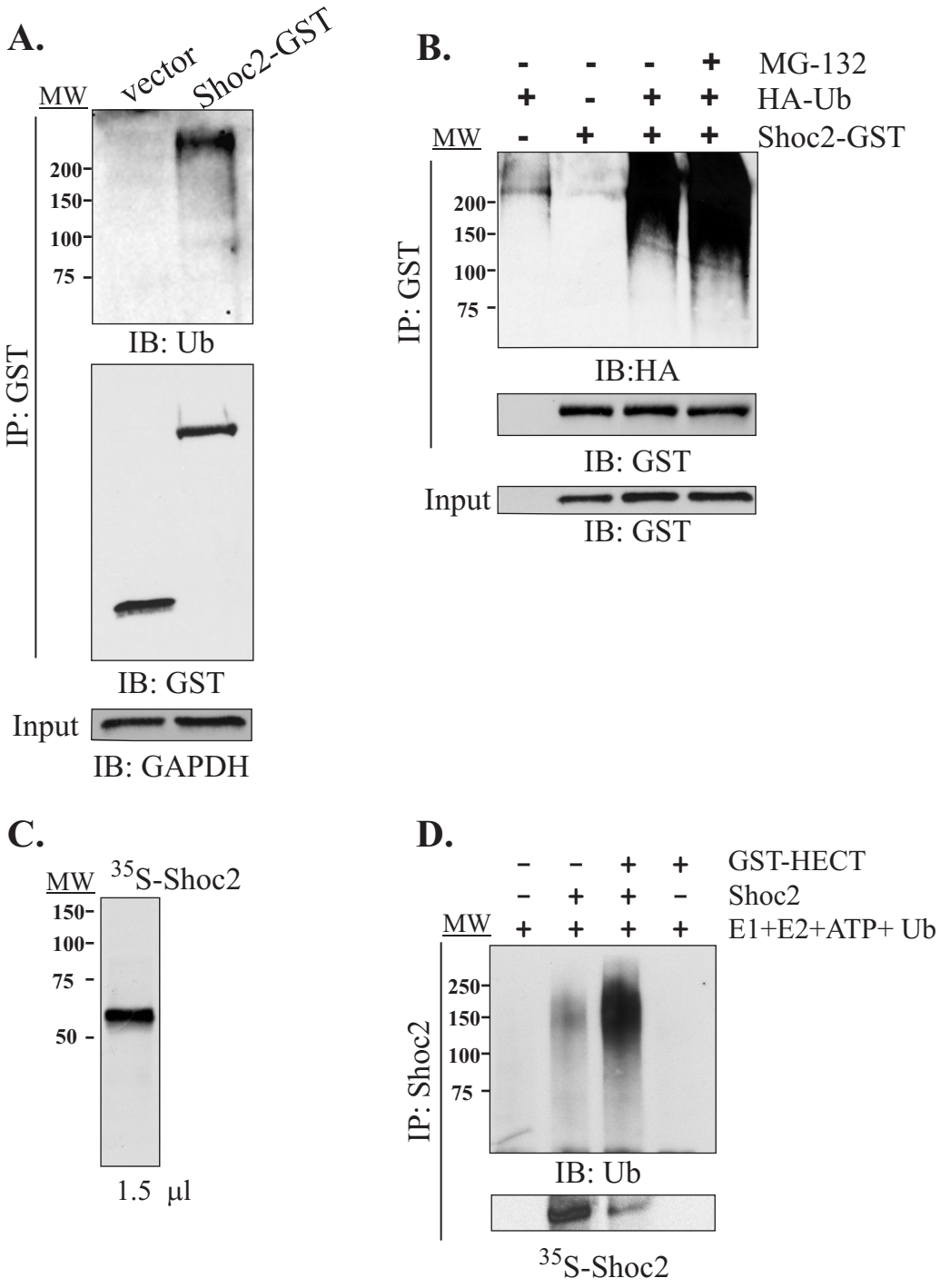
(D) Equal numbers of Cos-SY cells constitutively expressing either Shoc2(WT)-YFP or Shoc2(7KR)-YFP mutant of Shoc2 were plated onto 24 wells, and the numbers were counted 24, 48, 72 hours after seeding. The graph depicts the mean number of triplicate experiments ±SD.

