Supplemental Data

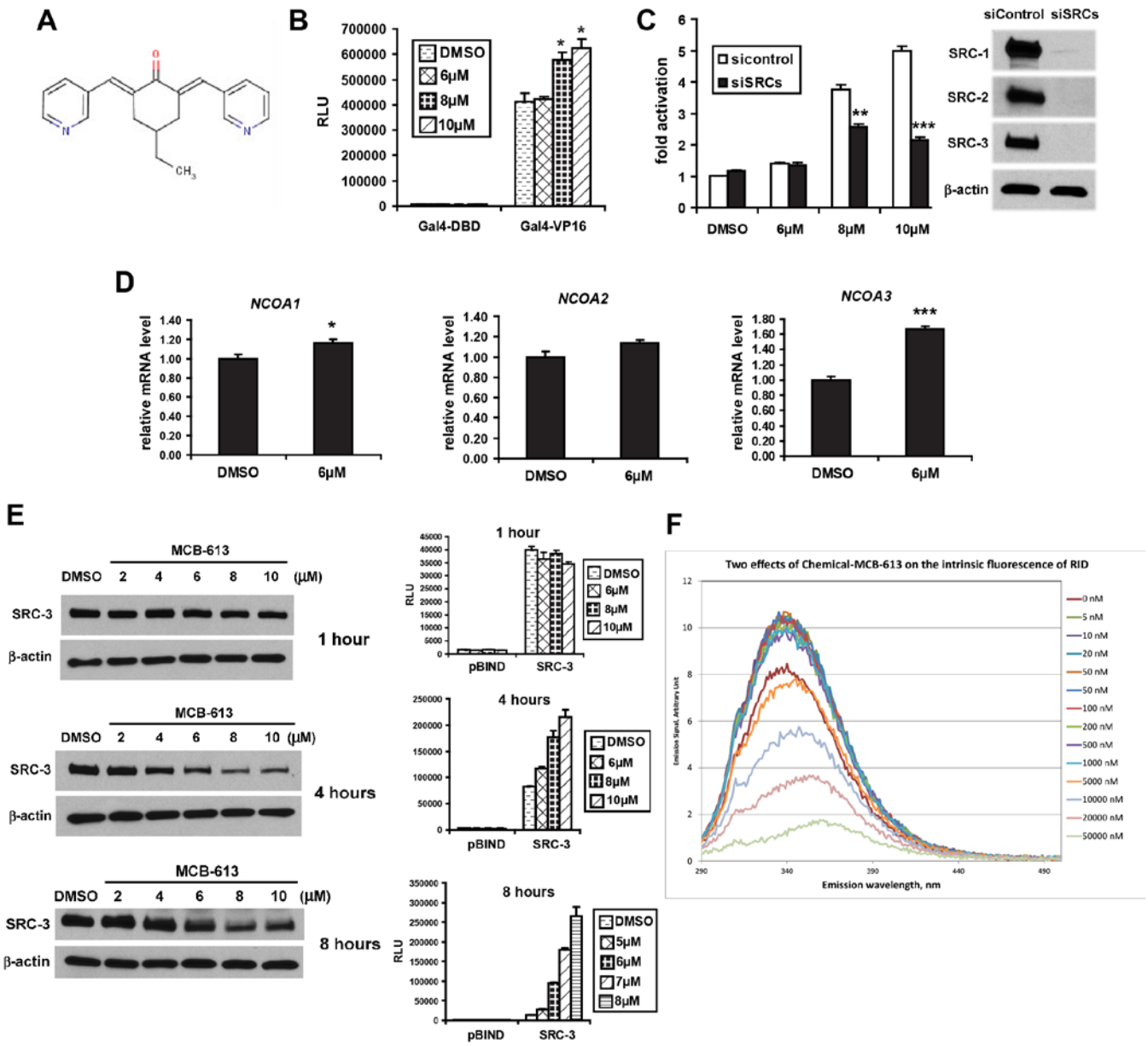


Figure S1, related to Figure 1. MCB-613 selectively activates SRCs.

(A) Structure of MCB-613.

(B) Luciferase assays on HeLa cells co-transfected with expression vectors for Gal4-DBD or Gal4-VP16 and the pG5-luc reporter followed by MCB-613 treatment for 24 hours.

(C) HeLa cells transfected with control siRNA or siRNAs targeting all three SRCs were transfected with MMP2-luc and subjected to MCB-613 treatment at the indicated concentrations for 24 hours followed by luciferase assays. Knock down efficiency for SRC-1, SRC-2 and SRC-3 was shown by Western blot in the right panel.

