SUPPLEMENTARY INFORMATION

Persistent Reduction of Oxysterol-binding Protein Caused by Compound Treatment Induces Prophylactic Anti-Viral Activity

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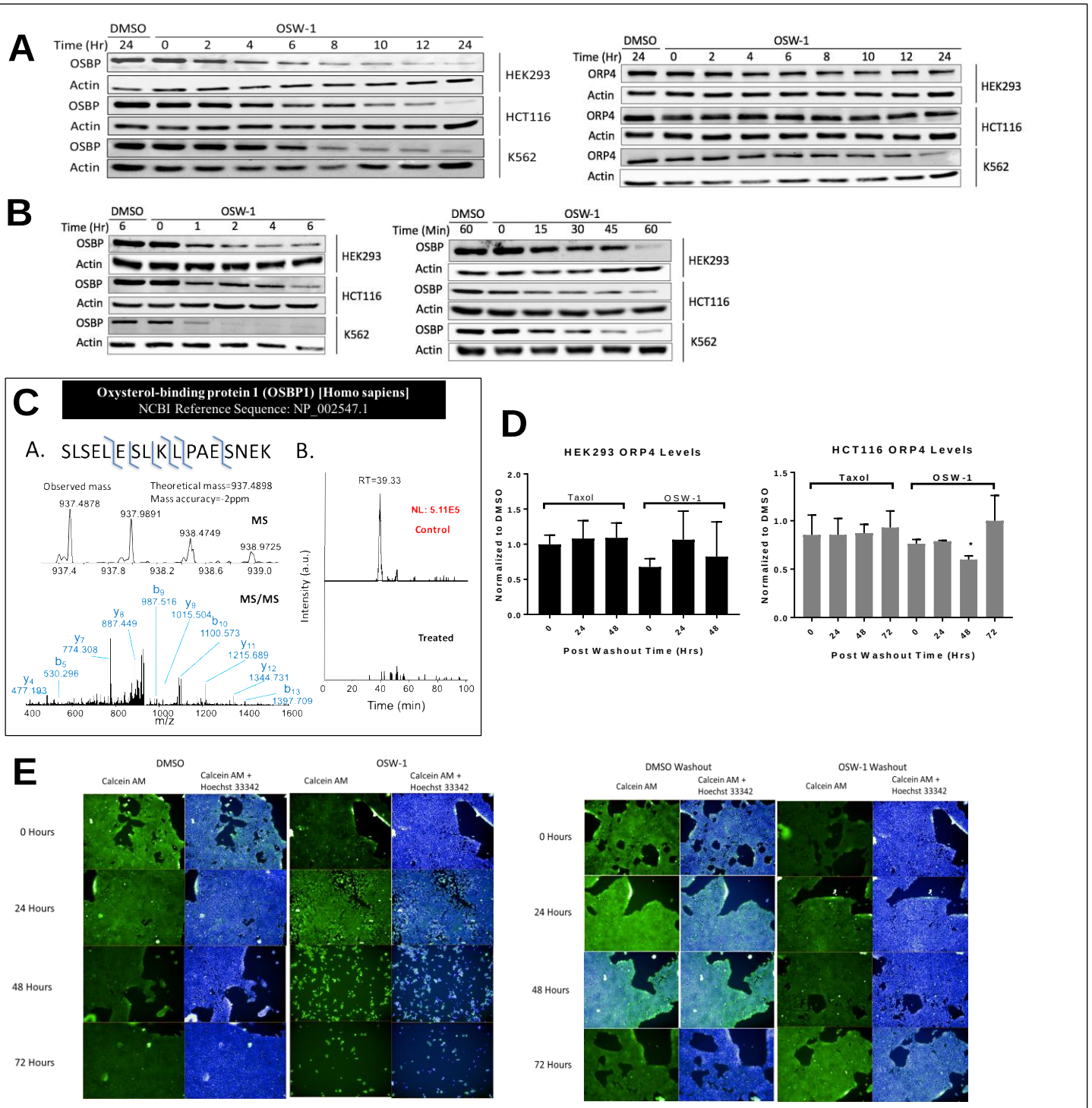
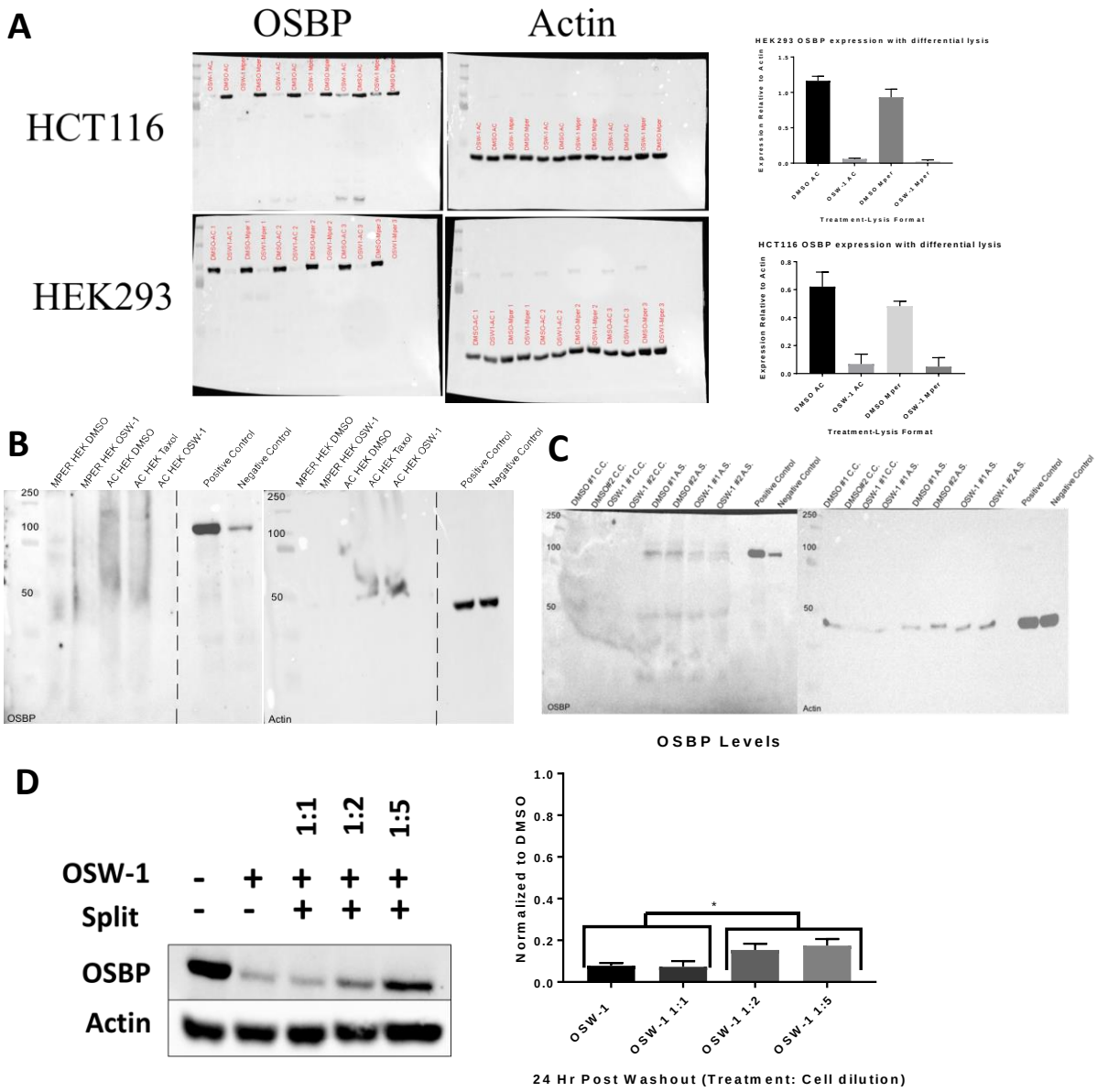
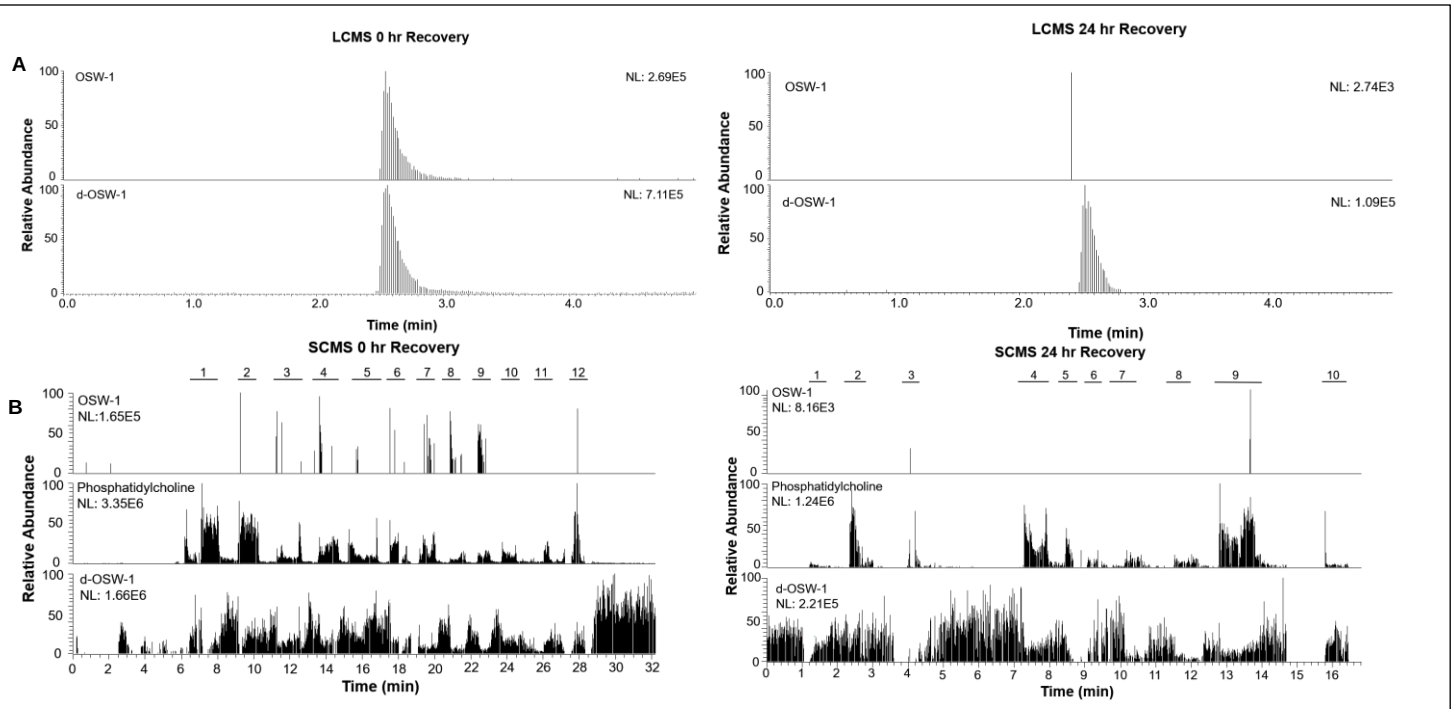


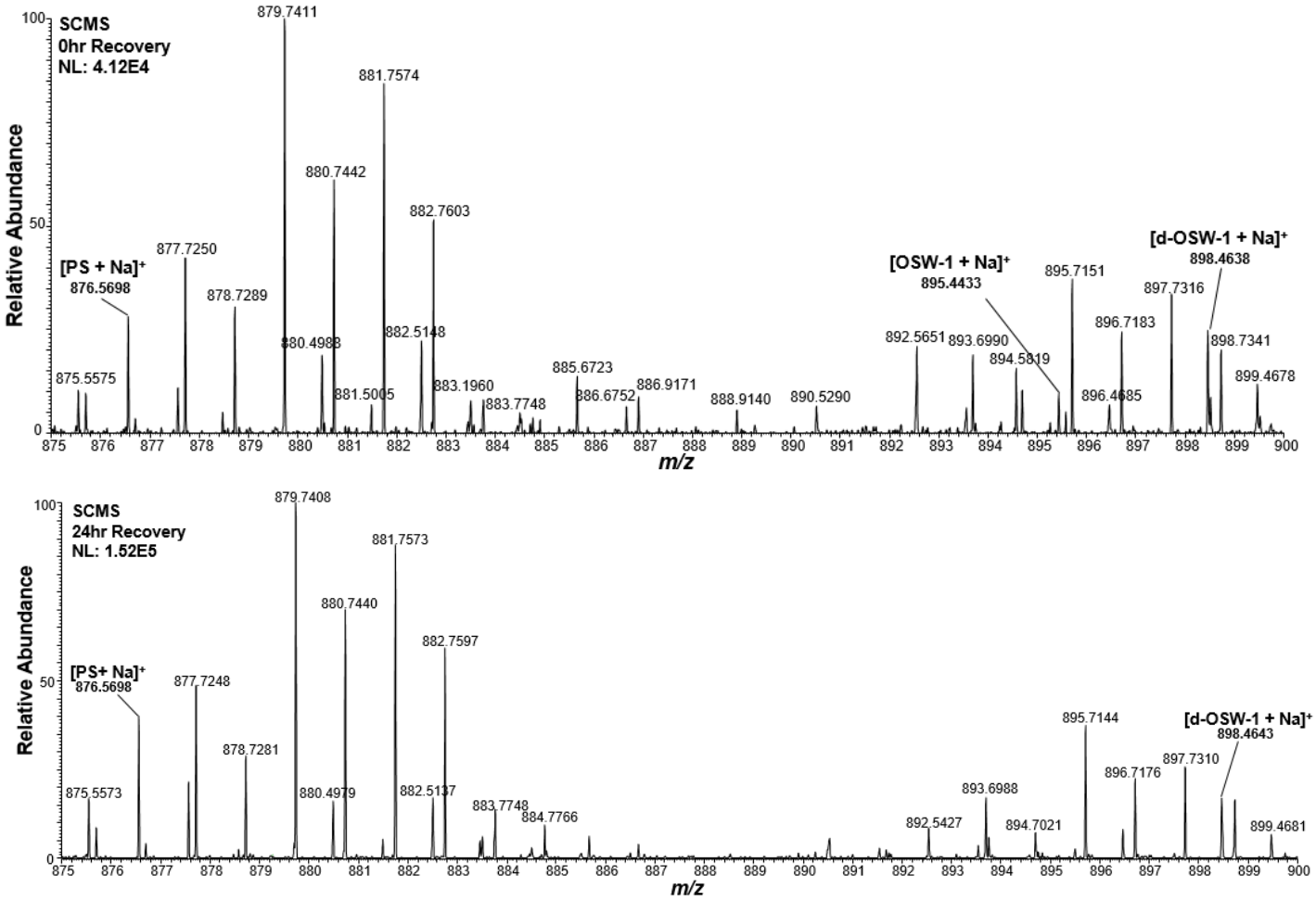
Supp. Figure 1) Effects of OSW-1 Compound Treatment on OSBP Levels and Cells. ***(A)*** *Determination of OSBP and ORP4 levels via Western blots after 0-24 hr continuous 1 nM OSW-1-compound treatment in HEK-293, HCT-116, and K562 cell lines.* ***(B)*** *Three independent OSBP Western blots of OSW-1-compound washout experiments show as a graph in* ***Figure 1E****.* ***(C)*** *Bottom-up proteomic mass spectrometry analysis of vehicle control or 1 nM OSW-1-compound treated for 6 hours followed by washout and a 24 hr recovery period. Shows loss of detectable OSBP peptide (i.e., peak with retention time of 39.33 min) in washout cells as compared vehicle control. (****D****) ORP4 Western blot levels under washout conditions with 0-72 hr recovery. ORP4 levels are not as significantly affected under washout conditions as compared to OSBP levels.* ***(E)*** *Calcein AM and Hoescht 33342 staining of HEK293 cells imaged using Operetta High-Content Imaging System treated with 1 nM under continual treatment (left) or washout conditions (right). Continual treatment of OSW-1 induces cytotoxicity at 24 hr (left); washout cells look unchanged from DMSO vehicle control.*



