Supplemental Information

Autophagy Facilitates Macrophage Depots of Sustained Release Nanoformulated Antiretroviral Drugs

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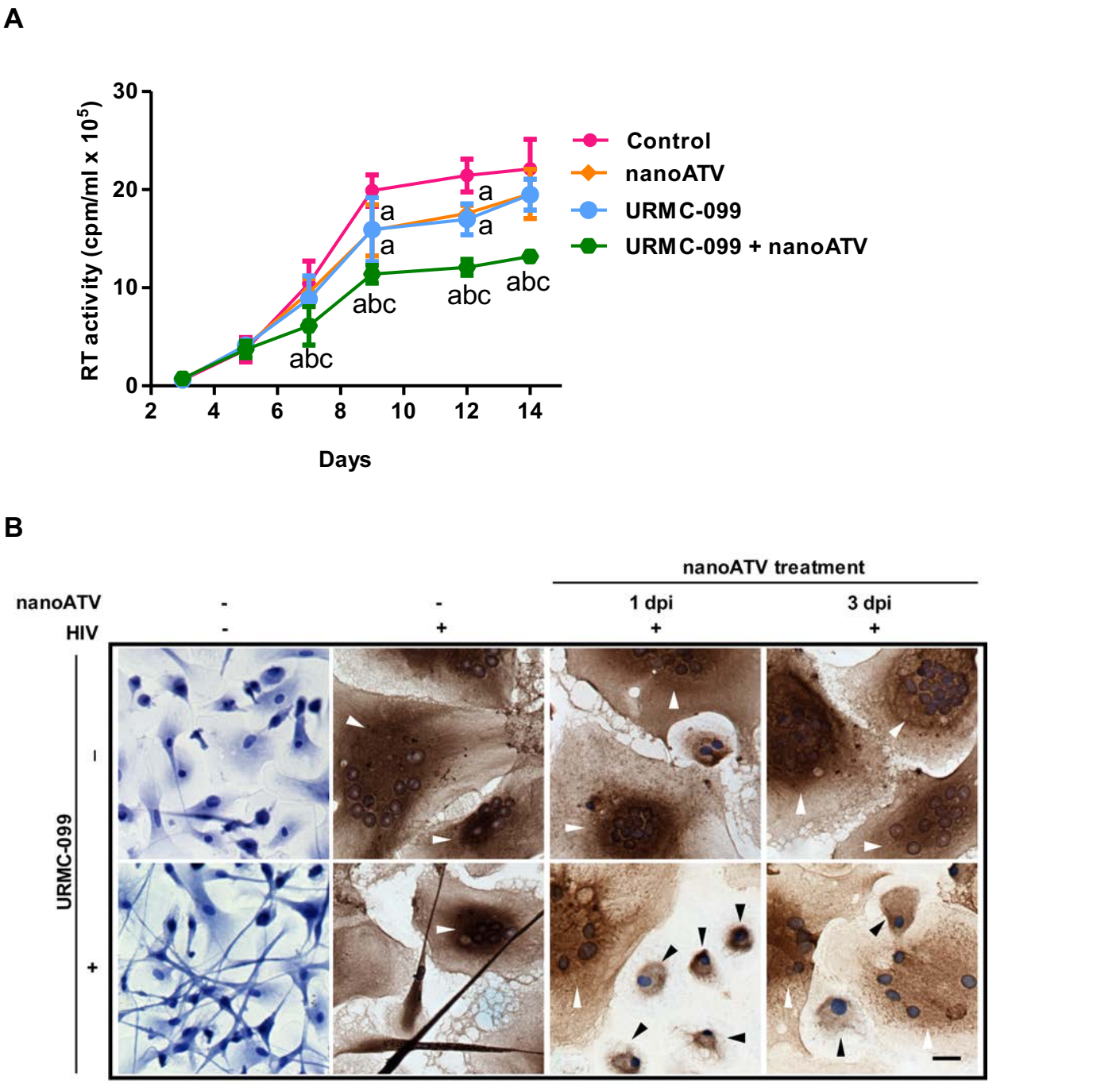
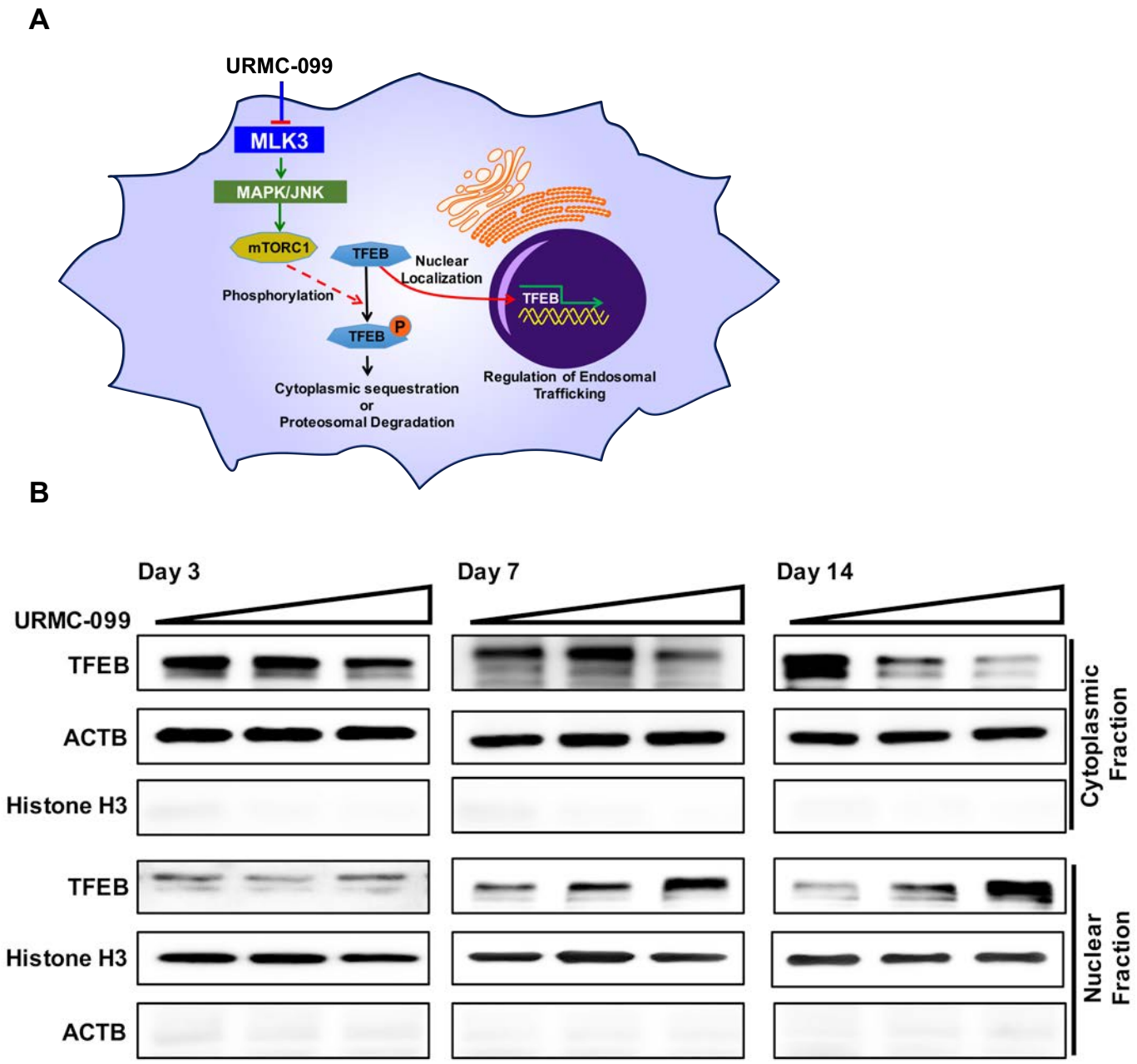
Figure S1



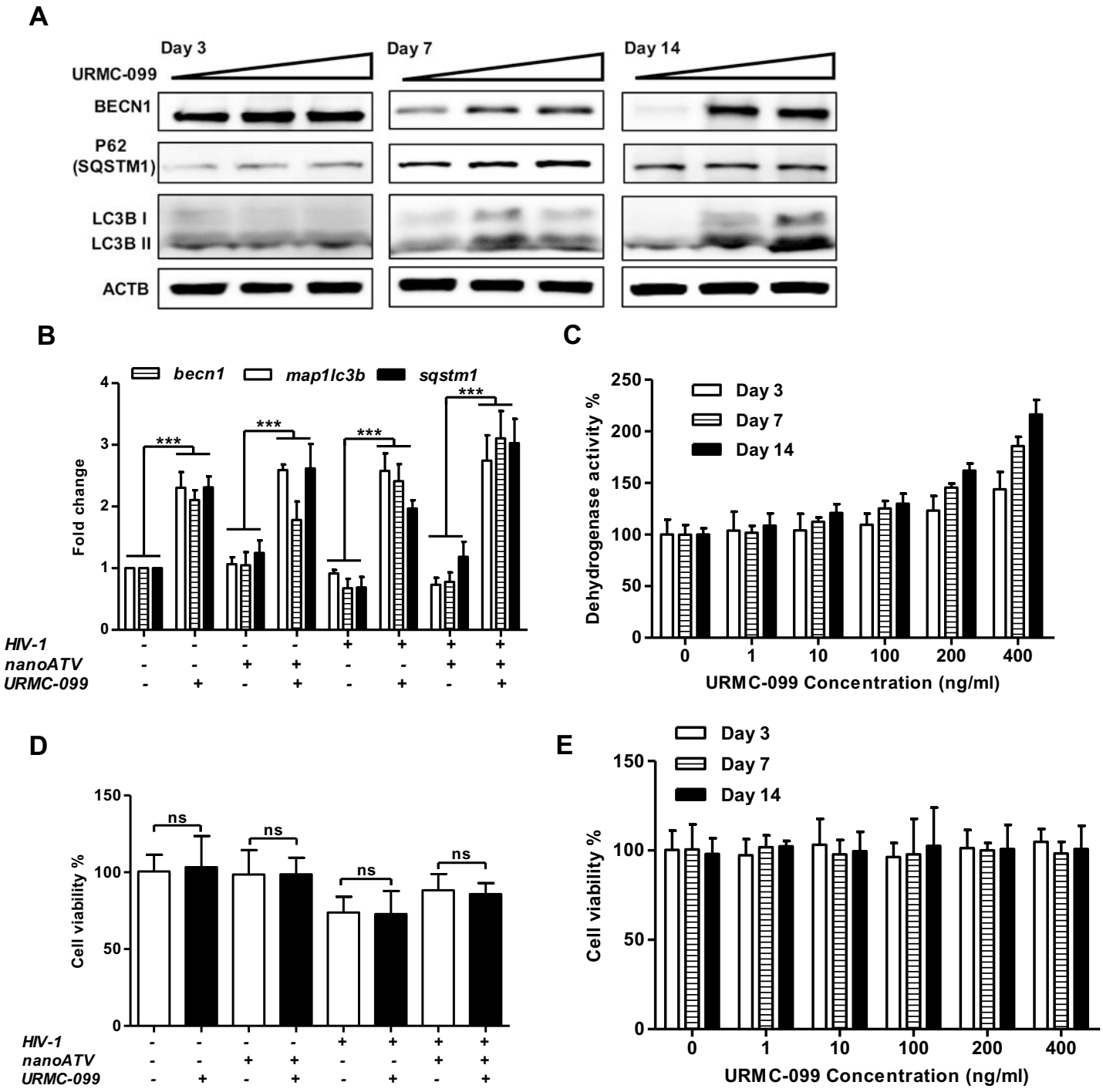
Supplemental Figure 1. URMC-099 potentiates the antiretroviral activity of long acting nanoART. (A) HIV-1_{ADA} infected human MDMs were treated with 1 μM nanoATV at day 5 after infection with or without 400 ng/ml URMC-099. Supernatants were collected at different days after infection for HIV-1 reverse transcriptase activity. Values represent mean ± SD (n=5). $p < 0.05$ compared to [a]Control, [b]nanoART, or [c]URMC-099 by two-way ANOVA. (B) At day 14 the cells were fixed and stained for HIV-1 p24 antigen and counter stained with hematoxylin. Scale bar = 20 μm.

