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Supplemental Material to:

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and Chi-Ming Liang

Recombinant protein rVP1 upregulates BECN1-independent autophagy, MAPK1/3 phosphorylation and MMP9 activity via WIPI1/WIPI2 to promote macrophage migration

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Figure S1.

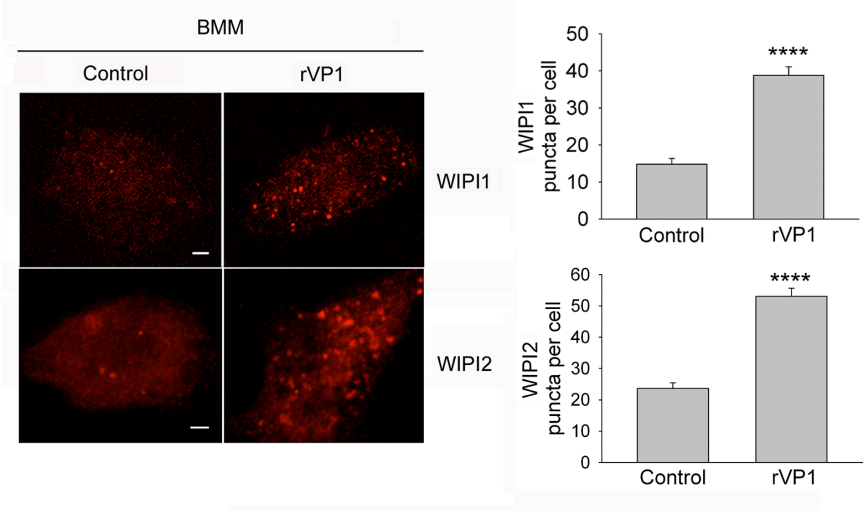


Figure S2.

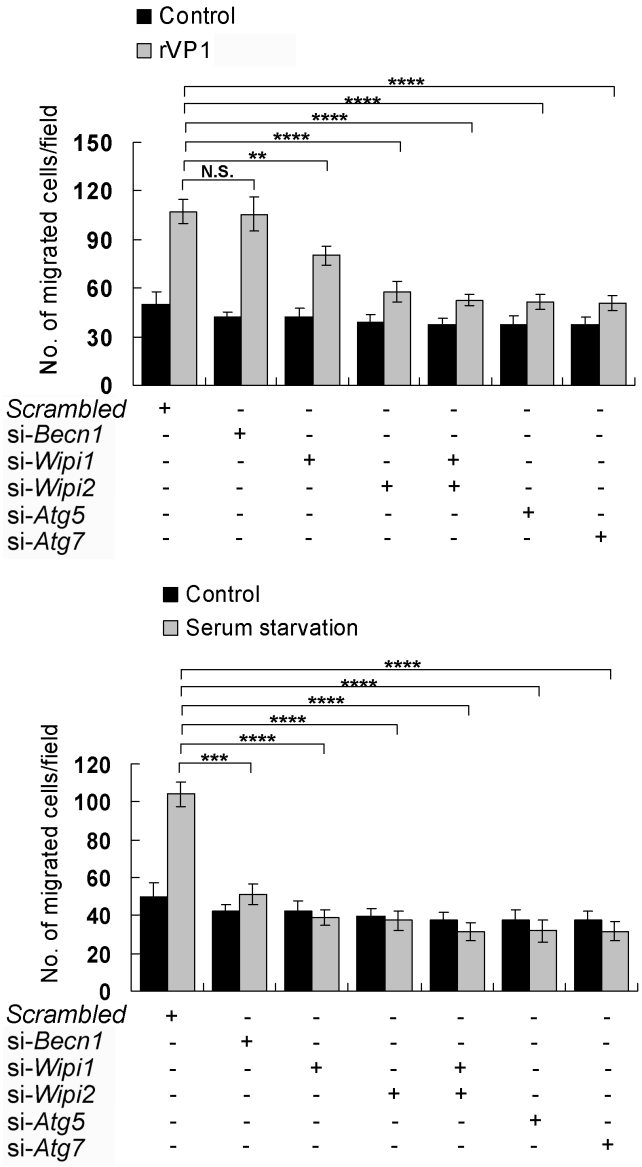


Figure S3.