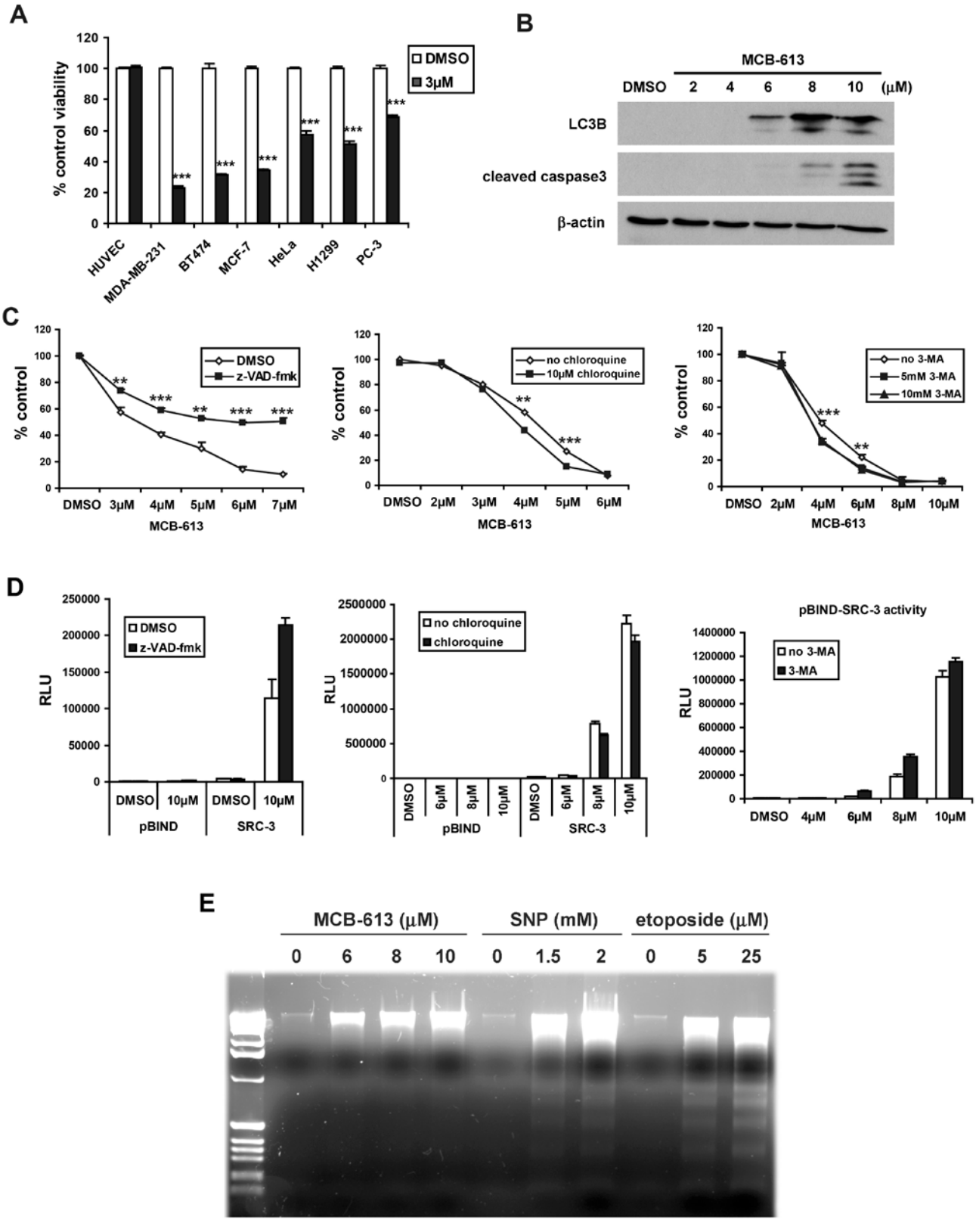
(D) qRT-PCR analysis of *SRC-1 (NCOA1), SRC-2 (NCOA2)* or *SRC-3 (NCOA3)* from HeLa cells treated with MCB-613 at the indicated concentration for 19 hours.

(E) HeLa cells were treated with MCB-613 for 1 hour, 4 hours and 8 hours. Protein levels of SRC-3 and β-actin were shown by Western blot in the left panels. HeLa cells co-transfected with pBIND or pBIND-SRC-3 expression vectors and the pG5-luc reporter were treated with MCB-613 for 1 hour, 4 hours and 8 hours, followed by luciferase assays, which were shown in the right panels.

(F) Fluorescence spectroscopy showing the direct binding of MCB-613 to the receptor interacting domain (RID) of SRC-3.

(G-H) Immunoblotting of CBP and CARM1 in the coIP complex from HeLa cells transfected with FLAG or FLAG-SRC-3 expression vectors and treated with the indicated concentrations of MCB-613 for 4 hours (G) or 8 hours (H).

Data are presented as mean ± SEM. *$p<0.05$, ** $p<0.01$, *** $p<0.001$.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% control viability
DMSO
3μM
HUVEC
MDA-MB-231
BT474
MCF-7
HeLa
H1299
PC-3
B
MCB-613
DMSO 2 4 6 8 10 (μM)
LC3B
cleaved caspase3
β-actin
C
% control
DMSO
z-VAD-fmk
MCB-613
no chloroquine
10μM chloroquine
no 3-MA
5mM 3-MA
10mM 3-MA
D
RLU
pBIND
SRC-3
chloroquine
pBIND-SRC-3 activity
3-MA
E
MCB-613 (μM)
SNP (mM)
etoposide (μM)