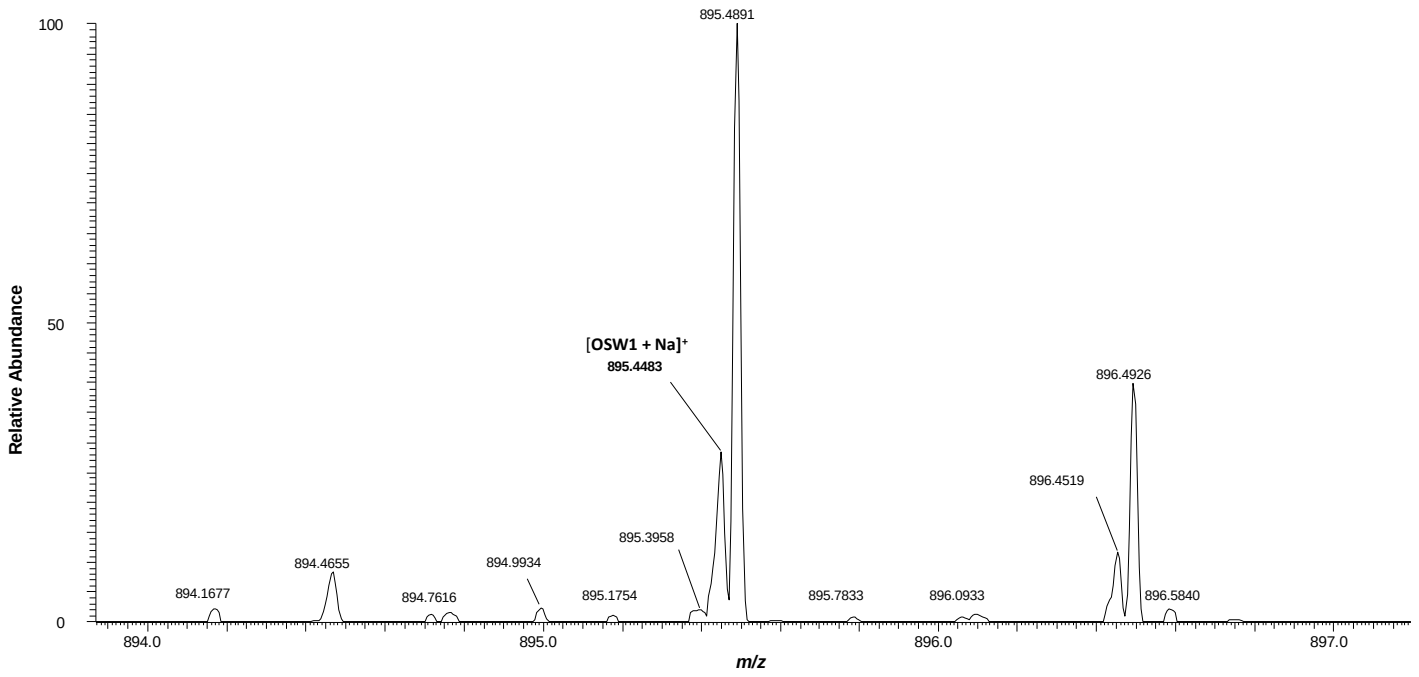
Supp. Figure 2: Reduction of OSBP Levels Due to OSW-1 Compound Washout is Not an Experimental Artifact. ***(A)*** *Two different cell lysis protocols produce the same reduction of OSBP levels in the OSW-1 washout experiment. HEK293 and HCT116 cell lysates were prepared by either the freeze/thawing AC lysis method or the M-PER extraction lysis method.* ***(B)*** *OSBP is not in the lysis cell pellet in the OSW-1 washout cells. Western blot analysis of cell lysis pellets after lysis using both MPER and AC lysis from HEK293 cells. Pellets showed no detectable signs of residual OSBP in the cell pellet.* ***(C)*** *OSBP is not excreted from cells during the OSW-1 washout experiment. Western blots of DMEM media collected after DMSO or OSW-1-compound treatment from two separate cultures for each treatment (i.e. #1, #2). Media was either centrifuge column concentrated (C.C.) to a final volume of 500 µL, or ammonia sulfate precipitated (A.S.). Media shows no detectable OSBP in C.C. concentrated media and very little detectable OSBP in A.S. samples. Faint bands in A.S. concentrated media can be explained by the much higher concentration of protein loaded for Western blot as a result of the protein precipitation.* ***(D)*** *Splitting OSW-1-washout cells has little effect of the reduction of OSBP levels in the OSW-1 washout cells. The adherent HEK293 cells were treated with under washout conditions (i.e., 1 nM OSW-1 for 6 hr, followed by washout). Then, the OSW-1-washout HEK293 cells were split (see SI for protocol), diluted with complete media as indicated (i.e., 1:1, 1:2 or 1:5), and re-plated in new plasticware for 24 hr, at which point the cells were lysed and analyzed via Western blot. An example OSBP Western blot and the average of a triplicate experiment is shown.*



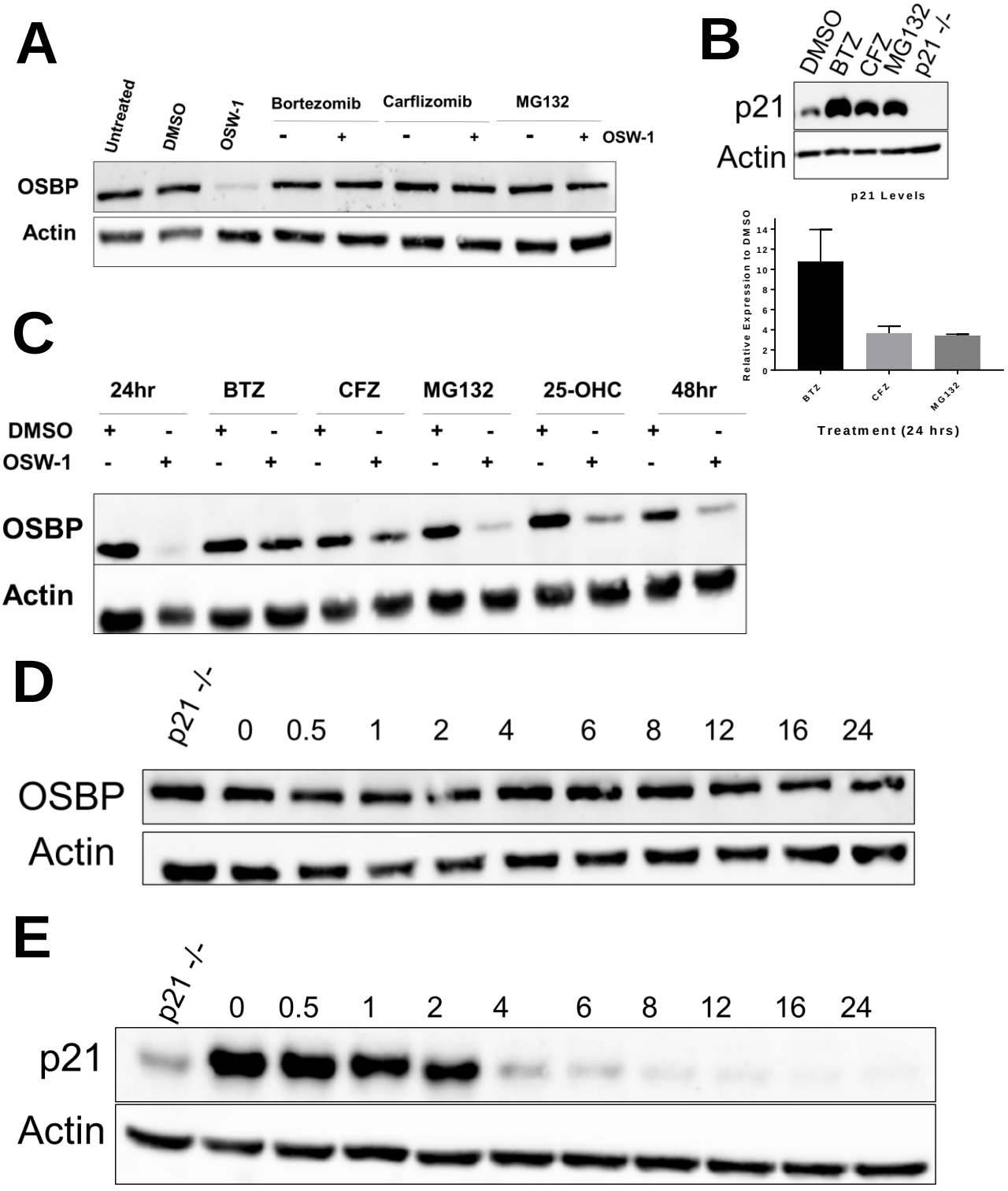
Supp. Figure 3: Full Chromatogram of LCMS and SCMS 100 nM OSW-1 Treatment of HCT116 Cells: *(**A**) Chromatogram of Liquid Chromatography Mass Spectrometry (LCMS) quantification of intracellular OSW-1 concentrations. HCT116 cells were treated with 100 nM OSW-1 for 1 hr, followed by washout, and either 0 or 24 hr recovery. The deuterated OSW-1 analog (**Fig. 1**, **2**) is used as an internal standard for LCMS quantification; 50 nM of deuterated OSW-1 **2** is spiked into lysate prior to LCMS analysis. (**B**) Chromatogram of Single Cell Mass Spectrometry (SCMS) quantification of OSW-1. HCT116 cells were treated with 100 nM of OSW-1 for 1 hr, followed by washout, and either 0 or 24 hr recovery. For the SCMS 0 hr recovery, 12 individual cells were analyzed; for the 24 hr recovery, 10 individual cells were analyzed. Cell number is indicated by the bars above the chromatogram. The total MS signals detected for each cell are integrated, and the intracellular OSW-1 levels compared to deuterated OSW-1 standard which is consistently present in the sampling solution at 50 nM. The intracellular lipid, phosphatidylcholine, is used to report the presence of cellular content in these SCMS experiments. See Method and SI for additional experimental details.*