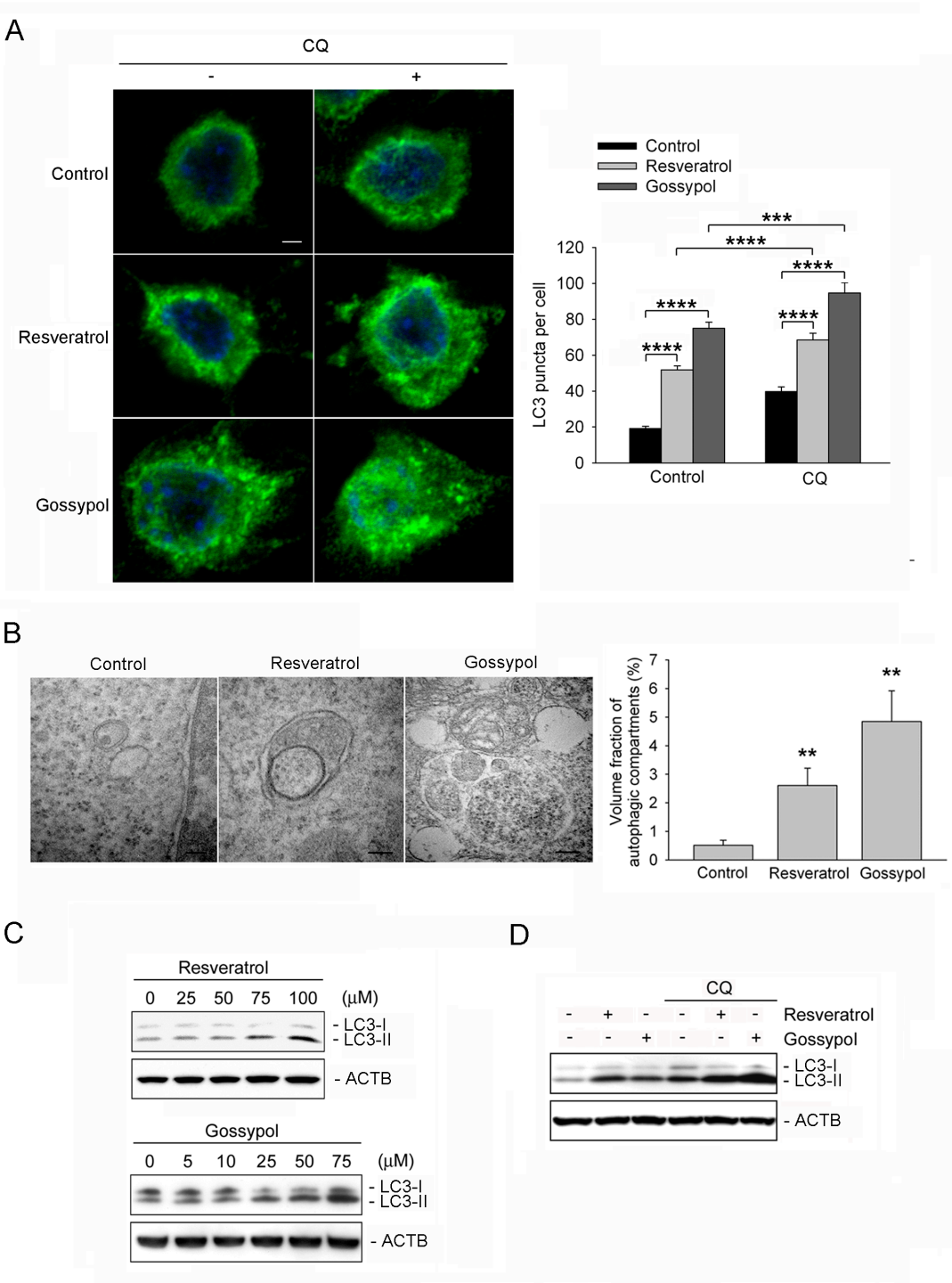

A
CQ
-
+
Control
Resveratrol
Gossypol
Control
Resveratrol
Gossypol
LC3 puncta per cell
0
20
40
60
80
100
120
****
***
****
****
****
****
Control
CQ
B
Control
Resveratrol
Gossypol
Volume fraction of autophagic compartments (%)
0
1
2
3
4
5
6
7
**
**
Control
Resveratrol
Gossypol
C
Resveratrol
0 25 50 75 100 (μM)
- LC3-I
- LC3-II
- ACTB
Gossypol
0 5 10 25 50 75 (μM)
- LC3-I
- LC3-II
- ACTB
D
CQ
- + - - + - Resveratrol
- - + - - + Gossypol
- LC3-I
- LC3-II
- ACTB

Figure S4.

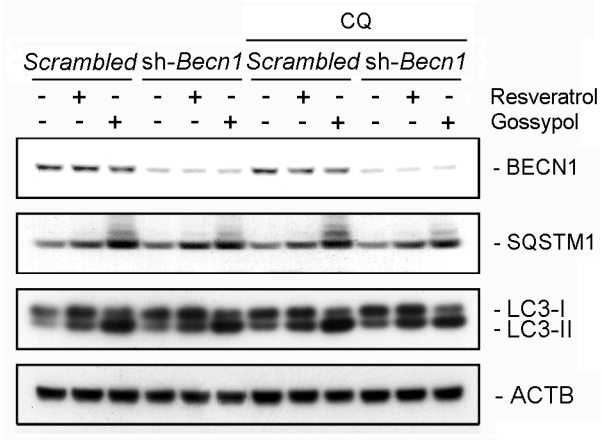
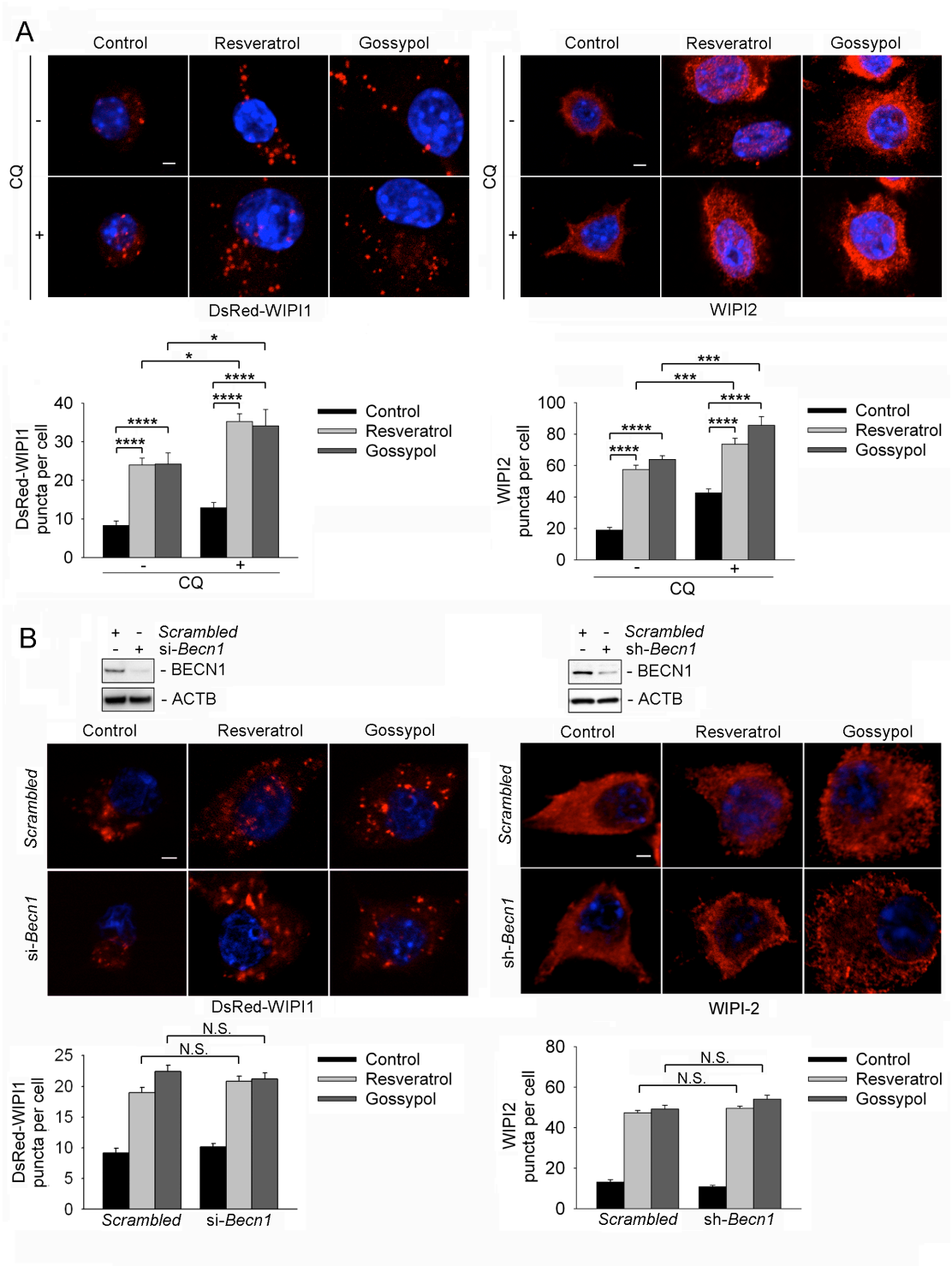