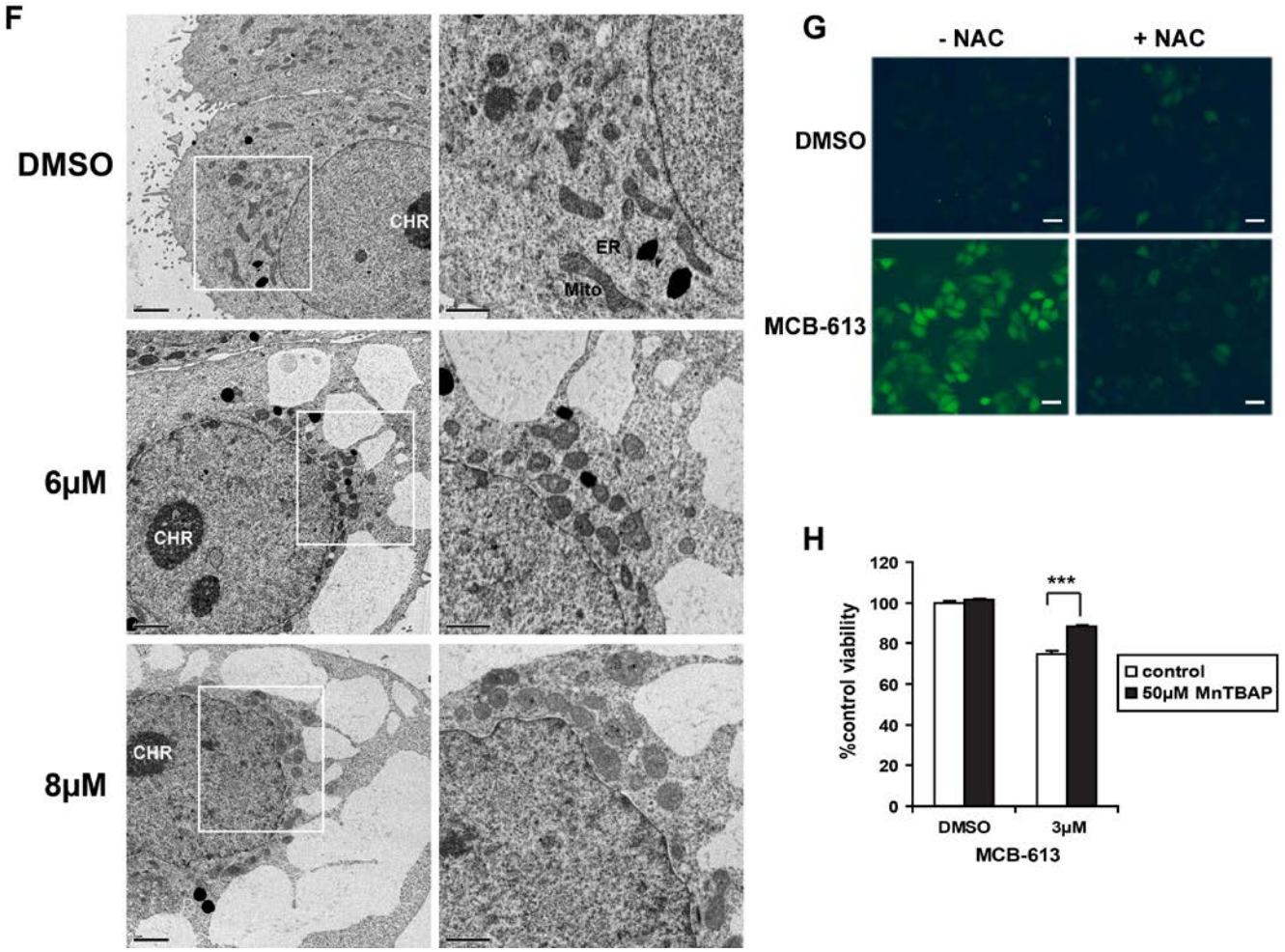


Figure S2, related to Figure 2. MCB-613 induces complex cytotoxicity with characteristics of paraptotic-like cell death.

(A) Viability of primary HUVEC and a panel of human cancer cell lines treated with MCB-613 at the indicated concentration for 48 hours. Data are presented as mean ± SEM. *** $p<0.001$.

(B) Immunoblotting of LC3B and cleaved caspase-3 from HeLa cells treated with MCB-613 for 24 hours.

(C) HeLa cells were treated with MCB-613 for 24 hours, in the presence or absence of caspase inhibitor z-VAD-fmk (left panel), autophagy inhibitor chloroquine (middle panel) or 3-MA (right panel). Cell viability was determined by MTS assay. Data are presented as mean ± SD. ** $p<0.01$, *** $p<0.001$.