Figure S2



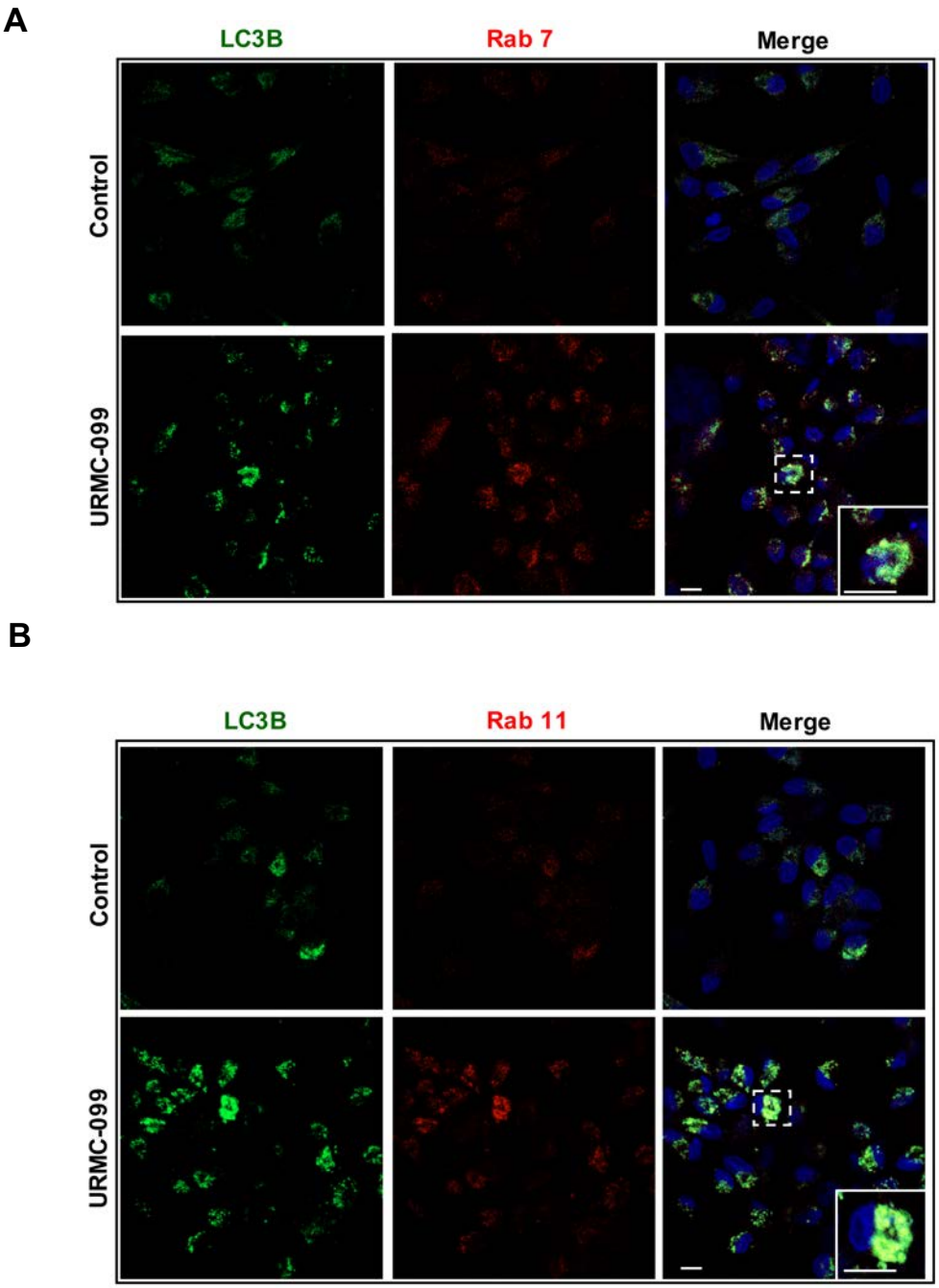
Supplemental Figure 2. URMC-099 regulates Transcription Factor EB (TFEB). (A) Proposed mechanism of URMC-099 induced cellular trafficking. Since URMC-099 inhibits MLK-3 and JNK phosphorylation, it may block mTORC1 induced phosphorylation of TFEB. Nuclear translocation of non-phosphorylated TFEB facilitates nanoART retention by regulating endosomal trafficking. (B) URMC-099 showed delayed response in MDMs. Human MDMs were treated with 0, 100 or 400 ng/ml of URMC-099. MDMs were fractionated to cytoplasmic and nuclear fraction at different days. Western blot was performed to check TFEB expression.