CQ
Scrambled
sh-Becn1
Scrambled
sh-Becn1
Resveratrol
Gossypol
- BECN1
- SQSTM1
- LC3-I
- LC3-II
- ACTB

Figure S5.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Control
Resveratrol
Gossypol
CQ
DsRed-WIPI1
WIPI2
DsRed-WIPI1 puncta per cell
WIPI2 puncta per cell
Control
Resveratrol
Gossypol
B
Scrambled
si-Becn1
sh-Becn1
- BECN1
- ACTB
DsRed-WIPI1
WIPI-2
N.S.

Figure S6.

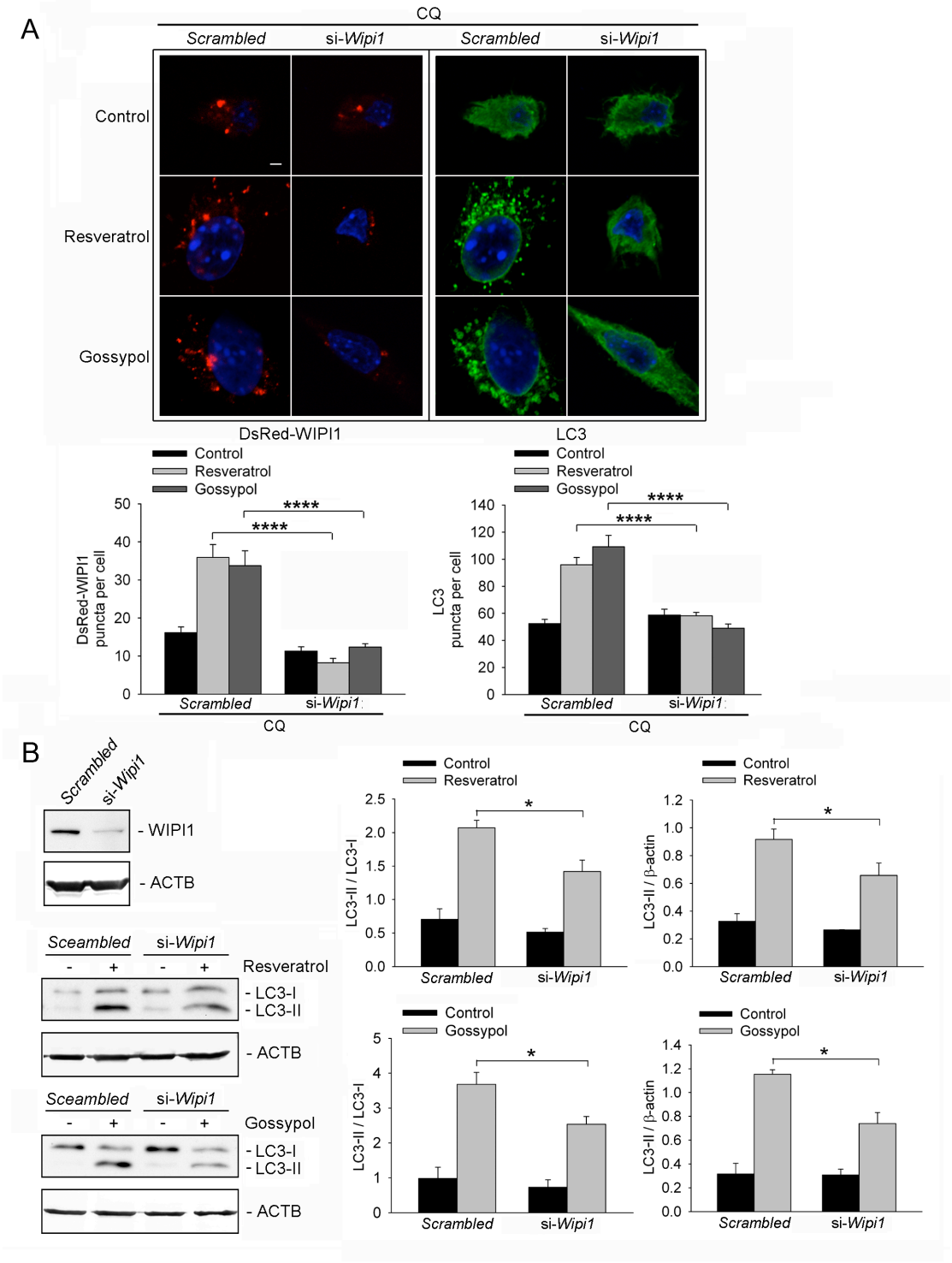
A
CQ
Scrambled
si-Wipi1
Scrambled
si-Wipi1
Control
Resveratrol
Gossypol
DsRed-WIPI1
LC3
Control
Resveratrol
Gossypol
DsRed-WIPI1 puncta per cell
LC3 puncta per cell
****
****
Scrambled
si-Wipi1
CQ
B
Scrambled
si-Wipi1
- WIPI1
- ACTB
Sceambled
si-Wipi1
Resveratrol
- LC3-I
- LC3-II
- ACTB
Sceambled
si-Wipi1
Gossypol
- LC3-I
- LC3-II
- ACTB
Control
Resveratrol
LC3-II / LC3-I
LC3-II / β-actin
Control
Gossypol
LC3-II / LC3-I
LC3-II / β-actin
*
Scrambled
si-Wipi1

Figure S7.