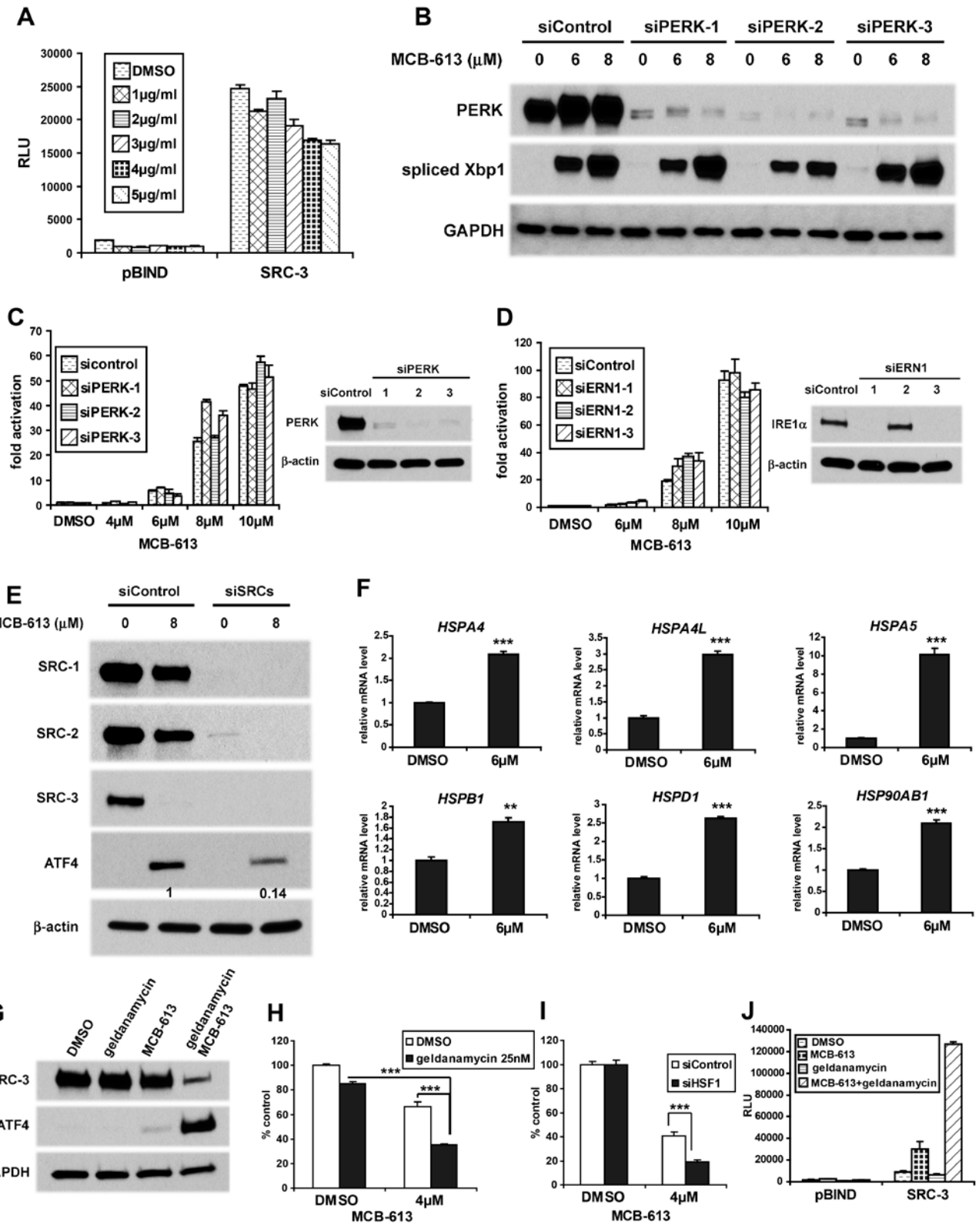
(D) Luciferase assays on HeLa cells transfected with pBIND or pBIND-SRC-3 expression vectors and the pG5-luc reporter and treated with different concentrations of MCB-613 for 24 hours in the presence or absence of z-VAD-fmk (left), chloroquine (middle) or 3-MA (right). Data are presented as mean ± SEM.

(E) Agarose gel electrophoresis of the soluble DNA from HeLa cells treated with different concentrations of MCB-613, SNP or etoposide for 20 hours.

(F) Transmission electron microscopy for HeLa cells treated with DMSO or different concentrations of MCB-613 for 8 hours. The areas within the white boxes in the left panels are magnified in the corresponding right panels. Scale bar: 1μm. CHR: chromatin; Mito: mitochondria.

(G) HeLa cells were treated with DMSO or MCB-613 in the absence or presence of NAC for 30 minutes. Intracellular ROS level was assessed using the ROS indicator CM-H2DCFDA. Scale bar: 40μm.

(H) HeLa cells were treated with MCB-613 in the absence or presence of MnTBAP for 24 hours. Cell viability was assessed by MTS assay. Data are presented as mean ± SD. *** $p<0.001$.


A
RLU
DMSO
1μg/ml
2μg/ml
3μg/ml
4μg/ml
5μg/ml
pBIND
SRC-3
B
siControl
siPERK-1
siPERK-2
siPERK-3
MCB-613 (μM)
PERK
spliced Xbp1
GAPDH
C
fold activation
sicontrol
siPERK-1
siPERK-2
siPERK-3
DMSO
4μM
6μM
8μM
10μM
MCB-613
siPERK
PERK
β-actin
D
fold activation
siControl
siERN1-1
siERN1-2
siERN1-3
MCB-613
siERN1
IRE1α
β-actin
E
siControl
siSRCs
MCB-613 (μM)
SRC-1
SRC-2
SRC-3
ATF4
β-actin
F
HSPA4
HSPA4L
HSPA5
HSPB1
HSPD1
HSP90AB1
relative mRNA level
DMSO
6μM
G
DMSO
geldanamycin
MCB-613
geldanamycin MCB-613
SRC-3
ATF4
GAPDH
H
% control
DMSO
geldanamycin 25nM
4μM
MCB-613
I
siControl
siHSF1
J
RLU
DMSO
MCB-613
geldanamycin
MCB-613+geldanamycin
pBIND
SRC-3