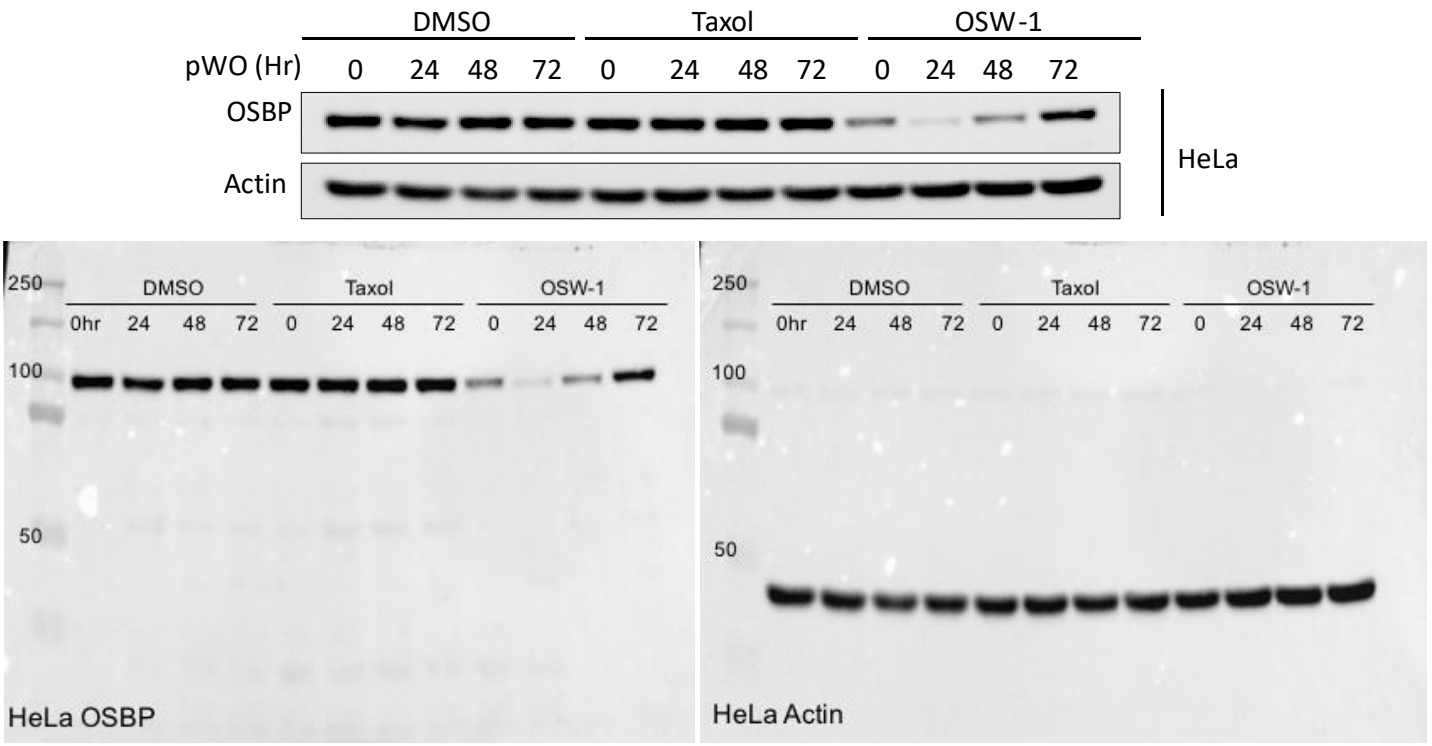
Supp. Fig 4: Spectra of SCMS at 0 and 24 hr Recovery: Zoomed out spectra of OSW-1 washout HCT116 cells shown in **Fig. 2A** and **2B.** *The cellular lipid phosphatidylserine (PS) is detected signifying cellular content is being analyzed in both the 0 hr recovery and 24 hr washout recovery timepoints. The OSW-1 signal at 895.4433 Da is absent in the 24 hr recovery time point. No other OSW-1-derived signals, including signals consistent with possible OSW-1 metabolites, are detected.*



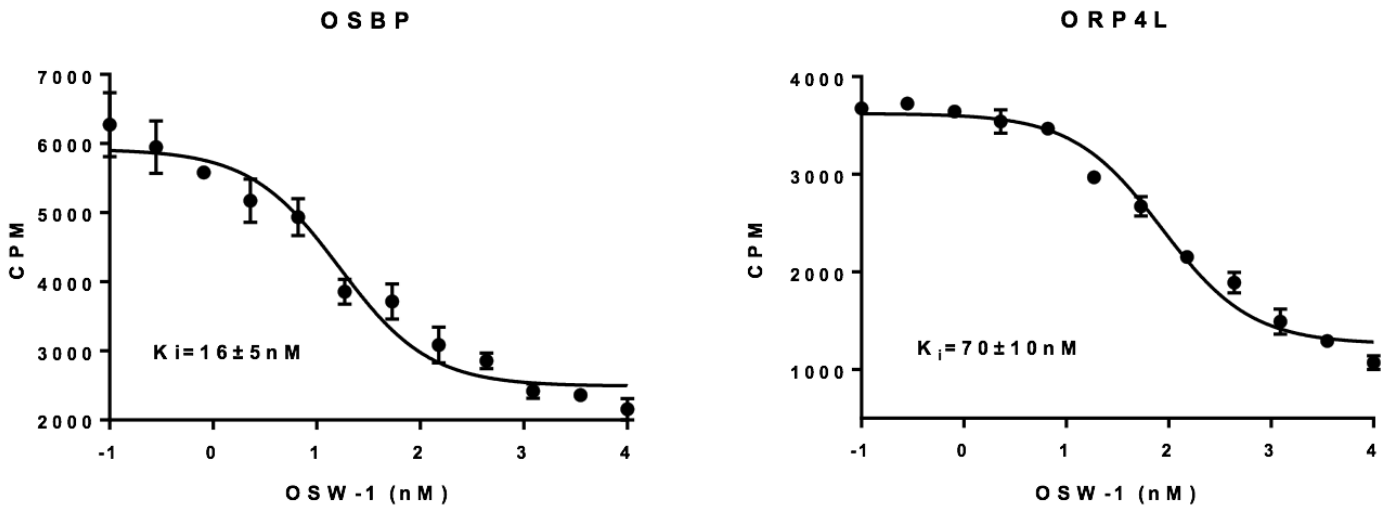
Supp. Figure 5: LCMS Limit of OSW-1 Quantification: *LCMS analysis of HCT116 cell lysate spiked with 100 pM for determination of sample for limit of quantification. 100 pM OSW-1 detected threshold is~ 4-fold ratio between signal to noise. Experiment was run in two independent replicates (n=2).*



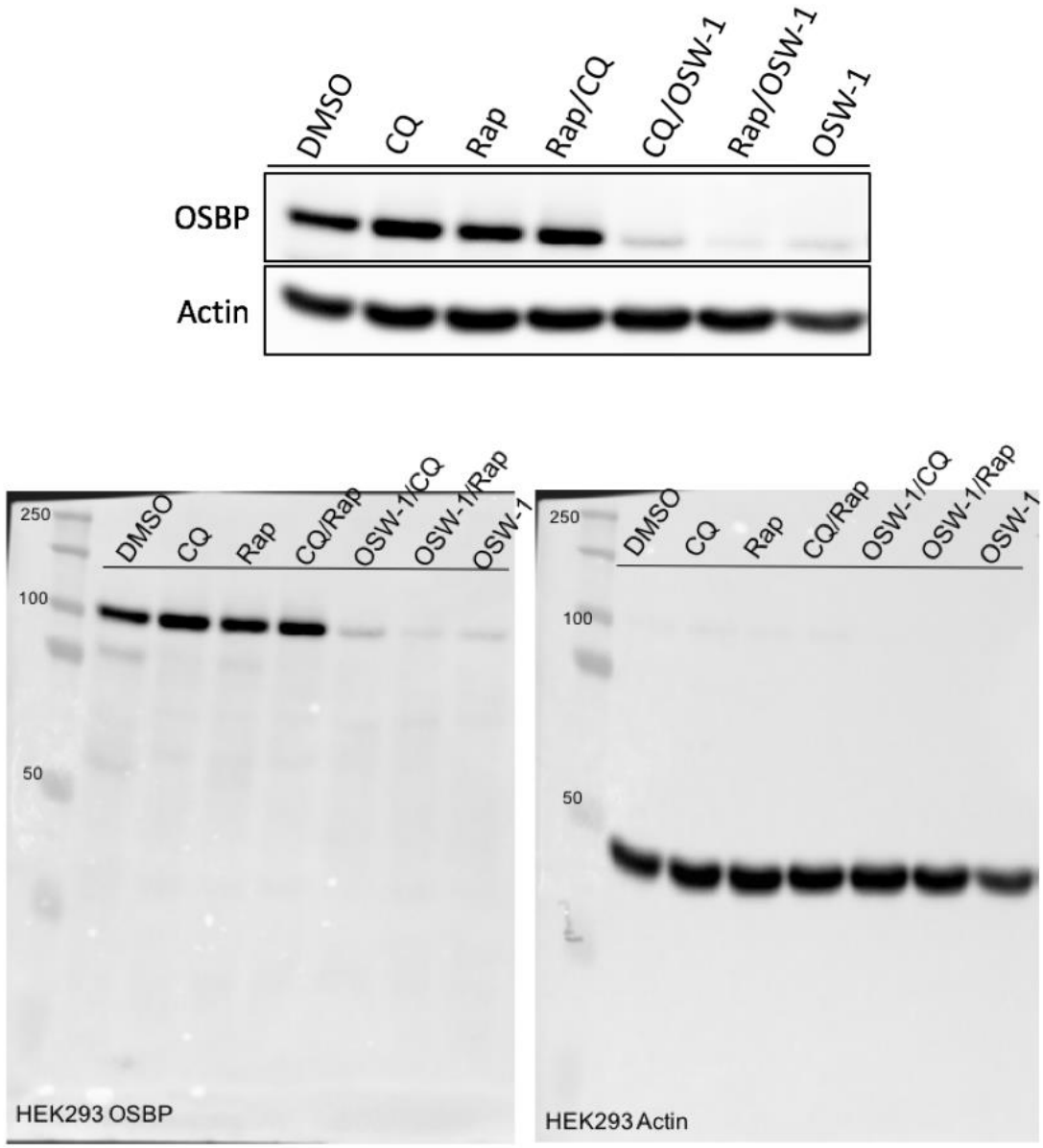
Supp. Figure 6: ***(A)*** *Example OSBP Western blot of co-incubation of 1nM OSW-1 and proteasome inhibitors 24 hr treatment in HCT116 cells; averaged graph in* ***Fig. 4A.*** ***(B)*** *p21 levels of HCT116 cells treated with proteasome inhibitor for 24 hr; control to demonstrate inhibition of the proteasome. Example p21 Western blot and averaged graph of independent experiments (n=2).* ***(C)*** *Example OSBP Western blot of proteasome inhibitor treatment on HCT-116 OSW-1 washout cells showed as graphical average in* ***Fig. 4B****.* ***(D&E)*** *Example Western blots of OSBP (****D*** *and p21* ***(E)*** *of HCT116 cells treated with cycloheximide for the indicated times; results shown as a graphical average of three independent experiments in* ***Fig. 4C****.*



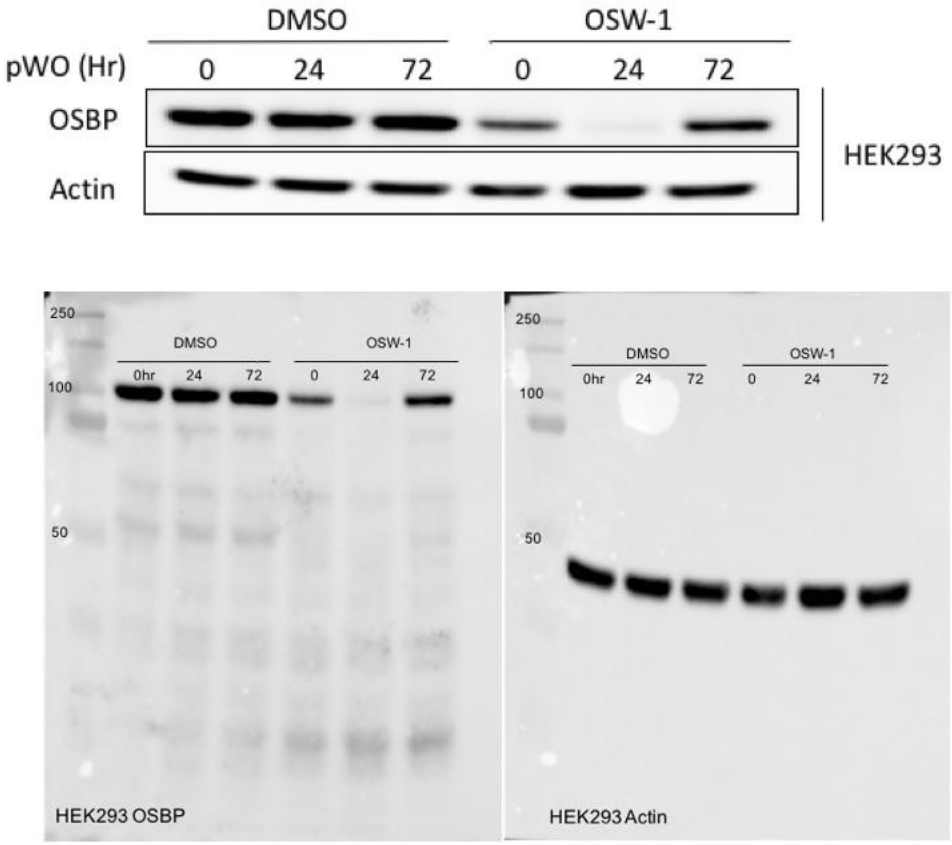
Supp. Figure 7: OSW-1 Washout in HeLa Cells Induces Persistent Reduction in OSBP Levels. *Western blot of OSBP from HeLa OSW-1 washout experiment (i.e., 1 nM, 6 hr treatment followed by washout) cells display the same OSBP reduction as the HEK293, HCT116, and K562 cell lines (**Fig. 1C**). Similar to HEK293 but not HCT116 or K562 (**Fig. 1C**), the OSBP levels in HeLa cells return to normal levels at 72 hr post-washout. The OSW-1 washout viral inhibition experiments (**Fig. 6**) were performed in HeLa cells.*



Supp. Figure 8: Representative Inhibition Binding Curves of OSBP and ORP4 for OSW-1 Compound. *96-well in vitro binding experiments in lysate made of HEK293T cells overexpressing human OSBP or human ORP4. The inhibition binding experiment measures the OSW-1-compound competing for OSBP or ORP4 binding to radiolabeled 25-hydroxycholesterol (see SI for experimental details).*