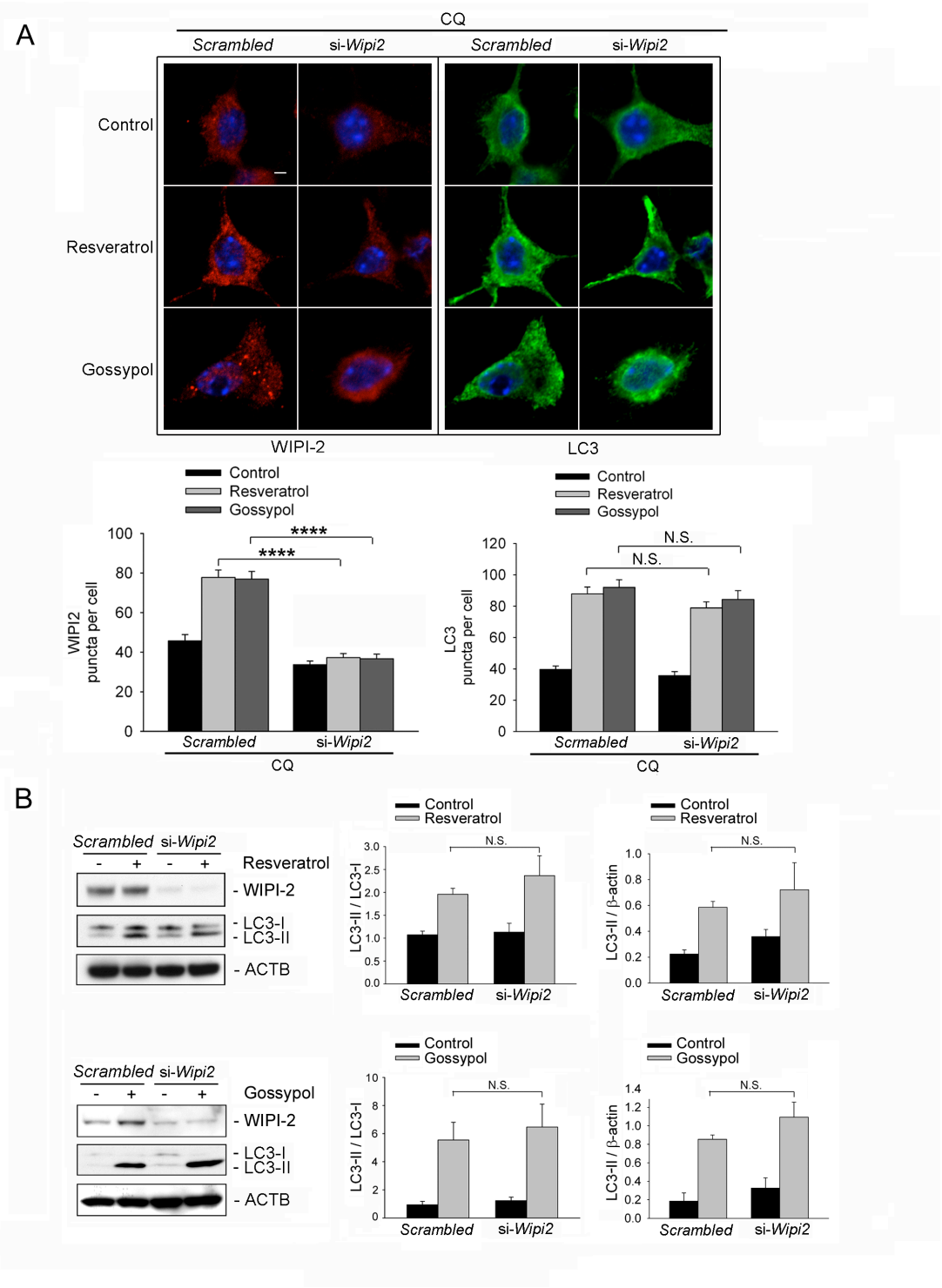
A
CQ
Scrambled
si-Wipi2
Scrambled
si-Wipi2
Control
Resveratrol
Gossypol
WIPI-2
LC3
Control
Resveratrol
Gossypol
WIPI2 puncta per cell
****
****
Scrambled
si-Wipi2
CQ
Control
Resveratrol
Gossypol
LC3 puncta per cell
N.S.
N.S.
Scrmabled
si-Wipi2
CQ
B
Scrambled
si-Wipi2
Resveratrol
- WIPI-2
- LC3-I
- LC3-II
- ACTB
Control
Resveratrol
LC3-II / LC3-I
N.S.
Scrambled
si-Wipi2
Control
Resveratrol
LC3-II / β-actin
N.S.
Scrambled
si-Wipi2
Scrambled
si-Wipi2
Gossypol
- WIPI-2
- LC3-I
- LC3-II
- ACTB
Control
Gossypol
LC3-II / LC3-I
N.S.
Scrambled
si-Wipi2
Control
Gossypol
LC3-II / β-actin
N.S.
Scrambled
si-Wipi2

Figure S8.

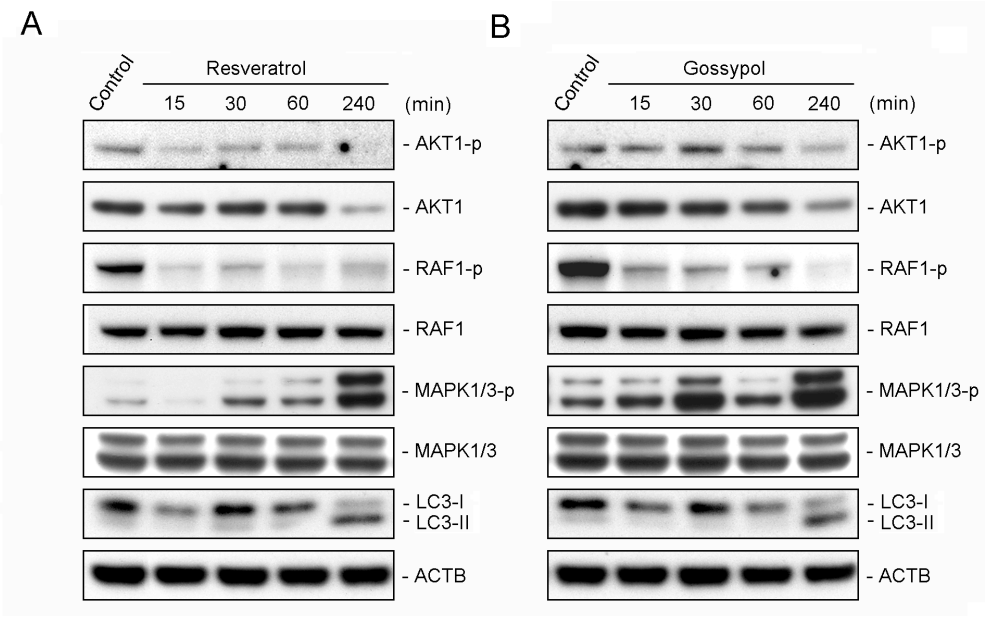

Figure S9.