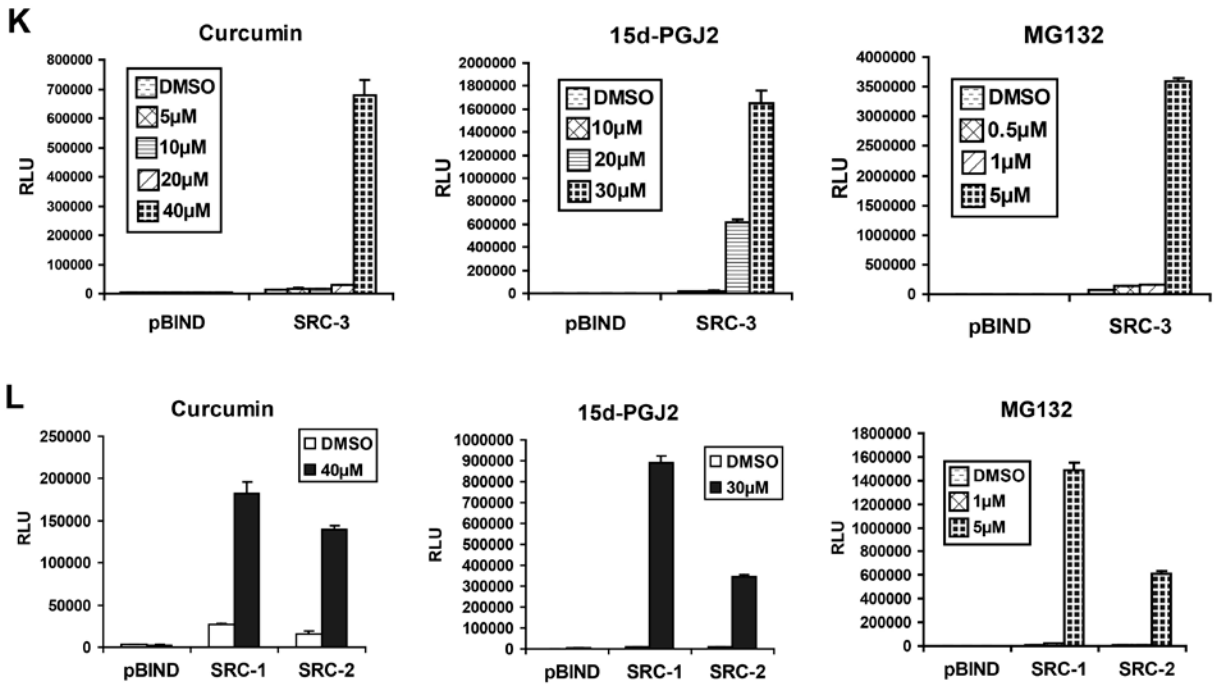


Figure S3, related to Figure 3. SRC activation by MCB-613 is upstream of UPR induction and critical for the paraptotic-like cell death.

(A) Luciferase assays on HeLa cells transfected with pBIND or pBIND-SRC-3 expression vectors and the pG5-luc reporter and treated with increasing concentrations of tunicamycin for 24 hours. Data are presented as mean ± SEM.

(B) Immunoblotting of PERK and spliced Xbp1 from HeLa cells transfected with control siRNA or PERK-targeting siRNAs and treated with MCB-613 for 20 hours.

(C) HeLa cells transfected with control siRNAs or siRNAs targeting PERK were transfected with the pBIND-SRC-3 expression vector and the pG5-luc reporter followed by the treatment of increasing concentrations of MCB-613 for 24 hours and luciferase assays. Knock down efficiency is shown by Western blot in the right panel. Data are presented as mean ± SEM.

(D) HeLa cells transfected with control siRNAs or siRNAs targeting ERN1 (IRE1α) were transfected and treated as in C. Knock down efficiency is shown by Western blot in the right panel. Data are presented as mean ± SEM.

(E) HeLa cells in which all three SRCs were simultaneously knocked down by siRNAs were treated with MCB-613 for 15 hours and immunoblotted for SRCs and ATF4. Numbers indicate the quantification of ATF4 in the Western blot.

(F) qRT-PCR analysis of the indicated heat shock proteins from HeLa cells treated with DMSO or MCB-613 for 19 hours. Data are presented as mean ± SEM. ** $p<0.01$; *** $p<0.001$.

(G) Immunoblotting of SRC-3, ATF4 and GAPDH from HeLa cells treated with 6μM MCB-613, or 50nM geldanamycin, or both for 24 hours.

(H) Viability of HeLa cells treated with MCB-613 in the presence or absence of geldanamycin at the indicated concentrations for 48 hours. Data are presented as mean ± SD. *** $p<0.001$.

(I) Viability of HeLa cells in which *HSF1* was knocked down by siRNAs after the treatment of MCB-613 for 48 hours. Data are presented as mean ± SD. *** $p<0.001$.

(J) Luciferase assays on HeLa cells co-transfected with pBIND or pBIND-SRC-3 expression vectors and the pG5-luc reporter and treated with 6μM MCB-613, 50nM geldanamycin, or both for 24 hours. Data are presented as mean ± SEM.

(K-L) Additional agents can activate SRC-3 (K), SRC-1 and SRC-2 (L), leading to paraptosis. HeLa cells co-transfected with pBIND or pBIND-SRC-3 (pBIND-SRC-1, pBIND-SRC-2) expression vectors and the pG5-luc reporter were treated with curcumin, 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), or MG132 at the indicated concentrations for 24 hours. Data are presented as mean ± SEM.