Supp. Figure 9: Chemical Inhibition of Autophagy Does Not Rescue OSBP Levels in OSW-1-Compound Washout Cells. *Western blots of HEK293 cells treated with 1 nM OSW-1 under the 6 hr washout conditions are shown as cropped (top) or full blots (below). Chloroquine (CQ) blocks autophagy-induced proteolysis, and therefore co-incubation with CQ is used to determine if a protein is degraded during autophagy. 25 μM of CQ was used. Cells were also treated with 100 nM of rapamycin (Rap), which is known to activate autophagy. Co-treatment of OSW-1 washout cells with 25 μM CQ did not rescue OSW-1 levels. Also, Rap treatment or co-treatment of Rap with the OSW-1-compound has no effect on OSBP levels.*



Supp. Figure 10: OSBP Levels in iTRAQ Lysate Samples. *Western blots of the DMSO vehicle control and OSW-1 washout HEK293 lysates used for iTRAQ analysis (**Fig. 5C** & **5D**).*

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Plasmids and Cloning

Human OSBP cDNA was obtained in a pOTB7 vector from the Mammalian Gene Collection (Thermo). PCR using 5'-GCTAGCATGGCGGCGACGGAG-3' forward and 5'-AAGCTTGAAAATGTCCGGGCATGAGC-3' reverse primers amplified a 2.44 kb Nhe I-HindIII fragment containing a full-length hOSBP cDNA, which was subcloned into pJET1.1 (Thermo) and sequence verified. The fragment was then cloned into the pcDNA™ 3.1/myc-His(-) C mammalian expression vector (Sigma) Nhe I-Hind III double-cut sites. The reverse primer does not include an additional nucleotide between HindIII cut site and the last OSBP codon resulting in an OSBP-tagless protein. ORP4L was cloned from HCT-116 with cDNA with a NheI forward primer 5'-GCTAGCATGGGGAAAGCG-3' and a HindIII reverse primer 5'-AAGCTTCGAAGATGTTGGGGCACATATG-3'. LacZ was PCR amplified from K-12 *E. coli* with NotI forward 5'- GCGGCCGCATGCCCG TCGTTTTA-3' and BamHI reverse primer 5'-GGGCGGATCCTTTTTGAC ACCAGACCAA-3'. To generate the proteins expressing tags, the MCS of the completed vector was changed through site-directed mutagenesis with a forward 5'-AAGCTTACGTACGAACAAAAACTCATCTCAGAAGAG-3' and reverse 5' CTCTTCTGAGATGAGTTTTTGTTCGTACGTAAGCTT-3'. The plasmid was expanded in *E. Coli* DH5α, and isolated through miniprep and maxiprep kits (Thermo). Gene and plasmid MCS were sequence verified through Oklahoma Medical Research Foundation (OMRF).

Cell Lines and Viruses

HEK293 STF (ATCC CRL-3249) and HeLa (ATCC CCL-2) were cultured in DMEM (Thermo 11995073) supplemented with 10% Hyclone (Fisher Sci SH3006603) and 1% penicillin-streptomycin (Thermo 15140122). HCT116 (ATCC CCL-247) was cultured in McCoy 5A media (Thermo 16600108) supplemented with 10% Hyclone and 1% penicillin streptomycin. HCT116 $p21^{-/-}$ cells were a gift from the Vogelstein Laboratory (Johns Hopkins University) and cultured in McCoy 5A media (Thermo 16600108) supplemented with 10% Hyclone and 1% penicillin streptomycin. K-562 (ATCC CCL-243) was cultured in RPMI 1640 (Thermo 22400105) media supplemented with 10% Hyclone and 1% penicillin streptomycin. MCF-7 cells were a gift from R. Cichewicz (University of Oklahoma, Norman) and cultured in MEM (Thermo 11095114) media 10% Hyclone, 1% penicillin streptomycin and 0.2 mg/mL insulin (A11382II). MRC-5 cells were a gift from E. Blewett (Oklahoma State University- Center for Health Sciences, Tulsa) and cultured in MEM media supplemented with 10% Hyclone and 1% penicillin streptomycin. RD, (rhabdomyosarcoma) cells (ATCC-CCL-136) were cultured in DMEM (Fisher Sci SH30081.0) with 10% FBS (Atlanta Biological S11550) and 1% penicillin-streptomycin (Gibco 15140-122). Coxsackievirus A9 (strain CoxA9-01) and Echovirus 2 (strain Echo2-01) were obtained from the Oklahoma State Department of Health Laboratory. They are clinical isolates, obtained from OK residents and typed by the OK State Department of Health and/or the Center for Disease Control and Prevention. All other identifiers have been stripped off. These viruses were passaged twice in RD cells, aliquoted in 1.0 mL amounts and stored in complete medium at -80 ºC. Each virus was titered on RD cells using a TCID-50 assay[1]. To allow m.o.i. to be determined a conversion factor of 0.7 was used to change TCID-50 to pfu/ml.

General Cell Culture:
All mammalian cell lines were cultured at 37 ºC in 5% CO_2. All handling of the mammalian cell culture was performed in a standard tissue culture hood using standard aseptic technique. Cell lines were cultured in the complete media described above. Cell culture stocks were aliquoted in complete media with 10% DMSO in 2 mL cryogenic vials (Corning 430659) and stored in liquid nitrogen vapor phase. Before beginning a new culture, the freezer stocks were thawed, diluted in 9 mL complete media and plated in Nunclon Delta 10 cm^2 dishes (VWR 10171744) or T25 flask (CellStar 690160) for suspension cell lines . After allowing ~16 hours for the revived cells to attach, the DMSO containing media was replaced with DMSO free complete media. All revived cultures were split at least twice prior to use in an experiment. Cell cultures were restarted approximately every 3-4 weeks. ***All cell based experiments reported used multiple restarted cell cultures in the independent experiments that make up the replicate results.*** For experiments, cell cultures were used with a confluency of ~70%. The cell cultures were not allowed to ever become superconfluent, and the cellular morphology and proliferation rate of the cell culture was carefully tracked to identify any abnormalities; any cell culture showing the slightest abnormalities were discard and the cell line restarted from frozen stocks. For experiments, cells were allowed to recover from splitting and replating a minimum of 16 hr prior to the start of an experiment.

The adherent mammalian cell lines are split every ~3 days with the following general procedure: the complete media is removed via aspiration and the cells are gently washed with 5 mL of 1X PBS. TrypLE trypsin reagent (2.5 mL for 10 cm^2 plate) is added and incubated for approximately 10 min at 37 ºC. After 10 mins 7.5 mL of the complete culture media is added to inactivate the TrypLE reagent. Cells were counted using a TC20™ Automated Cell Counter (BioRad), by combining 10 µL of cell solution with 10 µL Trypan Blue stain (Thermo 15250061).

The K562 leukemia suspension cell line was handled as described for the adherent cell lines except for the splitting and seeding procedure. For K562 cells, the cells were spun down at 200 x g for 5 minutes and the media was aspirated from the cell culture carefully so as not to disturb the cell pellet and replaced with 10 mL of complete media. The cell pellet was then resuspended and diluted to the desired seeding density using complete media.

OSW-1 Compound: The OSW-1 compound used was obtained through total synthesis in the Burgett lab or from isolation from the natural source. OSW-1 used in the experiments was of >95% purity as determined with ^{1}H-NMR and LCMS analysis. Solid OSW-1 compound was dissolved in analytical grade DMSO solution to produce 10mM stocks for experimentation. The 10 mM OSW-1 stock solution was aliquoted into Eppendorf brand 1.5 mL centrifuge tubes; Each individual 10 mM OSW-1 aliquots were thawed no more than three times. Additional cycles of freeze/thaws caused partial loss of OSW-1 compound in the aliquots.

Cell Lysis
Cell Lysis Method 1 (AC Freeze/Thaw Lysis): Adherent cells were cultured in Nunclon Delta 10 cm^2 dishes (VWR 10171744) and lysed by removing the media, washing with 1X PBS, followed by addition of 1 mL PBS and scraping. Cells were collected in a 1.5 mL

Eppendorf brand centrifuge tubes and spun down at 14,000 x g for 45 seconds. Supernatant was removed, and the cells were resuspended in 50 µL of AC Lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 0.8% NP40, 1mM DTT, 50 mM HEPES, 25 mM NaF, 1 mM Na_3PO_4) with 3X HALT/EDTA protease inhibitor (Thermo 78438) and 0.2 mM phenylmethanesulfonylfluoride (Goldbio). The cells were then frozen in liquid nitrogen and thawed in a 37°C bead bath three times, followed by a 14,000 x g spin for 15 minutes. Supernatant was transferred to a new tube and a portion was taken for protein quantification using a Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate #5000006, BSA-Santa Cruz sc-2323). After protein quantification, the lysates were diluted to the desired concentration using AC lysis buffer and 4X Laemmli buffer (1 M Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.2% bromophenol blue), followed by dry bath heating at 95°C for 10 minutes.

Adherent cells cultured on 6-well plates (Greiner 657160) were lysed by removing media, washed with 1X PBS, followed by adding 0.5 mL TrypLE™ Express (Gibco 12605-010) and incubated at 37°C for 5 minutes. TrypLE™ was neutralized using 0.5 mL of media and cells were then transferred to a 1.5 mL Eppendorf tube spun down at 14,000 x g for 45 seconds. Supernatant was removed, and 1 mL of PBS was added to wash the cells. Cells were spun down at 14,000 x g for 45 seconds, supernatant was removed, and the cells were resuspended in 50 µL of AC lysis buffer. Freeze/thaw method was continued as described above.

For suspension cell lines, cells were spun down at 200 x g for 5 minutes and the supernatant removed. Cell pellet was resuspended in 1 mL of PBS and spun down at 14,000 x g for 45 seconds, supernatant was removed, and cells were resuspended in 50 µL of AC lysis buffer. Freeze/thaw method continued as above.

Cell Lysis Method 2 (MPER Extraction): Mammalian protein extraction reagent, MPER, (Thermo 78501) was used as an alternative lysis method for 10 cm^2 dishes. Media was removed from the cells, and 5 mL of 1X PBS was added to wash cells. 1 mL of MPER was added to the plate and was shaken in a room temperature (Innova 42 incubator) at 250 rpm for 5 minutes. The solution was collected and spun down at 14,000 x g for 10 minutes. Supernatant was placed in a new tube and the protein concentration and sample preparation was conducted as described above.

Western Blotting

SDS-PAGE gels (8.5 or 12%) containing 25 µg of protein per well were transferred to 0.45 µm nitrocellulose (Bio-Rad 1620115) using constant voltage (100V) for 1 hr at 4°C in 1X transfer buffer with 10% ethanol. After transferring, the nitrocellulose membrane was blocked with 5% milk 1X TBST at room temperature for 30 minutes. The membranes were then washed three times, five minutes each, with 1X TBST. Primary incubation with antibodies was done overnight at 4°C. After primary incubation, the blots were washed five times, five minutes each, with 1X TBST and then incubated in secondary antibody in 1% milk TBST for thirty minutes at room temperature. After secondary antibody incubation, the blots were washed five times, five minutes each, with 1X TBST and then once with 1X TBS for ten minutes. TBS was removed, and the blots were incubated in Clarity™ Western ECL substrate (Bio-Rad 1705061) and imaged on the Bio-Rad ChemiDoc™ Touch Imaging System using the chemiluminescence setting with 2x2

binning. Ladder images were taken using the colorimetric setting. After development, the membranes were washed with 1X TBST twice for five minutes each. 1:1000 β-actin HRP (Santa Cruz sc-47778 HRP) in 1% milk TBST was added and incubated for 1.5 hr at room temperature. Developing occurred the same as after secondary antibody incubation. Primary antibodies used were 1:500 OSBP A-5 (Santa Cruz sc-365771), 1:500 p21 C-19 (Santa Cruz sc-397), 1:1000 OSBP2 B-1 (Santa Cruz sc-365922), 1:100 SQSTM1 (p62) D-3 (Santa Cruz sc-28359) and 1:1000 LC3A/B D3U4C XP® (Cell Signaling 12741). Secondary antibodies used were 1:1000-1:3000 goat anti-mouse IgG_1-HRP (Santa Cruz sc-2060), 1:3000 goat anti-rabbit IgG-HRP (Santa Cruz sc-2004) and 1:2000 goat anti-rabbit IgG-HRP (Cell Signaling 7074S).

Washout Experiments

Cells were treated with 1 nM OSW-1, 1 nM Taxol, or DMSO media for 6 hr or the indicated time period. Media was removed and the cells were gently washed with 5 mL of complete media 3 times and then 10 mL of fresh, OSW-1-compound free media was added back to the cells. The cells were then allowed to recover for the indicated times (0-72 hrs) and were lysed as described above and analyzed by Western blot.

Trypan Blue Staining

HEK293 and HCT116 cells were seeded out at 0.85 X 10^5 cells/mL into 10 cm^2 dishes and left to recover for 20 hrs. Cells were treated with 1 nM OSW-1, 1 nM Taxol, or DMSO for 6 hrs, followed by washout procedure and recovery for 0-72 hrs. After recovery time, cells were washed with 1X PBS and then incubated in 2.5 mL TrypLE™ for 5 minutes at 37°C. Reaction was neutralized using 7.5 mL of fresh media and cells were counted on a TC20™ Automated Cell Counter (BioRad) by combining 10 µL of cell solution with 10 µL Trypan Blue stain (Thermo 15250061).

Calcein AM and Hoechst Staining

HEK293 cells were seeded out at 10,000 cells per well into a 24-well plate and left to recover for 20 hrs. Half the plate of cells was treated with 1 nM OSW-1, 1 nM Taxol, or DMSO for 6 hrs, followed by washout while the other half was treated with the same concentration continuously. Washout and continual time points were 0, 24, 48, and 72 hrs, with the cells being treated on subsequent days, therefore, all time points ended collectively. Once the treatments were finished, the media was removed and a solution of 5 µM Calcein AM (Thermo C1430) and 5.5 mg/mL Hoechst 33342 (Thermo H1399) was added to the cells and incubated at 37°C for 1 hr. Plate was imaged using an Operetta High-Content Imaging System (PerkinElmer) using brightfield, 488, and Hoechst settings.