(E) Cell viability of Cos-SY cells constitutively expressing either Shoc2(WT)-YFP or Shoc2(7KR)-YFP mutant of Shoc2 was measured using the MTS assay 24, 48, 72, 96 hours after seeding. Cell viability was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay. The graph depicts the mean number of triplicate experiments ±SD.

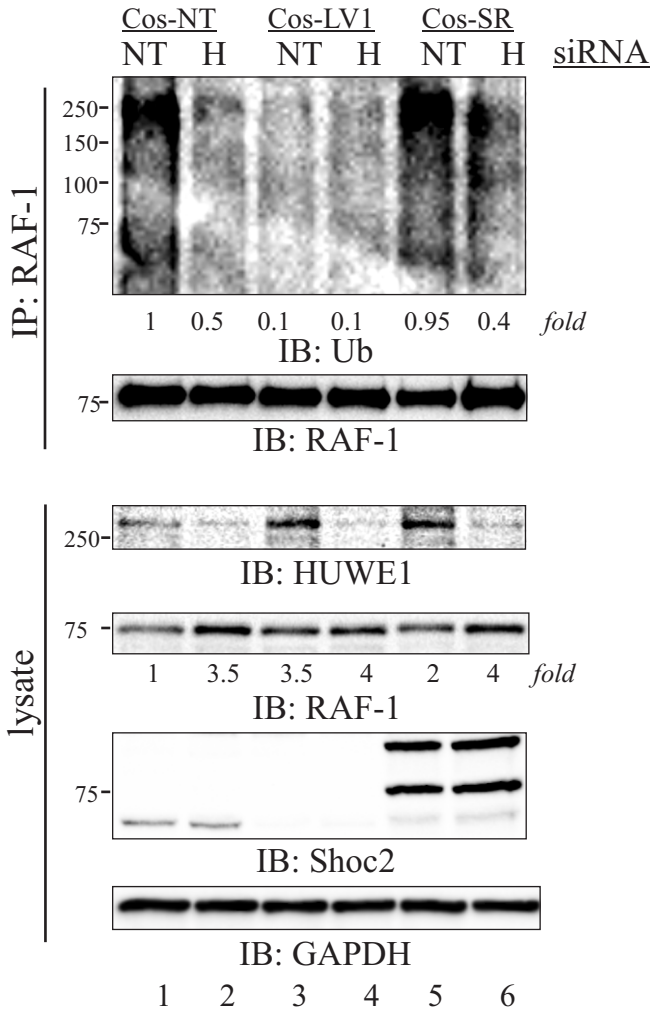
Supplemental Figure 1



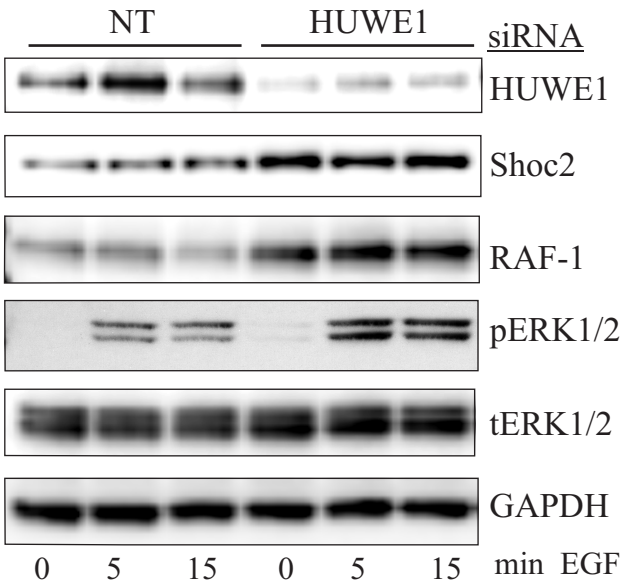
Supplemental Figure 2



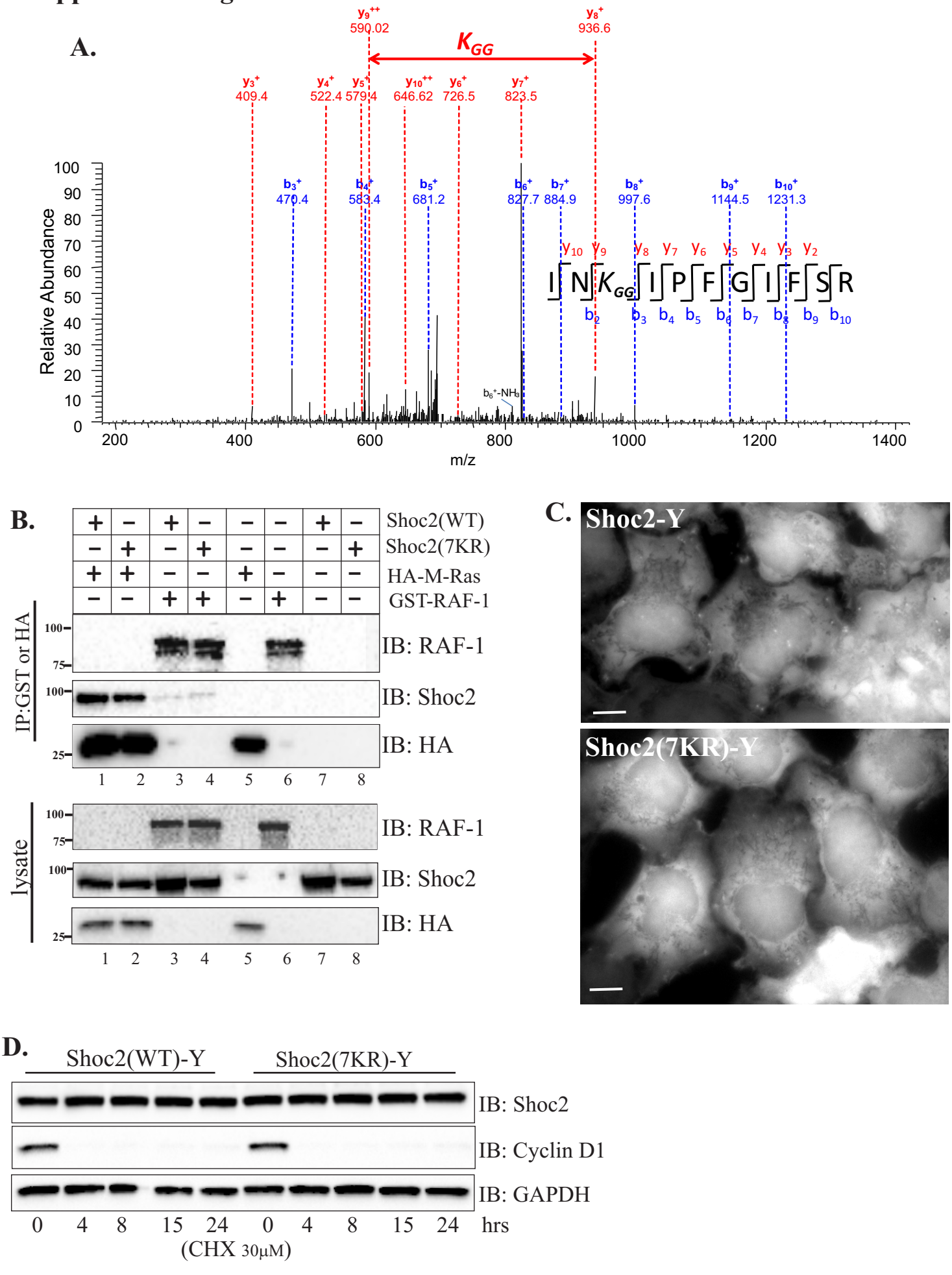
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6