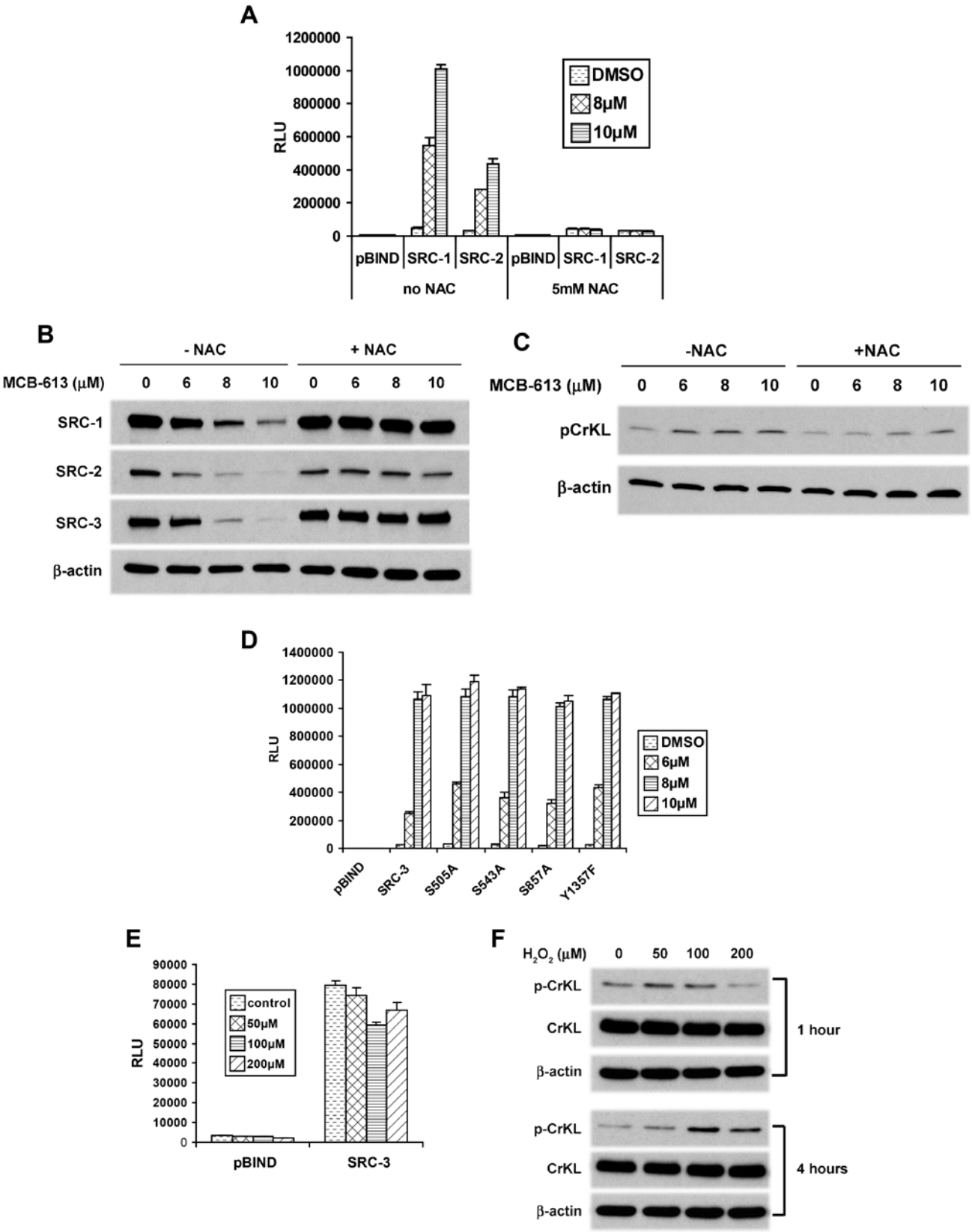

A
RLU
DMSO
8μM
10μM
pBIND
SRC-1
SRC-2
no NAC
5mM NAC
B
- NAC
+ NAC
MCB-613 (μM)
SRC-1
SRC-2
SRC-3
β-actin
C
-NAC
+NAC
pCrKL
β-actin
D
RLU
DMSO
6μM
8μM
10μM
pBIND
SRC-3
S505A
S543A
S857A
Y1357F
E
RLU
control
50μM
100μM
200μM
pBIND
SRC-3
F
H2O2 (μM)
p-CrKL
CrKL
β-actin
1 hour
4 hours

Figure S4, related to Figure 4. ROS induction by MCB-613 contributes to SRC activation via the Abl kinase signaling pathway.

(A) Luciferase assays on HeLa cells co-transfected with pG5-luc and pBIND, pBIND-SRC-1 or pBIND-SRC-2 expression vectors and treated with MCB-613 for 24 hours in the presence or absence of NAC. Data are presented as mean ± SEM.

(B) Immunoblotting of SRCs from HeLa cells treated with the indicated concentrations of MCB-613 in the absence or presence of NAC for 24 hours.

(C) Immunoblotting of phosphorylated CrKL from HeLa cells treated with increasing concentrations of MCB-613 in the absence or presence of NAC for 4 hours.