Cell Pellet Analysis for OSBP Levels

Cell pellets were procured from HEK293 cells and lysed using either the AC lysis (Lysis Method 1) or the MPER method (Lysis Method 2) of cell lysis described previously (1nM OSW-1, 24hr treatment). Pellets were gently washed with PBS and resuspended to remove any residual lysate from the exterior of the pellet. The samples were then re-pelleted using a microcentrifuge centrifuge (14,000 x g for 45 seconds). The PBS was removed and the cell pellets were resuspended in 29 microliters of Fast Digest buffer

(Thermo B64) and 1 µL of DNAase and incubated at 65°C for 5 minutes (twice the volume was used for MPER lysed pellets due to larger pellet size). 10 µL of Laemmli buffer was then added to the samples and samples were incubated at 95°C for 10 minutes. 18 µL of each sample was loaded into an SDS PAGE gel (8.5%). Samples were loaded immediately off the heat block to increase solubility. 25µg of protein was added for positive and negative controls (1nM DMSO and 1 nM OSW-1 treated lysates). Western blots were performed as described previously.

Cell Media Analysis for OSBP Levels

Media was aspirated from four HEK293 cell tissue culture plates (two DMSO treated and two OSW-1 treated (1nM, 24hr continuous treatment)), the media from the two DMSO plates were combined and the media for the two OSW-1 treated plates were combined for a total of 20 mL media for each treatment condition. The media was then separated into two aliquots for each treatment (two 10 mL DMSO media aliquots and two 10mL OSW-1 media aliquots). Media was filtered using a 0.2 µm pore filter (VWR 28145-501) to remove any cells that may have detached and were removed with the media. One aliquot for each treatment was centrifuge filter concentrated using a 10 kDa cut off filter (Sigma Z648027) to a final volume of 500µL. The remaining aliquot was concentrated using ammonium sulfate precipitation (2.91g ammonium sulfate per 10mL media). Samples were then diluted in Laemmli buffer and 18 µL of each sample was added to each well of the 8.5% SDS PAGE gel; 25µg of protein was added for positive and negative controls (same control lysates as the cell pellet analysis). Western blot was performed as previously described.

Synthesis of Deuterated OSW-1 Analog:

The deuterated OSW-1 analog (**Fig. 1A**, **2**) was produced via total synthesis of OSW-1 adapted from literature procedure.[2,3] During the synthesis of the xylose component, a benzoate group containing the deuterated methyl substituent was introduced.

Analytical Data on Deuterated OSW-1 Analog (2) (see appendix for spectrum)

1H NMR (400 MHz, Acetonitrile-d3) δ 8.02 (d, J = 9.0 Hz, 2H), 7.02 (d, J = 8.9 Hz, 2H), 5.31 (d, J = 4.9 Hz, 1H), 4.84 (t, J = 8.2 Hz, 1H), 4.73 (dd, J = 8.3, 6.2 Hz, 1H), 4.61 (d, J = 7.7 Hz, 1H), 4.20 (s, 1H), 4.01 (d, J = 6.2 Hz, 1H), 3.92 (dd, J = 11.5, 4.9 Hz, 1H), 3.88 (s, 1H), 3.76 (dd, J = 12.4, 4.2 Hz, 1H), 3.67 (dt, J = 8.2, 4.2 Hz, 2H), 3.64 – 3.50 (m, 2H), 3.42 (dd, J = 12.4, 2.3 Hz, 1H), 3.38 – 3.22 (m, 2H), 2.86 (q, J = 7.3 Hz, 1H), 2.69 (s, 1H), 2.46 (ddd, J = 18.2, 9.0, 6.5 Hz, 1H), 2.23 – 2.08 (m, 5H), 1.99 – 1.90 (m, 1H), 1.85 – 1.76 (m, 1H), 1.75 – 1.68 (m, 2H), 1.67 (s, 3H), 1.64 – 1.38 (m, 5H), 1.32 (dt, J = 13.1, 6.6 Hz, 1H), 1.29 – 1.14 (m, 3H), 1.07 (d, J = 7.4 Hz, 3H), 1.04 – 1.01 (m, 1H), 1.00 (s, 3H), 0.94 – 0.83 (m, 1H), 0.82 – 0.74 (m, 9H). 13C NMR (101 MHz, Acetonitrile-d3) δ 220.09, 169.95, 165.66, 164.73, 142.44, 132.79, 123.64, 121.86, 114.81, 102.92, 100.98, 88.19, 86.20, 80.82, 75.74, 74.47, 71.90, 71.72, 70.49, 67.67, 66.46, 65.22, 50.89, 49.20, 46.94, 46.54, 43.07, 39.71, 38.13, 37.35, 35.74, 33.19, 32.86, 32.69, 32.63, 32.37, 28.19, 22.88, 22.67, 21.34, 21.04, 19.79, 13.56, 12.16. HRMS calcd for C47H65D3O15Na: 898.4639 [M + Na]+, found 898.4611.

Intracellular OSW-1 Quantification Using LC-MS and Single Cell MS Methods
nano-UPLC/MS: HCT-116 cells ($1.5x10^5$) were seeded in a 6-well plate. Upon 60% confluency, cell lysate was created following a 1 hr treatment of 100 nM OSW-1, with or without a 24 hr post wash recovery. Trypsin (0.5 mL) was used to detach the cells, with additional McCoy's media (0.5 mL) to stop digestion. Cell count was performed using a Bio-Rad TC20™ Automated Cell Counter with trypan blue viability staining. Cells were spun at 500 xg for 5 min followed by a 1-mL PBS wash. The cell pellet was lysed using 1 mL of 50 nM d-OSW-1 dissolved in cold acetonitrile and methanol (1:1) with brief vortexing on ice for 10 min. The cell pellet was spun at 15000 xg at 4°C for 15 min. The supernatant was transferred to a new tube and dried using a speed vacuum (Savant SPD11V, Thermo Scientific) at 70°C. Prior to analysis, cells are resuspended in 150 µL of ACN: H_2O (1:10). Analysis was performed using a Waters nanoAQUITY BEH C-18 column (100 µm x 100 mm, 1.7 µm) coupled with a mass spectrometer (Thermo LTQ Orbitrap XL, Waltham, MA) using a flow rate of 0.3 uL/min. Mobile phase A is ACN with 0.1% formic acid, and mobile phase B is H_2O with 0.1% formic acid. The time/%A are as follows: 0/0, 1/50, 2/100, 3/100, and 4/0 for a total runtime of 5 minutes.

Single Cell Mass Spectrometry HCT-116 cells ($1.5x10^5$) were seeded on to a glass microchip (18 mm diameter) with chemically-etched microwells (55 µm diameter; 25 µm deep) placed into each well of a 6-well plate. Upon 60% cell confluency, cells were treated as described for nano-UPLC/MS. Following treatment, the microchip was washed with 5 mL of FBS-free McCoy's media and placed on an X, Y, Z-translational stage for quantification. MS analysis was performed as previously described.[4] Briefly, single-probes were coupled to the mass spectrometer by using a flexible arm clamp to position the nano-ESI emitter in front of the inlet. The solvent-providing capillary was connected to the solvent through the conductive union. For quantification, 50 nM d-OSW-1 was added into the solvent. High voltage (~4.5 kV) was used for SCMS experiments in the positive ion mode with a mass resolution ($m/\Delta m$) of 60,000. A flow rate of ~5 nL/s was used (the actual flowrate is optimized for each Single-probe). Data was collected using Xcaliber software and exported into Excel for analysis.

Immunofluorescence
50,000 cells were seeded onto sterile 18 mm cover slips in 12 well plates for treatments lasting (0-24 hrs) or 25,000 cells for longer treatment. The cells rested for 24 hrs before treatment to ensure attachment of the cells. Once treatments were completed media was aspirated and cells were washed with warm 1X PBS. PBS was removed and 0.5 mL of freshly prepared 4% paraformaldehyde in PBS was added. Coverslips were at 37°C for 20 mins and then the paraformaldehyde was removed followed by three 1X PBS washes. Permeabilization of the cells was done with 0.5 mL of 0.5% Triton X-100 in PBS at room temperature for 10 minutes. 1X PBS was used to wash the cells three times. Image-iT FX signal enhancer (Thermo I36933) was added onto the cover slips and incubate at room temperature for 30 minutes followed by three 1X PBS washes. Coverslips were blocked with 0.5 mL of 1% BSA in PBS at room temperature for 30 minutes followed by three washes with 1X PBS. Primary antibody was added, and the slips were incubated overnight at 4°C. The primary antibody solution was removed and washed slips three times with 1X PBS. Secondary antibody solution was incubated in darkness at room

temperature for 1 hr. The secondary antibody solution was removed and washed three times with 1% BSA-PBS, three times with 1X PBS, and then soaked the cover slip in 300 nM DAPI (Thermo D1306) solution for 10 minutes Mounted the slips onto glass slides using VECTASHEILD HardSet Antifade mounting media (VECTOR labs H-1400). Stored slides at -20°C until imaging was conducted. Primary antibodies used were 1:100 OSBP1 1F2 (Novus NBP2-00935) and 1:500 TGN46 (Novus NBP1-49643). Secondary antibodies used were 1:500 goat anti-mouse IgG H&L Alexa Fluor® 488 (Abcam ab150113) and donkey anti-rabbit IgG H&L Alexa Fluor® 594 (Abcam ab150076). Imaging was done with a Lecia SP8 using a 63x objective with 2x digital zoom. Images were analyzed with ImageJ software.

Cycloheximide Chase Experiments

HCT116 and HEK293 cells were seeded at $1.6x10^5$ cell per well into six well plates (Greiner 657160) and left to rest for 20 hrs. Media containing 177 μM cycloheximide (Sigma C7698-1G) was added to the plates and the cells were incubated for the times indicated before the cells were lysed and analyzed by Western blotting.

Proteasome Inhibitor Assays

HCT116 cells were seeded out into plates and left to rest for 20 hrs. For the co-incubation experiments, the cells were treated with DMSO (Sigma 472301), 1 nM OSW-1, 25 nM Bortezomib (Sigma 5043140001), 25 nM Carfilzomib (AdooQ Bioscience A11278), 170 nM MG-132 (Sigma 474787), or a combination of treatments for 24 hrs. Cells were lysed and analyzed by western blotting. For the washout experiments, the cells were treated with media containing DMSO or 1 nM OSW-1 for 6 hrs, washed 3 times with 5 mL of media, and then allowed to recover for 24 hrs. After the 24 hr recovery, one set of treatments were lysed as a control to ensure OSPB loss while the other cells were treated with media containing 25 nM Bortezomib, 25 nM Carfilzomib, 170 nM MG-132, or DMSO for 24 hrs. Cells were lysed and analyzed by western blot.

Calpain Analysis

For the continual treatment, HeLa cells were seeded out in a 6 well plate. Upon 70% confluency, cells were treated with DMSO, DMSO and ALLN (10μM), OSW-1 (1nM), or OSW-1 (1nM) and ALLN (10μM) for 24 hr. Cells were lysed and analyzed following the 6-well lysis method and the western blot protocol. Under the washout experimental conditions, cells were treated with DMSO or OSW-1 (1nM) for 6 hr. Cells were washed out according to the washout experimental method. After 24 hr recovery, one set of DMSO and one set of OSW-1 was lysed following the 6 well lysis method. At the same time (24hr post-wash), ALLN (10μM) was added to one set of DMSO and one set of OSW-1. The cells continued to incubate until 48 hr post-wash, at which point they were lysed and analyzed using the western blot method.

RT-PCR Analysis

HCT116 and HEK293 cells were seeded at $0.85x10^5$ cells/mL into 10 cm^2 plates and left to rest for 20 hrs. Cells were treated in the same manner as the washout with 1 nM Taxol, 1 nM OSW-1, and DMSO and left to recover for 0-72 hrs. Once each time point was

reached, media was removed from cell plates and 1 mL of TRIzol (Thermo 15596026) was added to the plates and cells were scraped and collected in a 1.5 mL Eppendorf tube and incubated at room temperature for 5 minutes to ensure nucleoprotein complex dissociation. To each tube, 0.2 mL of chloroform was added and incubated for 2.5 minutes at room temperature. The samples were then spun down at 12,000 x g for 15 minutes at 4°C. After spinning the upper aqueous phase was transferred into a fresh 1.5 mL Eppendorf tube. 0.5 mL of 100% isopropanol was added and the tubes were mixed by inversion followed by a 10 minute incubation at room temperature. Samples were then spun down at 12,000 x g for 10 minutes at 4°C. Supernatant was discarded and 1 mL of 75% ethanol was added to the pellet to wash the RNA. The sample was vortexed briefly to dislodge pellet and then spun down at 7,500 x g for 5 minutes at 4°C. Supernatant was discarded and pellets were left to air dry for 5 minutes, after which 100 µL of MQ H_2O were added to resuspend the RNA. Samples were then heated at 60°C for 10 minutes. RNA concentration was taken using a nano-drop before being stored at -80°C. cDNA was made by using the Maxima First Strand cDNA Synthesis Kit (Thermo K1671). 4 µg of RNA was added to a PCR tube containing µ1 L of dsDNase, 1 µL of 10x dsDNase buffer, and MQ H_2O to 10 µL. PCR tube was then incubated at 37°C for 2 minutes, placed on ice, spun down briefly, and placed back on ice. 1 µL of 100 mM DTT was added to the tube and incubated at 55°C for 5 minutes, placed on ice, spun down briefly, and placed back on ice. 1 µL of 10 mM dNTPs, 1 µL random primers, and MQ H_2O to 15 µL. Tubes were briefly mixed and incubated at 65°C for 5 minutes, put on ice, spun down, and put back on ice. 4 µL of 5X RT buffer and 1 µL of Maxima enzyme were added to the tube and then incubated at 25°C for 20 minutes, 50° for 30 minutes, followed by an inactivation at 85°C for 5 minutes. cDNA was stored at -20°C. cDNA synthesis was confirmed by PCR with intron spanning β-Actin primers. Once verification was confirmed, RT-PCR was set up using Fast SYBR Green (Thermo 4385612) with intron spanning primers (OSBP, ORP4, and β-Actin). 10 µL of Fast SYBR Green was mixed with 0.3 µL of 100 µmoles forward and reverse primer solution, 1 µL of cDNA, and MQ H_2O to 20 µL. Each gene was done in triplicate for each time point. The plate was then run on a Roche LightCycler480 using SYBR green protocol.