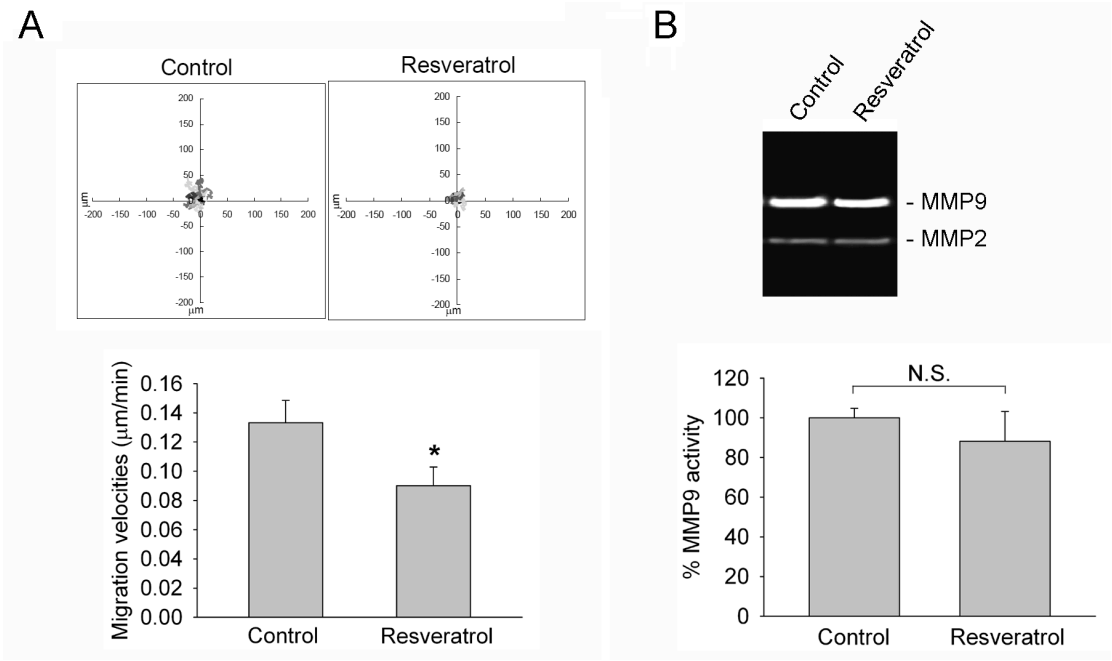

Figure S10.

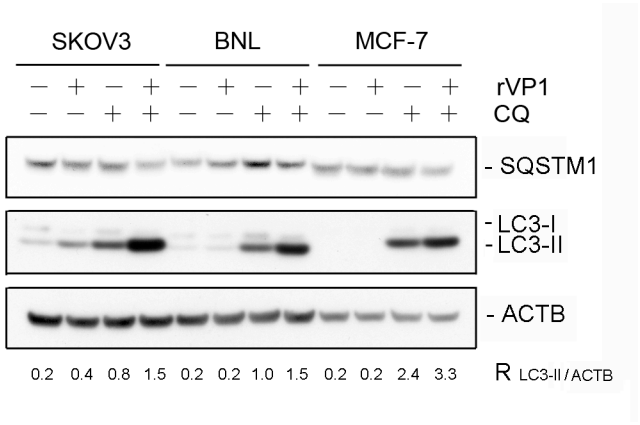

Figure S11.

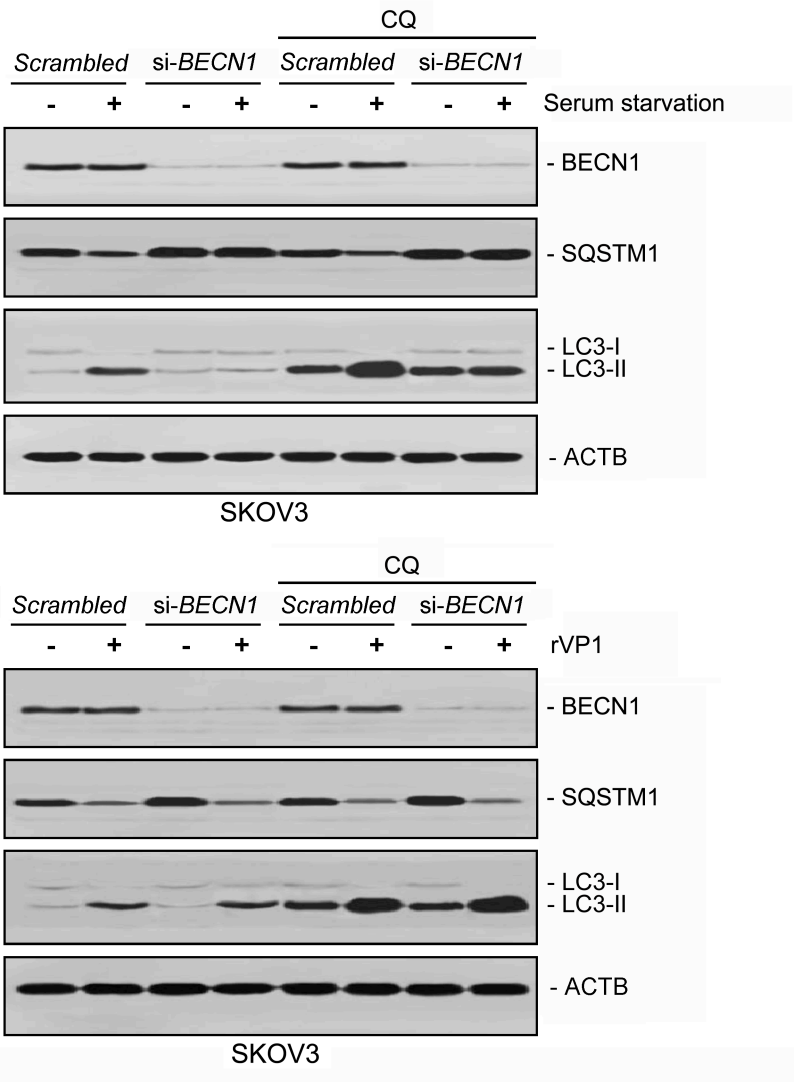

Figure S12.