(D) Luciferase assays on HeLa cells co-transfected with pG5-luc and pBIND, pBIND-SRC-3WT, or the indicated pBIND-SRC-3 phospho mutant expression vectors and treated with MCB-613 for 24 hours. Data are presented as mean ± SEM.

(E) Luciferase assays on HeLa cells co-transfected with pG5-luc and pBIND or pBIND-SRC-3 expression vectors and treated with H_2O_2 at the indicated concentrations for 24 hours. Data are presented as mean ± SEM.

(F) Immunoblotting of phosphorylated CrKL from HeLa cells treated with H_2O_2 as in E for 1 or 4 hours.

Supplemental Experimental Procedures

Chemicals and antibodies. MCB-613 (4-Ethyl-2,6-bis-pyridin-3-ylmethylene-cyclohexanone) was obtained from ChemBridge. N-Acetyl cysteine, cycloheximide, bufalin, chloroquine, tunicamycin, curcumin and H_2O_2 are from Sigma. MG132 and z-VAD-fmk are from EMD Millipore. 15d-PGJ2 is from Cayman Chemical. Kinase inhibitor library, AT9283, PHA7393598 and geldanamycin are from SelleckChem. CM-H2DCFDA is from Invitrogen. Phos-tag is from Wako. QuikChange Lightening site-directed mutagenesis kit is from Agilent. Antibodies against SRC-1, SRC-3, Calnexin, Abl, phospho-CrKL (Y207), phospho-eIF2α (S51), cleaved caspase-3, ubiquitin and spliced Xbp1 were purchased from Cell Signaling. Antibodies against CARM1 and SRC-2 were obtained from Bethyl Laboratories. FLAG antibody was obtained from Sigma. Antibody against phospho-IRE1α (S724) was purchased from Abcam. Antibodies against ATF4, GAPDH and CBP were purchased from Santa Cruz Biotechnology.

Cell culture. Cell lines were maintained in DMEM (HeLa, MCF-7, HepG2, MEF), RPMI 1640 (H1299), or DMEM/F12 (PC-3) supplemented with 10% fetal calf serum, penicillin and streptomycin (100 U/ml). Primary mouse hepatocytes were isolated and cultured as previously described (Louet et al., 2010). All cells were maintained at 37°C under 5% CO_2.

Plasmids and siRNA transfection. The constructs expressing the Gal4 responsive luciferase reporter (pG5-luc), Gal4 DBD fusion with SRC-1, SRC-2 and SRC-3 (pBIND-SRC-1/-2/-3), *MMP2* or *MMP13* promoter driven luciferase reporter (MMP2-luc, MMP13-luc) were described previously (Lonard et al., 2000; Yan et al., 2008). pBIND-SRC-3 phospho mutants were generated using the QuikChange Lightening site-directed mutagenesis kit according to the manufacturer's instruction. The indicated expression plasmids were transfected into cells using lipofectamine 2000 according to the manufacturer's instructions. Smart pools of siRNAs targeting SRC-1, SRC-2, SRC-3, PERK, or Abl1 were purchased

from Dharmacon and single siRNA against PERK or ERN1 was from Invitrogen. siRNAs were transfected into cells using lipofectamine RNAiMax (Invitrogen) according to manufacturer's instructions.

Surface plasmon resonance. Fragments of SRC-3 CID, RID, and bHLH were expressed as GST fusion proteins and purified as previously described (Wang et al., 2011). Surface Plasmon Resonance experiments of the bindings of MCB-613 to SRC-3 fragments were carried out in a Biacore 3000 (GE Health Bioscience). The proteins were immobilized on CM-5 chips according to manufacturer's instructions (GE Health Bioscience) to a level between 750 to 8000 RU (Resonance Unit). Reference cells were prepared according to manufacturer's instruction. Corrections of solvent effect were applied to the reference cell signals (Karlsson and Stahlberg, 1995). Signals from flow cells immobilized with a SRC3 fragment were subtracted with the solvent effect corrected reference cell signal to yield the binding signals.

Fluorescence spectrometry. Fluorescence spectrometric measurements were performed on a SLM 48000S fluorescence spectrophotometer (SLM-Aminco) and an Agilent Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies) using the GST fusion proteins of different portions of SRC-3 as described previously (Wang et al., 2011). A total of 1.5 μM of GST SRC-3 RID, CID or bHLH was placed in a fluorescence cuvette and excited by UV light at a wavelength of 278 nm with a 2 nm bandwidth and the emission spectra were recorded from 290nm to above 500 nm with a bandwidth of 4nm. The aliquot size of test compound was maintained below 5% of the total sample volume in order to minimize the effects of dilution.

Luciferase assays. After various compound treatments, cells were lysed in luciferase lysis buffer and assayed for luciferase activity using the ONE-Glo luciferase assay system (Promega). All luciferase activities were normalized to protein concentration determined by Bradford assay (Bio-Rad).