Autophagy Experiments

HEK293 cells were seeded into 10 cm^2 plates. Upon 70% confluency, cells were treated with DMSO as a vehicle control, 1 nM OSW-1, 25 µM chloroquine, or 100nM rapamycin, or a combination of treatments for 6 hrs in 10 mL DMEM media for each 10cm^2 plate. After 6 hrs, the media containing the OSW-1-compound was washed out with three separate 5 mL drug free media washes (same as the washout experimental protocol). Cells were then treated with either drug free media, 100 nM rapamycin, 25 µM chloroquine, or a combination of treatments, and allowed to recover from OSW-1 compound treatment for 6, 15, or 24 hrs. After the indicated post washout time point, the cells were lysed using AC lysis buffer according to the cell lysis protocol described previously. Lysates were analyzed via Western blot using OSBP, SQSTM1 (p62), and LC3-A/B antibodies, with β-actin antibody used as a loading control for quantification (antibody information can be found in ‘Western blotting’ experimental methods).

iTRAQ Experiments

HEK293 cells were seeded and treated according to the 0-72 hr recovery washout experimental procedure previously described (1nM OSW-1 or DMSO, 6 hr treatment). After the desired post washout time point, the cells were lysed according to the AC lysis protocol (lysis method 1) using modified AC lysis buffer. The modified AC lysis buffer contained no DTT and only 3X HALT (no EDTA or PMSF) for protease inhibitor. Free thiols can interfere with the cysteine blocking step prior to iTRAQ tagging and protease inhibitors were kept to a minimum to avoid inhibiting trypsin during the digestion process. Effective treatment was confirmed via Western blot using OSBP antibody and β-Actin antibody as a loading control. A Multiplex Buffer Kit (Sciex 4381664) was used for the denaturing, reducing, and blocking steps. Trypsin with $CaCl_2$ (Sciex 4352157) was used for digestion; and iTRAQ Reagent-8Plex Multiplex Kit (Sciex 4390812) was used for iTRAQ labeling. These kits were utilized according to the iTRAQ Reagents- 8plex Protocol. After tagging, the pH of the samples was lowered to approximately 3, using 1N Phosphoric Acid, and ran through a cation-exchange cartridge system (Sciex cation-exchange cartridge 4326747, cartridge holder 4326688, outlet connector 4326690, and needleport adapter 4326689) to remove any substances that could interfere with LC/MS/MS analysis. The protein eluate was quantified using a NanoDrop One^C (Thermo). Samples were normalized to a "mixed" sample that contained 5.5 µg of protein from each sample. Only shared proteins in all 3 biological replicates were used to test for changes in different conditions. P-values were generated using multiple t-tests in GraphPad Prism 7 ($P<0.05$ for significantly changed proteins). Volcano plots were also generated in GraphPad Prism 7 to display P values against fold changes between the two treatment conditions.

LC-MS/MS analysis of labeled peptides

Mixed peptide samples were analyzed using the LC-MS/MS following previously published protocol.[5,6] Peptide samples were desalted, dried in SpeedVac, and resuspended in buffer A (0.1% formic acid in water). 1 µg of the digested sample was injected onto a custom-packed C18 RPLC column (75 µm i.d., 150 mm length, 2 µm C18 resin, Thermo) using a Waters (Milford, MA, USA) nano-Acquity UPLC system, which is online coupled with a LTQ Orbitrap Velos Pro mass spectrometer (Thermo) through a custom nano-ESI interface. For peptide separation, a 100-min gradient was applied from 3% buffer A to 35% buffer B (0.1% formic acid in acetonitrile). Full MS spectra were acquired at a resolution of 60K (m/z range between 350 and 2000). The data-dependent higher-energy collisional dissociation (HCD) based MS/MS spectra were acquired at a resolution of 15K with a normalized collisional energy of 33% using the ten most abundant parent ions. Peptides were identified using MSGF+ to search LC-MS/MS against the annotated Uniprot human protein database.[7,8] Peptide identifications were filtered with a MSGF cut-off score lower than the calculated FDR<1% at the unique peptide level against decoy database. The iTRAQ reporter ion intensities of each HCD scan were extracted and analyzed using in-house developed software.

Mass Spec label-free 2D OSBP quantification

The first-dimension high-pH (pH=10) separation was performed on a Thermo Accela HPLC system (Thermo Scientific, Hanover Park, IL) with an ACQUITY UPLC BEH300 C18 column (50mm, Waters, Milford, MA, USA). The mobile phase A (MPA) was 20 mM ammonium formate in water and the mobile phase B (MPB) was 20 mM ammonium formate in acetonitrile. The mobile phases were adjusted to pH 10. A 60-min gradient from 3% to 70% (3% to 10% in a minute) mobile phase B was applied for peptide separation, and 60 fractions were collected (one minute per fraction). Fraction concatenation was performed following Yang's paper.[9] A total of 12 fractions were obtained for the second-dimension low-pH LC-MS/MS analysis.

([^{3}H]-25-OHC) Charcoal/Dextran Binding Assay

The [^{3}H]-25-OHC binding assay was run according to the protocol outlined by Burgett et al.[10]

Anti-Viral Experiments

HeLa cells were grown to <75% confluency (healthy log phase cells) in complete media, DMEM (Hyclone SH30081.0) with 10% FBS (Atlanta Biological S11550) and 1% penicillin-streptomycin (Gibco 15140-122). For experiments, cells were trypsinized, counted using a hemocytometer and seeded into 20 wells of two 24-well trays (Falcon 3047) with 2.0 x 10^5 cells per well, in 1.0 mL complete media. Each treatment is performed using quadruplicate wells (n=4) and each virus was on a separate plate. After seeding, cells were incubated 20 hr at 37 ºC, 5% CO_2, at which point cells have grown to a near confluent monolayer.

For the OSW-1 compound continual treatment experiments, (**Fig. 6A**), the media was gently removed from each well, and 1mL of media was added with the desired OSW-1-compound concentration to each well, without disturbing the cells. Cells were incubated for 6 hr, after which time the media was removed and cells were gently washed three times with 1.0 mL of FBS-free DMEM media. After the media was removed, CoxA9-01 or Echo2-01 viruses, diluted in serum-free DMEM with a M.O.I. of 1.0 was added to the culture. The 2.0 x 10^5 cells per well was assumed to double during incubation so 4.0 x 10^5 pfu/well of virus was used for an M.O.I. of 1.0. The virus and cells were incubated for 30 minutes at 37 ºC 5% CO_2. Then, the virus inoculum was removed, and the culture washed one time with 1.0 mL of serum-free media per well. Then, 1.0 mL of complete media with the indicated concentration of OSW-1 was added to the well, and the infected cells were then incubated for 10 hr at 37 ºC, 5% CO_2. After 10 hrs the plate was stored at -80 ºC until the TCID-50 titration. This experiment was performed independently three times to generate the data in the figure.

For the OSW-1 compound washout treatment experiments, (**Fig. 6B**), cells were seeded as above. After 20 hr incubation the media was gently removed from each well, and 1mL of media was added with the desired OSW-1-compound concentration to each well, without disturbing the cells. Cells were incubated for 6 hr, after which time the media was removed and cells were gently washed three times with 1.0 mL of FBS-free DMEM media. This was replaced with complete media and cells allowed to incubate for 20 hrs. After the media was removed, CoxA9-01 or Echo2-01 viruses, diluted in serum-free DMEM with a M.O.I. of 1.0 was added to the culture. The 2.0 x 10^5 cells per well was assumed to double and double again during incubation so 8.0 x 10^5 pfu/well of virus was

used for an M.O.I. of 1.0. The virus and cells were incubated for 30 minutes at 37 ºC 5% CO_2. Then, the virus inoculum was removed, and the culture washed one time with 1.0 mL of serum-free media per well. Then, 1.0 mL of complete media was added to the well, and the infected cells were then incubated for 10 hr at 37 ºC, 5% CO2. After 10 hr incubation, the plate was stored at -80 ºC until processing. Then, the plates were rapidly thawed, the cells in media were scrapped from the wells into sterile 1.5 mL centrifuge tubes and the suspension then centrifuged at 10,000 g at 4ºC to produce the virus containing supernatant, which is assayed for TCID-50 titration on sub-confluent RD cells. This experiment was performed independently three times to generate the data in the figure. The TCID-50 titration was performed according to the protocol described by Reed et al.[1] This experiment was performed independently three times to generate the data in the figure.

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APPENDIX

Full Western blots:

Figure 1B

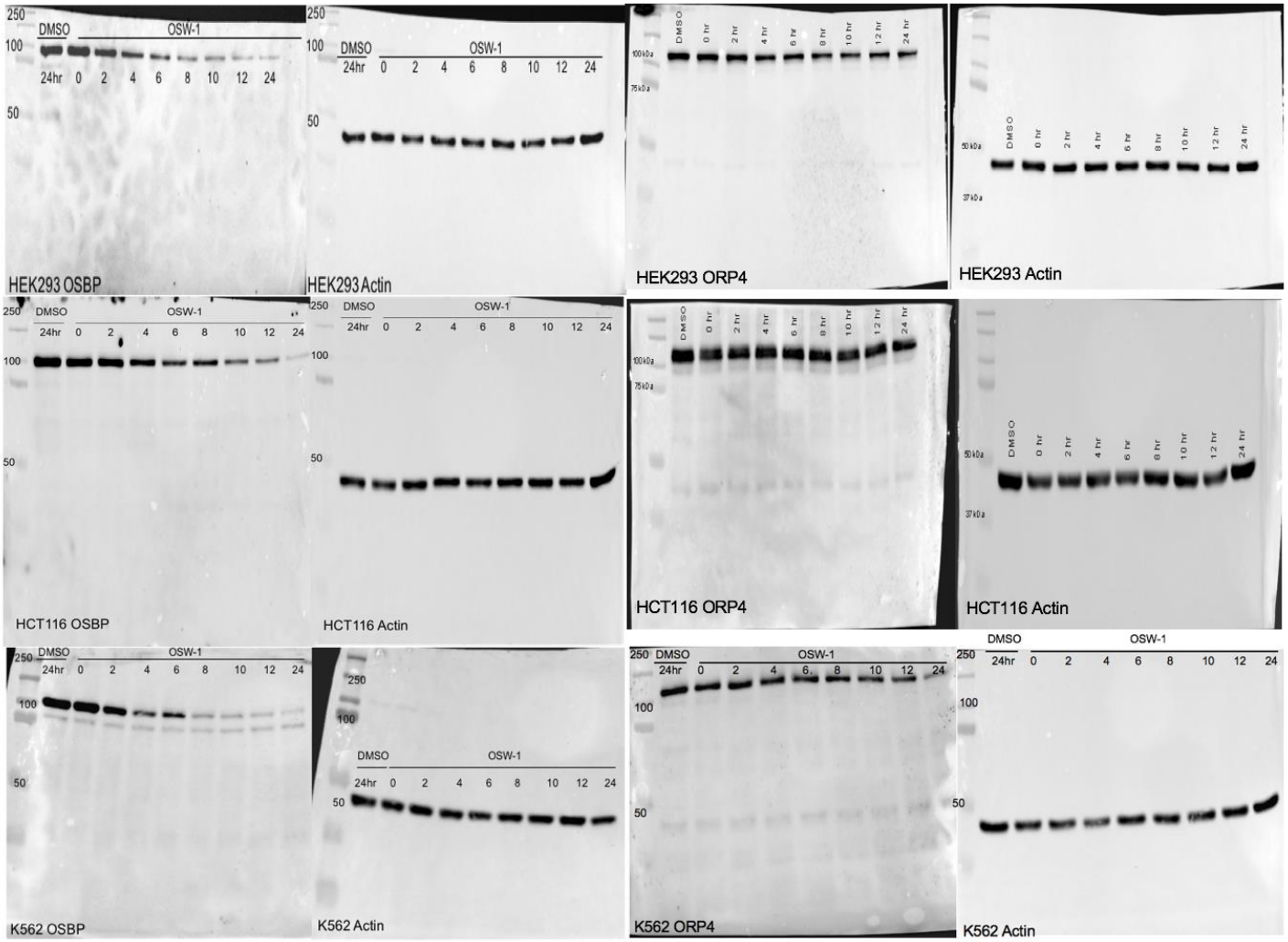


Figure 1C

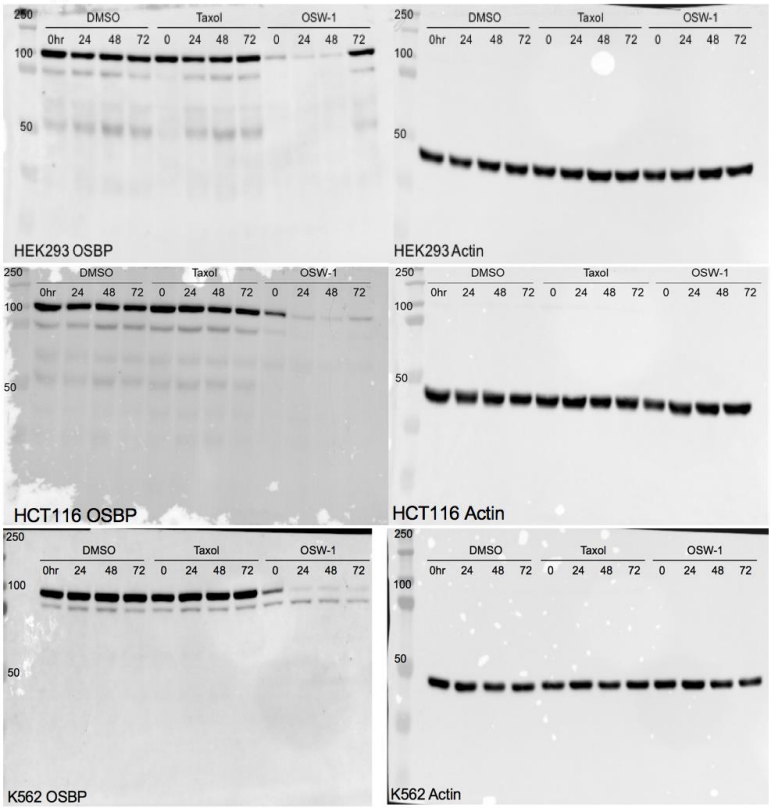


Figure 1D

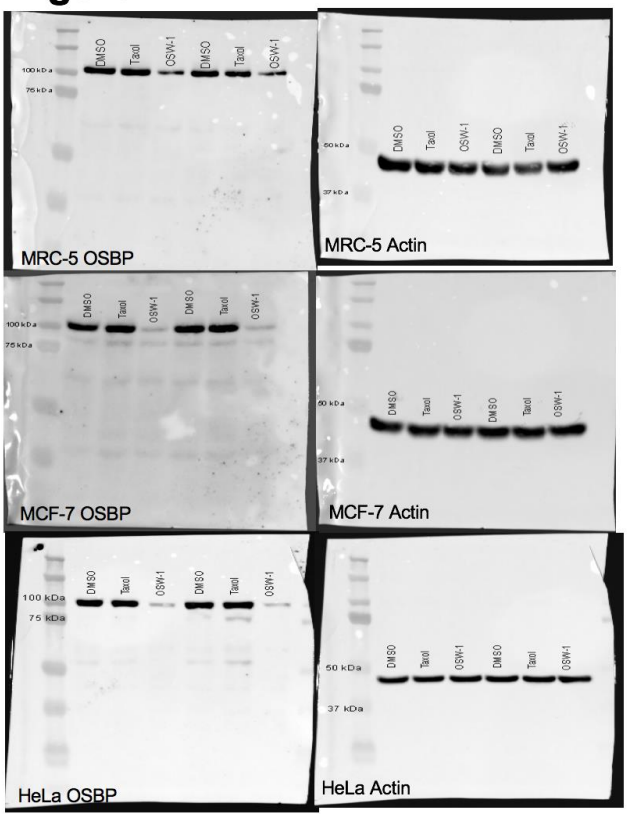
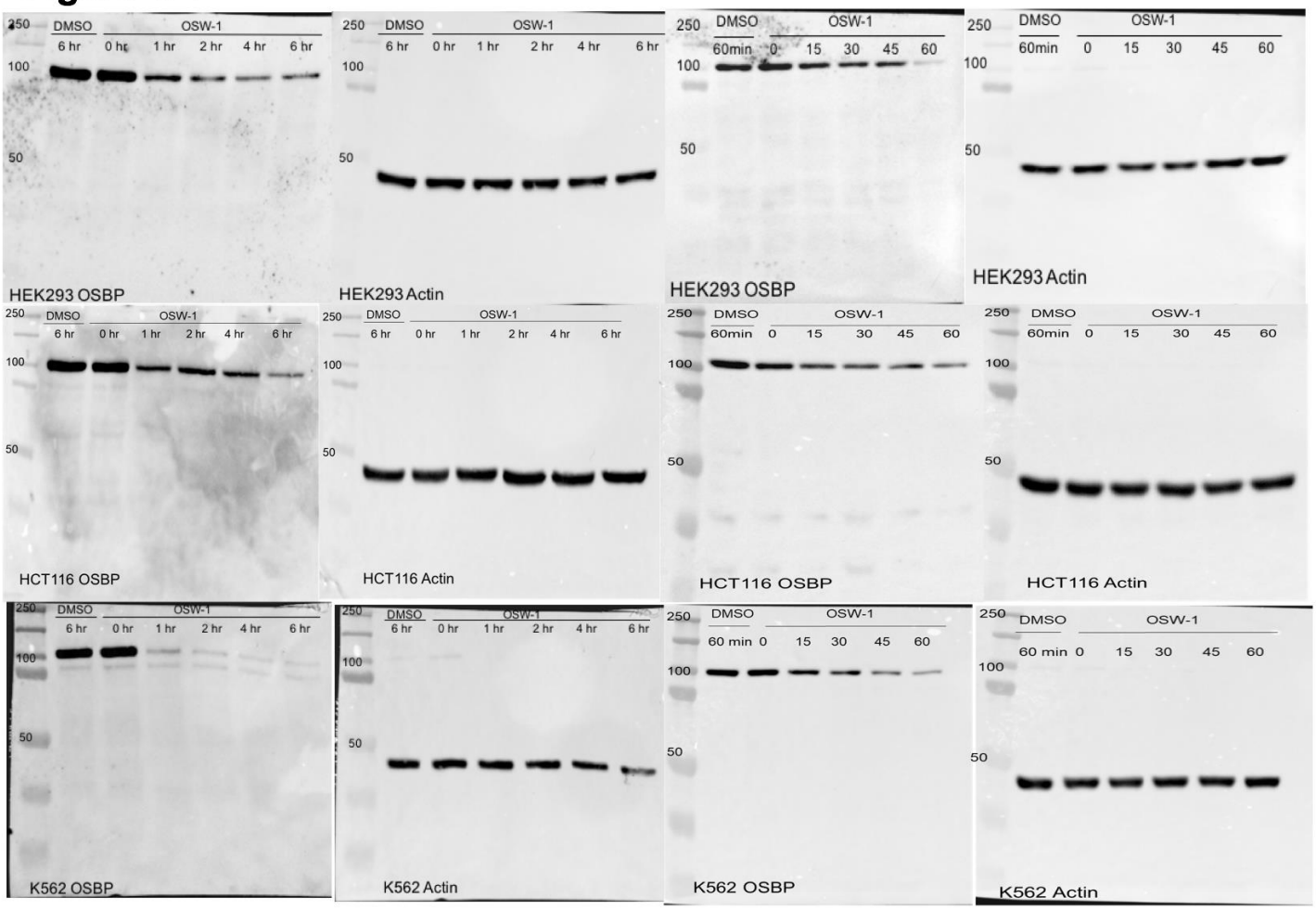


Figure 1E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100
50
DMSO
OSW-1
6 hr
0 hr
1 hr
2 hr
4 hr
6 hr
HEK293 OSBP
HEK293 Actin
60min
0
15
30
45
60
HEK293 OSBP
HEK293 Actin
HCT116 OSBP
HCT116 Actin
HCT116 OSBP
HCT116 Actin
60 min
K562 OSBP
K562 Actin
K562 OSBP
K562 Actin

Figure 4A and B

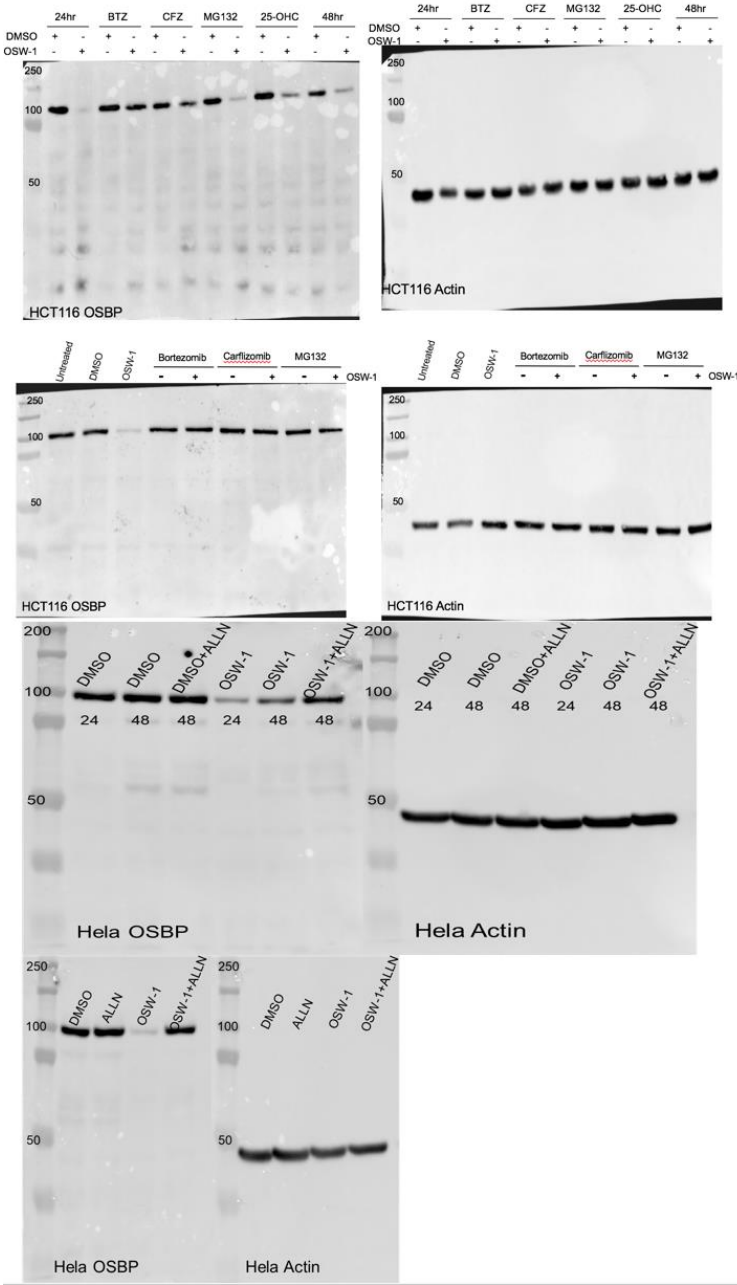


Figure 4C

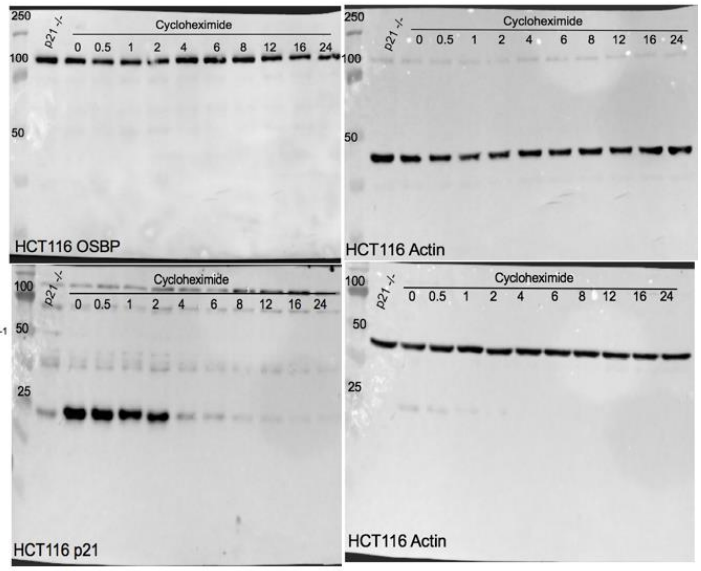
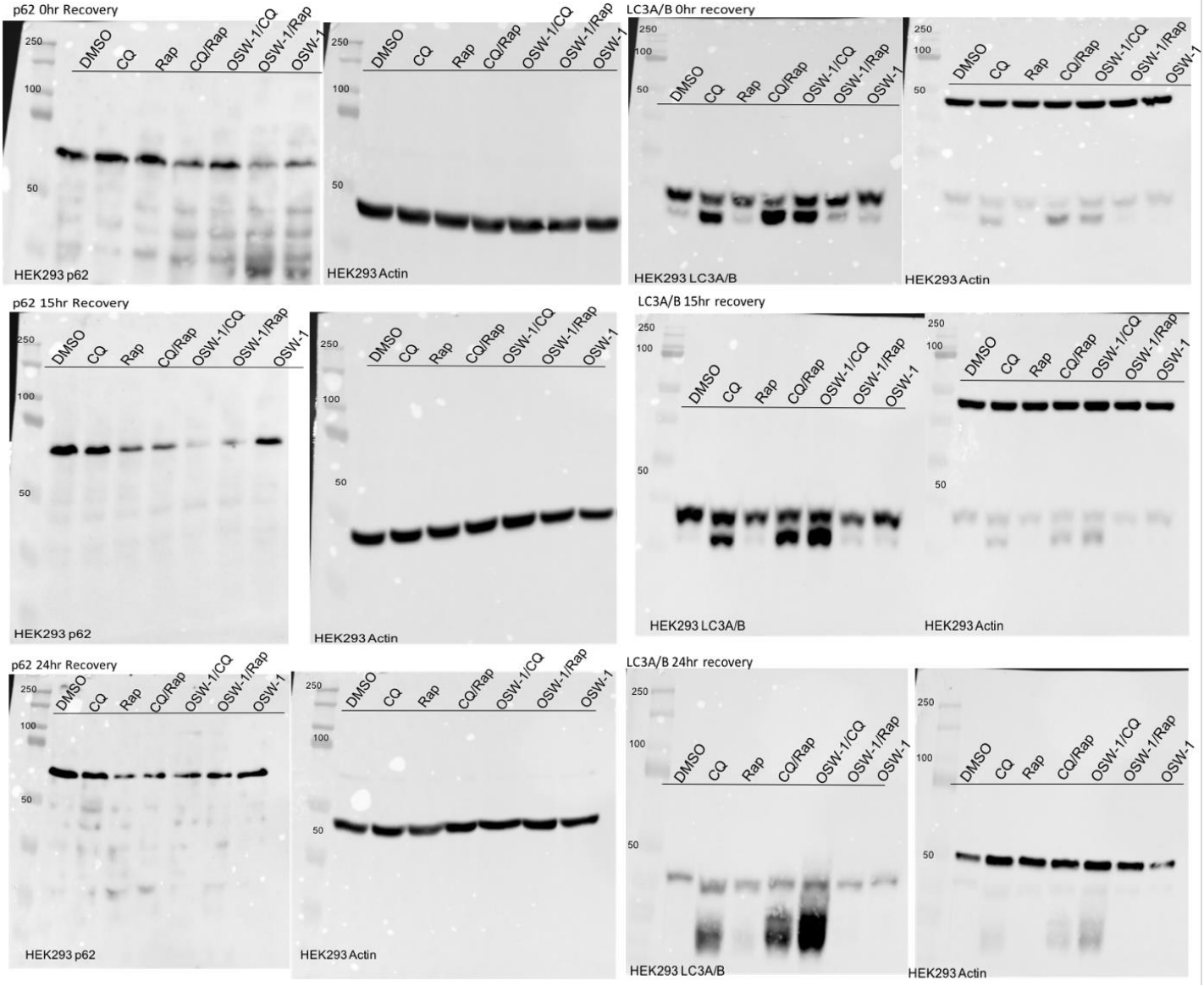
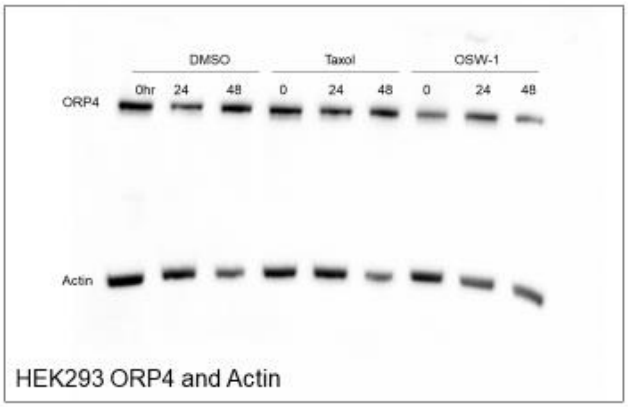