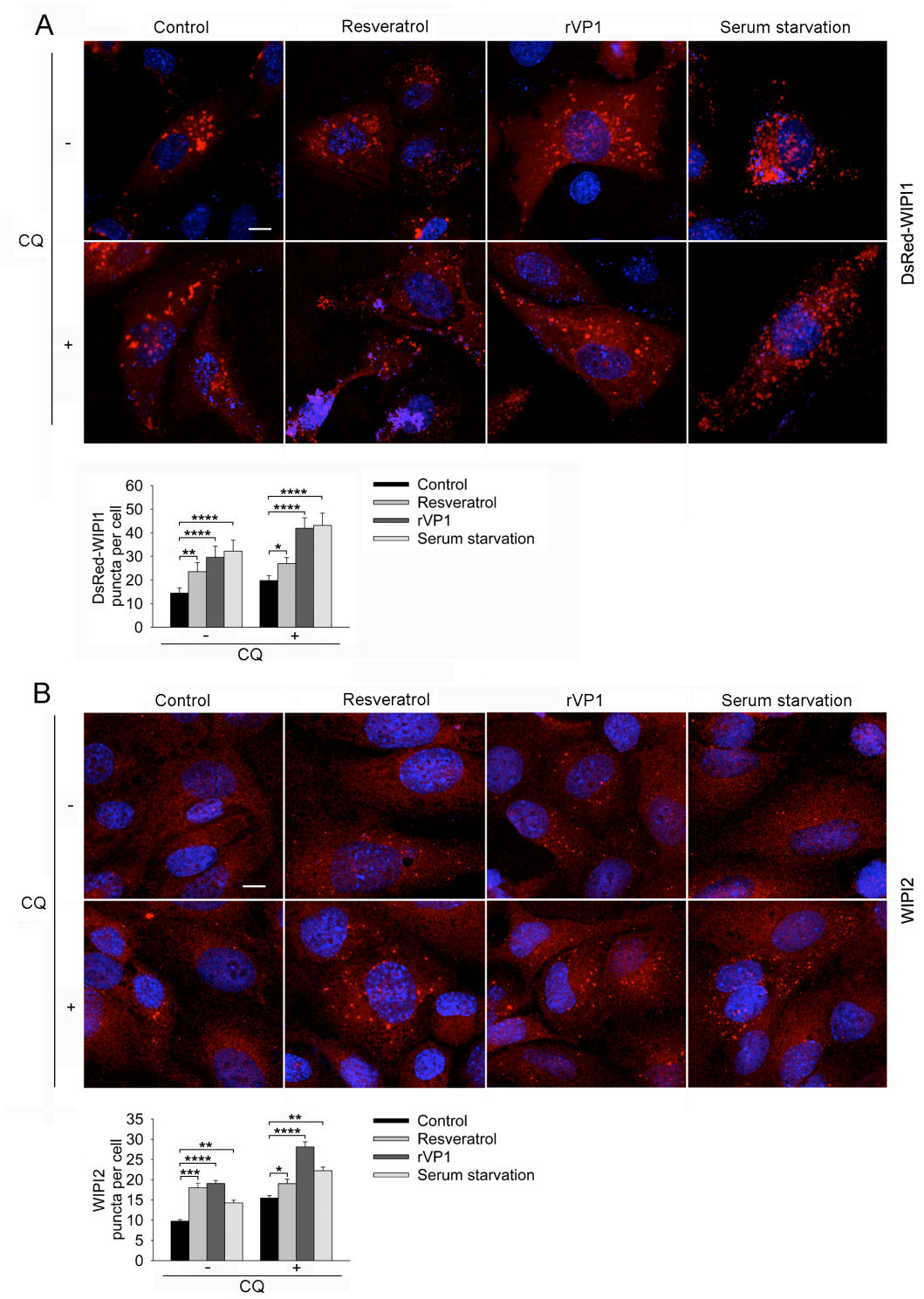
A
Control
Resveratrol
rVP1
Serum starvation
CQ
-
+
DsRed-WIPI1
DsRed-WIPI1 puncta per cell
Control
Resveratrol
rVP1
Serum starvation
CQ
B
Control
Resveratrol
rVP1
Serum starvation
CQ
-
+
WIPI2
WIPI2 puncta per cell
Control
Resveratrol
rVP1
Serum starvation
CQ

Figure S13.

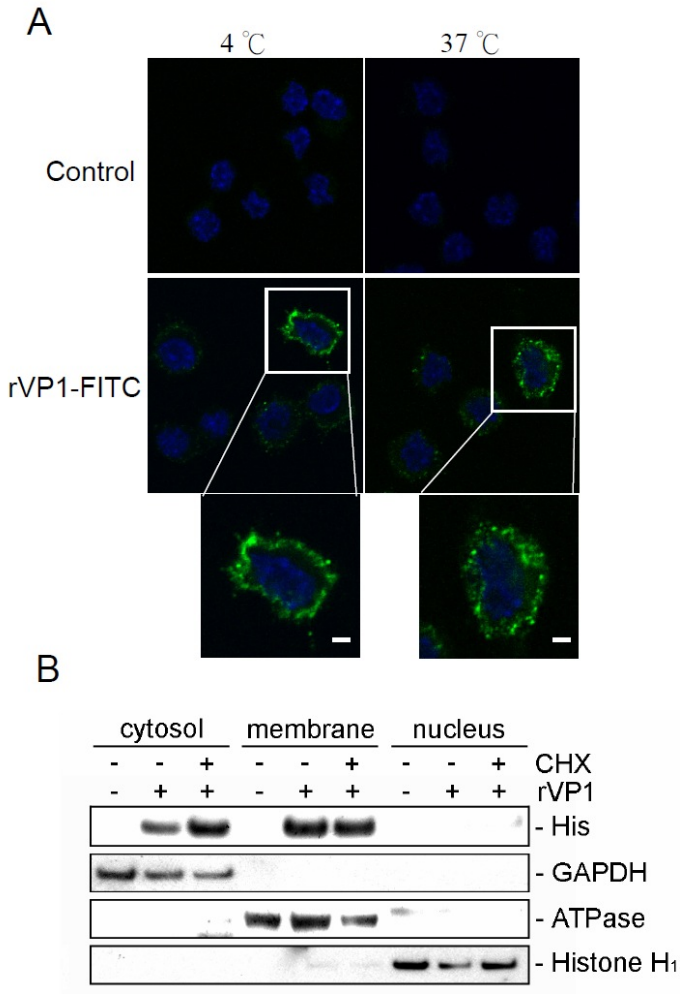

Figure S14.