Western blot and immunoprecipitation. For Western blotting, cells were harvested and lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% NP40, protease and phosphatase inhibitor) and then centrifuged for 15 min at 14000 rpm at 4°C. After total cellular protein concentration was determined by Bradford analysis (Bio-Rad), protein lysates were loaded onto and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (BioRad) which were blocked and incubated with indicated antibodies. For immunoprecipitation, 10 μg of plasmid encoding FLAG or FLAG-SRC-3 was transfected into HeLa cells at 90% confluence in one 10cm plate with lipofectamine 2000 (Invitrogen). 24 hours after transfection, the cells were treated with MCB-613 and lysed with lysis buffer (25mM Tris-Cl pH7.5, 150mM NaCl, 5% glycerol, 1 mM EDTA, 1% Triton X-100, protease and phosphatase inhibitor) at 4°C. After a 30-second sonication, the lysate was cleared by centrifugation at 14000rpm at 4°C for 15 minutes and was incubated with anti-Flag-M2 agarose affinity gel (Sigma) for four hours. The agarose gel was washed four times with washing buffer (25mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and resuspended with SDS loading buffer for Western blotting.

Cell viability assays. Cells were seeded in 96-well plates and allowed to reach 60% to 70% confluence. After indicated compound treatments, relative numbers of viable cells were measured by MTS assay using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Measurement of reactive oxygen species (ROS). Cells were loaded with 10 μM general ROS indicator CM-H2DCFDA in serum free DMEM for 30 minutes at 37°C in the dark. After a 5-minute recovery in complete DMEM, cells were treated with indicated concentrations of MCB-613 for 30 minutes, then examined by fluorescence microscopy.

Inter-nucleosomal DNA fragmentation. HeLa cells treated with different compounds were lysed in hypotonic buffer (10mM Tris, 10mM EDTA, pH 7.4) containing 0.5% Triton X-100. The resultant supernatant was treated with proteinase K and RNase A, extracted with phenol:chloroform and ethanol precipitated. DNA precipitates were dissolved in 10mM Tris, 1mM EDTA at pH 8.0 and analysed by 1.5% agarose gel electrophoresis.

Transmission electron microscopy (TEM). TEM analysis was performed in the Integrated Microscopy Core of Baylor College of Medicine using the Hitachi H-7500 Transmission Electron Microscope with Gatan 2Kx2K CCD.

Human Stress and Toxicity PathwayFinder qPCR array. *SRC-3 WT* and *KO* HeLa cells were treated with MCB-613 or DMSO for 24 hours, total RNA was extracted and reverse transcribed using the RT^2 First Strand Kit (SA Biosciences), followed by the Human Stress and Toxicity PathwayFinder qPCR Array (SA Biosciences) according to the manufacturer's instruction.

Quantitative PCR analysis. HeLa cell total RNA was extracted using the RNeasy mini Kit (Qiagen), which was then reverse transcribed into cDNA with the SuperScript VILO cDNA synthesis kit (Invitrogen). The Taqman-based quantitative PCR was performed using the ABI StepOnePlus real-time PCR system (Biosystems). Relative quantitation was obtained by normalizing to the internal control *GAPDH*.

Table. Primers and corresponding Roche universal probes used in qPCR

Gene	Primer sequence	Probe
MMP13	5'-ccagtctccgaggagaaaca-3' and 5'-aaaaacagctccgcatcaac-3'	#73
ATF6B	5'- caccacagtccttctgcagtc-3' and 5'-tcaggctggactcgaatagc-3'	#83
DDIT3	5'-aaggcactgagcgtatcatgt-3' and 5'- tgaagatacacttccttcttgaaca-3'	#21
DNAJC3	5'- gagctcatcagagatggcaga-3' and 5'-tgaacgaactgtatattcagcaatg-3'	#21
TNFRSF10B	5'-agacccttgtgctcgttgtc-3' and 5'-ttgttgggtgatcagagcag-3'	#18
LDHA	5'-gtccttggggaacatggag-3' and 5'- ttcagagagacaccagcaaca-3'	#47
GSR	5'- aacaacatcccaactgtggtc-3' and 5'-tccatatttatgaatggcttcatct-3'	#83
GAPDH	5'-agccacatcgctcagacac-3' and 5'-gcccaatacgaccaaatcc-3'	#60
NCOA1	5'-tgcagcagaatgtcttccag-3' and 5'-aaagttggcctcaccttgg-3'	#15
NCOA2	5'-aggcaacctgttcccaaac-3' and 5'-actggcttcagcagtgtcag-3'	#27
NCOA3	5'-agctgagctgcgaggaaa-3' and 5'-gagtccaccatccagcaagt-3'	#70
HSPA4	5'-cagcagacaccagcagaaaa-3' and 5'-ccttggatccagcttgagag-3'	#13
HSPA4L	5'-agacacagaaaattggctttatga-3' and 5'-gaataggctggccgtatttct-3'	#55
HSPA5	5'-agctgtagcgtatggtgctg-3' and 5'-aaggggacatacatcaagcagt-3'	#64
HSPB1	5'-tccctggatgtcaaccactt-3' and 5'-gatgtagccatgctcgtcct-3'	#22
HSPD1	5'-gcaaagttcctcagaagttggt-3' and 5'-gcatccaataaagcagttctca-3'	#19
HSP90AB1	5'-aaccgcatctatcgcatga-3' and 5'-catcaggaactgcagcattg-3'	#6

Supplemental References

Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Molecular cell *5*, 939-948.

Louet, J. F., Chopra, A. R., Sagen, J. V., An, J., York, B., Tannour-Louet, M., Saha, P. K., Stevens, R. D., Wenner, B. R., Ilkayeva, O. R., *et al.* (2010). The coactivator SRC-1 is an essential coordinator of hepatic glucose production. Cell metabolism *12*, 606-618.