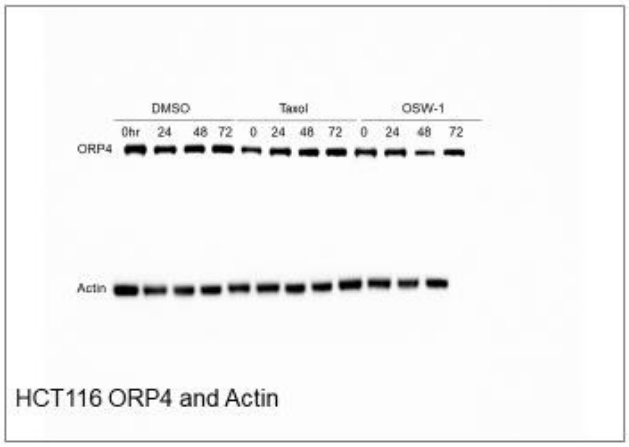
Figure 5B


p62 0hr Recovery
DMSO
CQ
Rap
CQ/Rap
OSW-1/CQ
OSW-1/Rap
OSW-1
250
100
50
HEK293 p62
HEK293 Actin
LC3A/B 0hr recovery
HEK293 LC3A/B
HEK293 Actin
p62 15hr Recovery
LC3A/B 15hr recovery
p62 24hr Recovery
LC3A/B 24hr recovery

Supp 1D

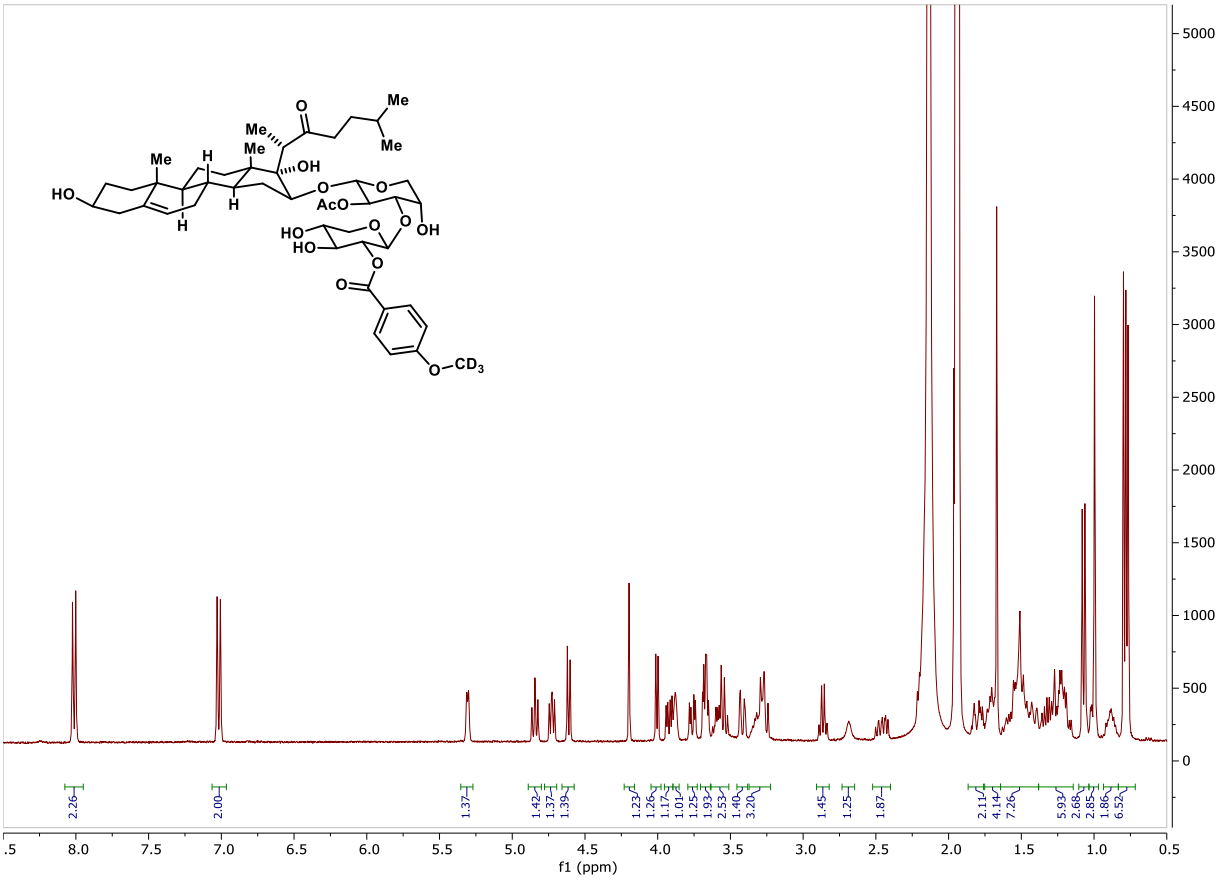


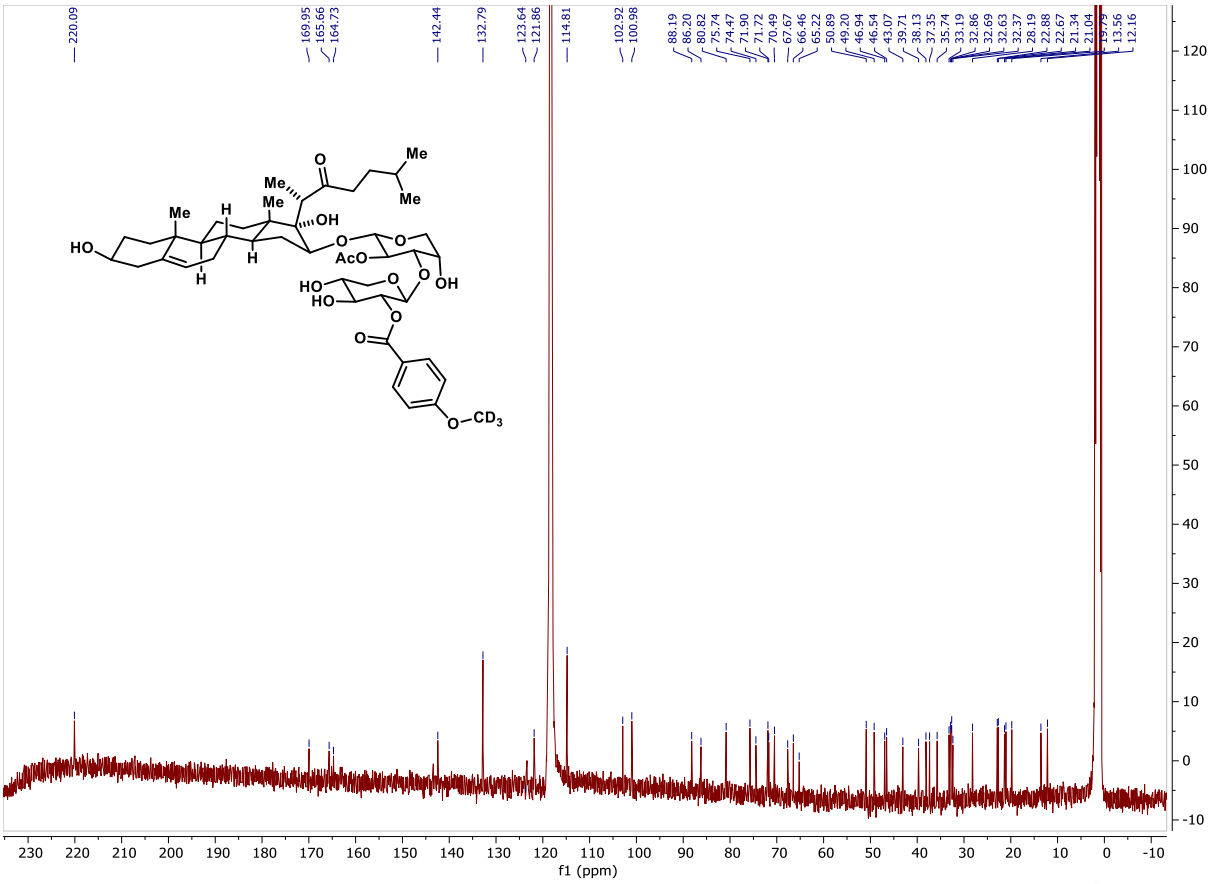
HEK293 ORP4 and Actin



HCT116 ORP4 and Actin

Deuterated OSW-1 data





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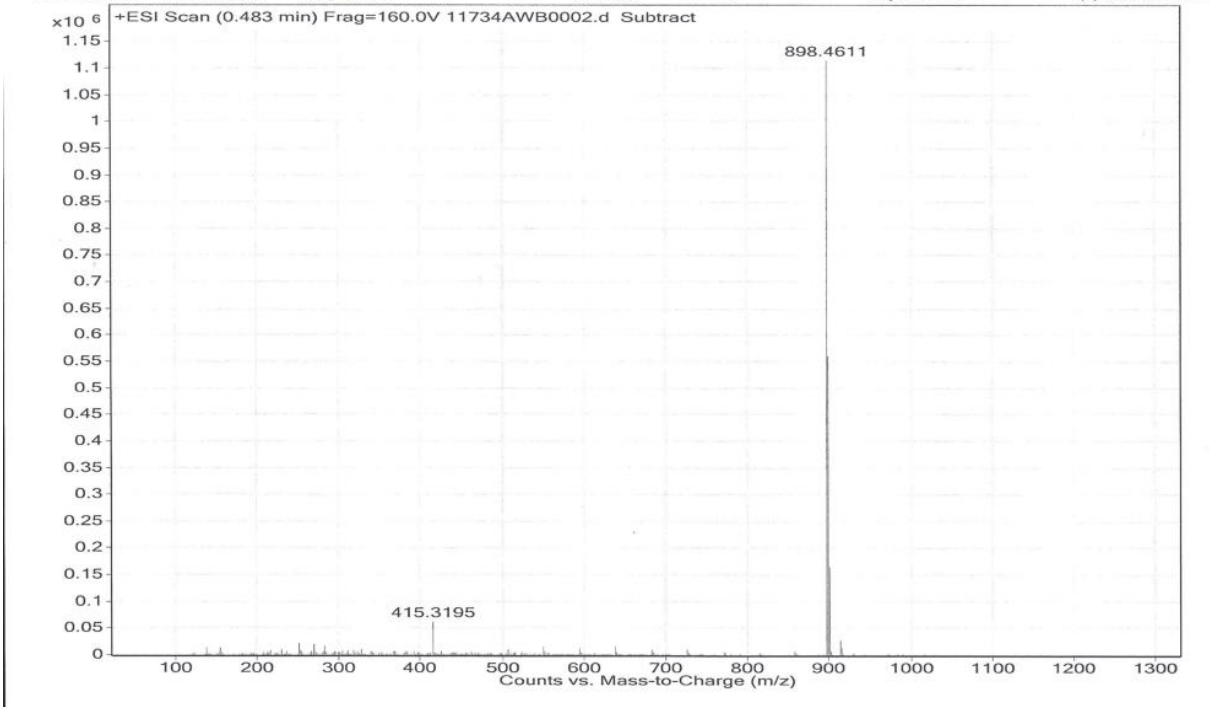

Sample Name	ATL-II-045+047	**Position**	P1-E1	**Instrument Name**	Instrument MS	**User Name**	Foster
Inj Vol	5	**InjPosition**		**SampleType**	Sample	**IRM Calibration Status**	All Ions Missed
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