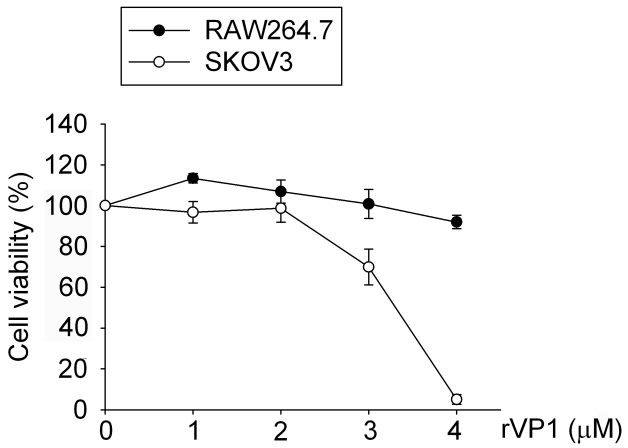

Figure S1. rVP1 induced WIPI1 and WIPI2 puncta formation in bone marrow derived macrophages. Bone marrow derived macrophage (BMM) cells were treated with or without 4 μM rVP1 for 4 h. Cells were fixed and immunolabeled with anti-WIPI1 or anti-WIPI2 primary antibodies followed by rhodamine-conjugated secondary antibodies (red). Fluorescent images were acquired by confocal microscopy. Scale bar, 2 μm. Data represent means ± SEM of quantitative analyses of WIPI1 and WIPI2 puncta per cell in at least 50 individual cells/experiment in three independent experiments; ****$P < 0.0001$.

Figure S2. WIPI1, WIPI2, ATG5 and ATG7 but not BECN1 were required for rVP1-mediated macrophage migration. Raw264.7 cells were transfected with *scrambled*, *Becn1*, *Wipi1*, *Wipi2*, *Atg5*, or *Atg7* siRNA and treated with or without serum starvation or 4 μM rVP1 for 24 h as indicated. Cell migration ability was examined by transwell migration assay. Data represent means ± SEM of three independent experiments; **$P < 0.001$, ***$P < 0.0001$, N.S., not significant.

Figure S3. Resveratrol and gossypol induced autophagy in macrophages. RAW 264.7 cells were pretreated with or without 2 μM chloroquine (CQ) and then 75 μM resveratrol or 50 μM gossypol for 4 h as indicated. (A) Resveratrol and gossypol

induced LC3 puncta formation. After treatment, cells were fixed, stained with DAPI (blue), and immunolabeled with LC3 antibody followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (green). Fluorescent images were acquired by confocal microscopy. Scale bar, 2 μm. Data represent means ± SEM of quantitative analyses of LC3 puncta per cell in at least 50 cells/experiment in three independent experiments; ***$P < 0.001$, ****$P < 0.0001$. (B) Resveratrol and gossypol induced formation of double-membrane autophagosomes. After treatment, cells were observed with transmission electron microscopy. Data represent means ± SEM of quantitative volume fraction of autophagic compartments; **$P < 0.01$. (C) Resveratrol and gossypol increased LC3 lipidation concentration-dependently. Cells were treated with serial concentrations of resveratrol and gossypol as indicated and their lysates were subjected to immunoblot analysis using antibodies against LC3. (D) Lysosomal degradation inhibitor CQ enhanced resveratrol- and gossypol-mediated LC3 lipidation. Cells were incubated with 20 μM CQ for 30 min and then 75 μM resveratrol or 50 μM gossypol for 4 h. Cell lysates were collected and analyzed by immunoblotting using anti-LC3 antibodies. ACTB was used as a loading control. Blots are representative of three independent experiments.

Figure S4. Resveratrol and gossypol induced autophagy in a BECN1 -independent manner. RAW264.7 cells stably knocked down with *scrambled* or *Becn1* shRNA were pretreated with or without 2 μM CQ for 30 min and then incubated with or without 75 μM resveratrol or 50 μM gossypol for 4 h as indicated. Cells were lysed and analyzed by immunoblotting using antibodies against BECN1, SQSTM1 and LC3, respectively. ACTB was used as a loading control. Blots are representative of three independent experiments.

Figure S5. Resveratrol and gossypol induced DsRed-WIPI1 and WIPI2 puncta formation independent of BECN1. To examine WIPI1 puncta formation, RAW264.7 cells were transfected with *DsRed-Wipi1* gene. The cells were then pretreated with or without 2 μM CQ for 30 min and subsequently treated with or without 75 μM resveratrol or 50 μM gossypol for 4 h as indicated. To examine WIPI2 puncta formation, RAW264.7 cells were immunolabeled with anti-WIPI2 antibody followed by rhodamine-conjugated goat anti-mouse IgG (red). (A) Resveratrol and gossypol increased DsRed-WIPI1 and WIPI2 puncta formation. (B) Resveratrol and gossypol induced DsRed-WIPI1 and WIPI-2 puncta formation regardless of whether BECN1 was knocked down. $RAW^{sh\text{-}scrambled}$, $RAW^{sh\text{-}Becn1}$ stable cell lines or RAW264.7 cells transfected with *DsRed-Wipi1* and *scrambled* or *Becn1* siRNA were treated with 2

μM CQ for 30 min and then with or without 75 μM resveratrol or 50 μM gossypol for 4 h as indicated. Fluorescent images were acquired by confocal microscopy. Scale bar, 2 μm. Data represent means ± SEM of quantitative analyses of DsRed-WIPI1 and WIPI2 puncta per cell in at least 50 cells/experiment in three independent experiments; *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, N.S., not significant.

Figure S6. WIPI1 was required for autophagy mediated by resveratrol and gossypol. RAW264.7 cells transfected with *DsRed-Wipi1* gene and *scrambled* or *Wipi1* siRNA were pretreated with 2 μM CQ and then incubated with or without 75 μM resveratrol or 50 μM gossypol for 4 h. (A) Knockdown of WIPI1 decreased LC3 puncta formation induced by resveratrol and gossypol. Cells were fixed, stained with DAPI (blue) and immunolabeled with anti-LC3 followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (green). Fluorescent images were acquired by confocal microscopy. Scale bar, 2 μm. Data represent means ± SEM of quantitative analyses of DsRed-WIPI1 and LC3 puncta per cell in at least 50 cells/experiment in three independent experiments; ****$P < 0.0001$. (B) Knockdown of WIPI1 decreased LC3 lipidation induced by resveratrol and gossypol. Cells transfected with *scrambled* or *Wipi1* siRNA were incubated with or without 75 μM resveratrol or 50 μM gossypol for 4 h. Cell lysates were collected and the expression levels of WIPI1, LC3-I and

LC3-II were determined by western blot analysis. ACTB was used as a loading control. Blots are representative of three independent experiments. Data represent the means ± SEM of densitometric ratio of LC3-II/LC3-I and LC3-II/ACTB from three independent experiments; **P* < 0.05.

Figure S7. Knockdown of WIPI2 did not affect resveratrol and gossypol-mediated autophagy. RAW264.7 cells transfected with *scrambled* or *Wipi2* siRNA were pretreated with 2 μM CQ and then incubated with or without 75 μM resveratrol or 50 μM gossypol for 4 h. (A) Knockdown of WIPI2 did not decrease LC3 puncta formation in resveratrol- or gossypol-treated cells. Cells were fixed, stained with DAPI (blue) and immunolabeled with anti-WIPI2 and anti-LC3 followed by rhodamine-conjugated goat anti-mouse IgG (red) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (green). Fluorescent images were acquired by confocal microscopy. Scale bar, 2 μm. Data represent means ± SEM of WIPI2 and LC3 puncta per cell in at least 50 cells/experiment in three independent experiments; *****P* < 0.0001, N.S., not significant. (B) Knockdown of WIPI2 did not decrease LC3 lipidation in resveratrol- or gossypol-treated cells. Cells transfected with *scrambled* or *Wipi2* siRNA were incubated with or without 75 μM resveratrol or 50 μM gossypol for 4 h. Cell lysates were collected and levels of WIPI2, LC3-I and LC3-II were

determined by western blot analysis. ACTB was used as a loading control. Blots are representative of three independent experiments. Data represent means ± SEM of the ratio of LC3-II/LC3-I and LC3-II/ACTB from three independent experiments; N.S., not significant.

Figure S8. Resveratrol and gossypol increased phosphorylation of MAPK1/3 but not AKT1 and RAF1. RAW 264.7 cells were treated with or without (A) 75 μM of resveratrol or (B) 50 μM of gossypol for 15 to 240 min as indicated. Cell lysates were collected and subjected to immunoblot analysis using antibodies against LC3, phosphorylated AKT1 at Ser473, phosphorylated RAF1 at Ser338, phosphorylated MAPK1/3 at Thr202/Tyr204 of MAPK3 (Thr185/Tyr187 of MAPK1) and their non-phosphorylation control. ACTB was used as a loading control. Blots are representative of three independent experiments.

Figure S9. Resveratrol did not increase MMP9 activity and migration ability of macrophages. RAW264.7 cells were incubated with or without 50 μM resveratrol for 22 h. (A) Time-lapse microscopy experiments were performed to obtain serial phase-contrast images every 15 min for 22 h. Trajectories and migration velocity of RAW264.7 cells treated with control medium or resveratrol were displayed after 22 h

of time-lapse. Trajectories of cells were displayed in diagrams drawn with the initial point of each trajectory placed at the origin of the plot. Data represent means ± SEM of migration velocities of 20 cells; *$P < 0.05$. (B) Resveratrol did not affect MMP2 and MMP9 activity of macrophage. RAW264.7 cells were incubated with or without 50 μM resveratrol for 22 h. MMP2 and MMP9 activity were examined by gelatin zymographic analysis. Data represent means ± SEM of three independent experiments; N.S., not significant.

Figure S10. rVP1 induced autophagy in cancer cell lines. SKOV3, BNL and MCF-7 cells were pretreated with or without 10 μM CQ and then incubated with or without 3 μM rVP1 for 8 h. Cell lysates were collected and analyzed by immunoblotting using anti-SQSTM1 and anti-LC3 antibodies. ACTB was used as a loading control. Blots are representative of three independent experiments. Ratios of densitometric LC3-II/ACTB are shown below the blot.

Figure S11. rVP1 induced BECN1-independent autophagy in SKOV3. SKOV3 cells transfected with *scrambled* or *BECN1* siRNA were pretreated with or without 10 μM CQ for 30 min and then incubated with serum depleted media or 3 μM rVP1 for 8 h as indicated. Cell lysates were collected and analyzed by immunoblotting using

anti-BECN1, anti-SQSTM1 and anti-LC3 antibodies. ACTB was used as a loading control. Blots are representative of three independent experiments.

Figure S12. Resveratrol, rVP1 and serum starvation induced DsRed-WIPI1 and WIPI2 puncta formation in SKOV3. To examine WIPI1 puncta formation, SKOV3 cells were transfected with *DsRed-WIPI1* gene. The cells were then pretreated with or without 10 μM CQ for 30 min and then treated with 64 μM resveratrol, 3 μM rVP1 or serum depletion for 8 h as indicated. To examine WIPI2 puncta formation, SKOV3 cells were immunolabeled with anti-WIPI2 antibody followed by rhodamine-conjugated goat anti-mouse IgG (red). (A) Resveratrol, rVP1 and serum starvation induced DsRed-WIPI1 puncta formation in SKOV3 cells. (B) Resveratrol, rVP1 and serum starvation induced WIPI2 puncta formation in SKOV3 cells. Fluorescent images were acquired by confocal microscopy. Scale bar, 10 μm. Data represent means ± SEM of quantitative analyses of DsRed-WIPI1 and WIPI2 puncta per cell in in at least 50 cells/experiment in three independent experiments; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $***P < 0.0001$.

Figure S13. Binding and internalization of rVP1 in RAW264.7 cells. (A) RAW264.7 cells were pretreated with 10 μM cycloheximide (CHX) for 30 min and then

incubated with or without 3 μM rVP1-FITC at 4°C for 30 min or at 37°C 10 min as indicated. Cells were fixed and stained with DAPI. Fluorescent images were acquired by confocal microscopy. Scale bar, 2 μm. (B) RAW264.7 cells were pretreated with or without 10 μM cycloheximide (CHX) for 30 min and then incubated with or without 3 μM rVP1 for 10 min at 37°C. Protein fractions of cytosol, membrane and nucleus were isolated using FractionPREP Cell Fractionation system (BioVision, #K270-50). As rVP1 contains His-tag,[27] the level of rVP1 was examined by immunobloting using the anti-His antibody (His). GAPDH, ATPase and Histone H_1 were used as loading controls for fractions of cytosol, membrane and nucleus, respectively. Blots are representative of three independent experiments.

Figure S14. rVP1 did not cause severe cytotoxicity to RAW264.7 macrophages. RAW264.7 and SKOV3 cells were incubated with or without 1 μM to 4 μM rVP1 for 24 h. The cell viability was examined by WST-1 assay.