Figure S3



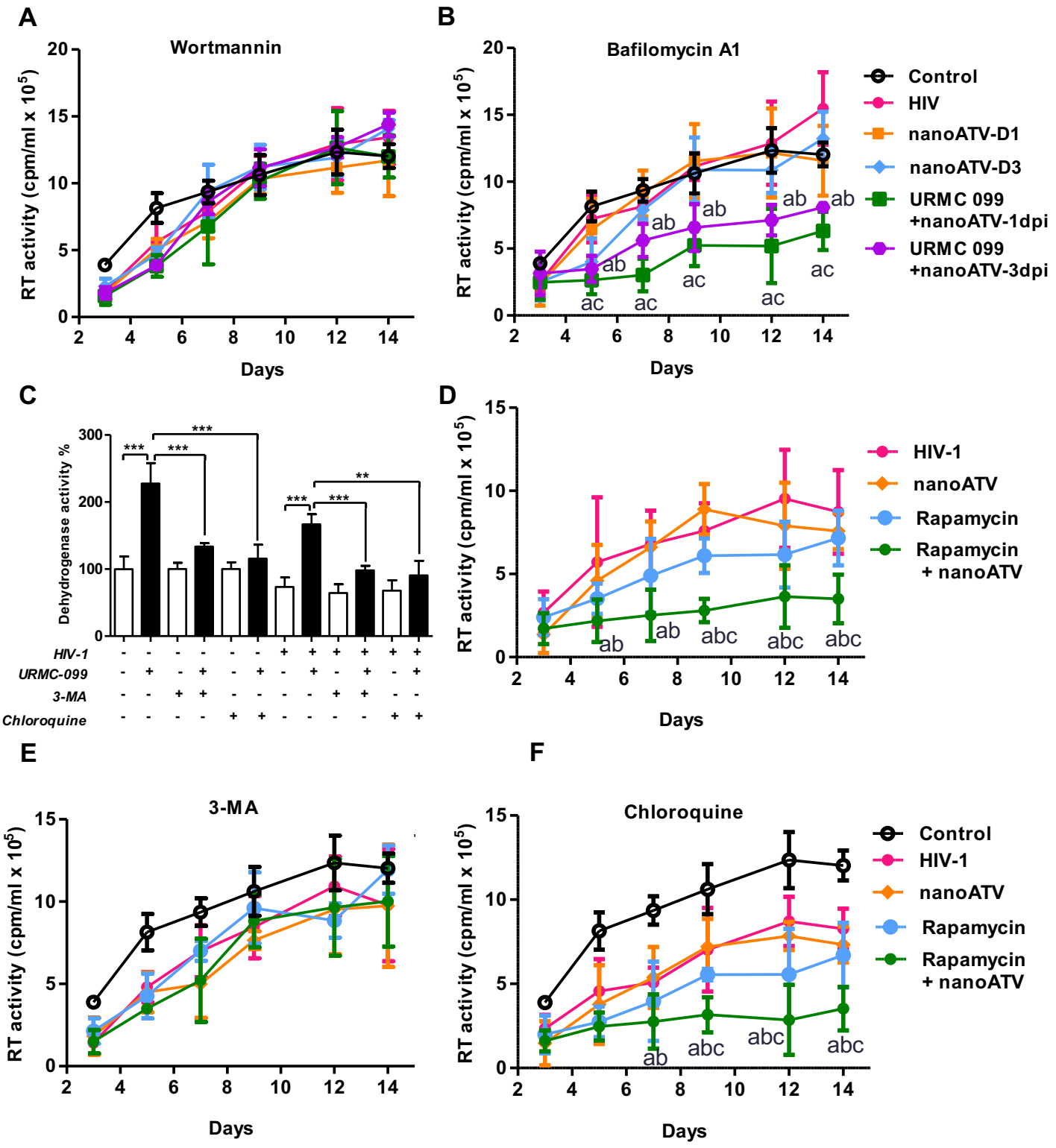
Supplemental Figure 3. URMC-099 induces autophagy and mitochondrial activity. (A) Human MDM were treated with 0, 100 or 400 ng/ml of URMC-099. At different days total cell lysate was analyzed for different autophagy markers by western blot. (B) Human MDM were treated with 400 ng/ml of URMC 099 in the presence or absence of nanoATV (100 μM) with or without HIV-1_{ADA} infection for 7 days. Total RNA as isolated at day 7 and real-time qPCR was performed for map1lc3b, becn1 and sqstm1 expression (n=3). (C) Human MDM were treated with 400 ng/ml of URMC-099. At days 3, 7, and 14 MTT assay was performed to check the mitochondrial activity (n=5). (D and E) Cell viability was determined using trypan blue exclusion assay (n=3). The data is normalized to actual cell number plated. Values represent mean ± SD. ***, $p \leq 0.001$, Students' t-test.

Figure S4



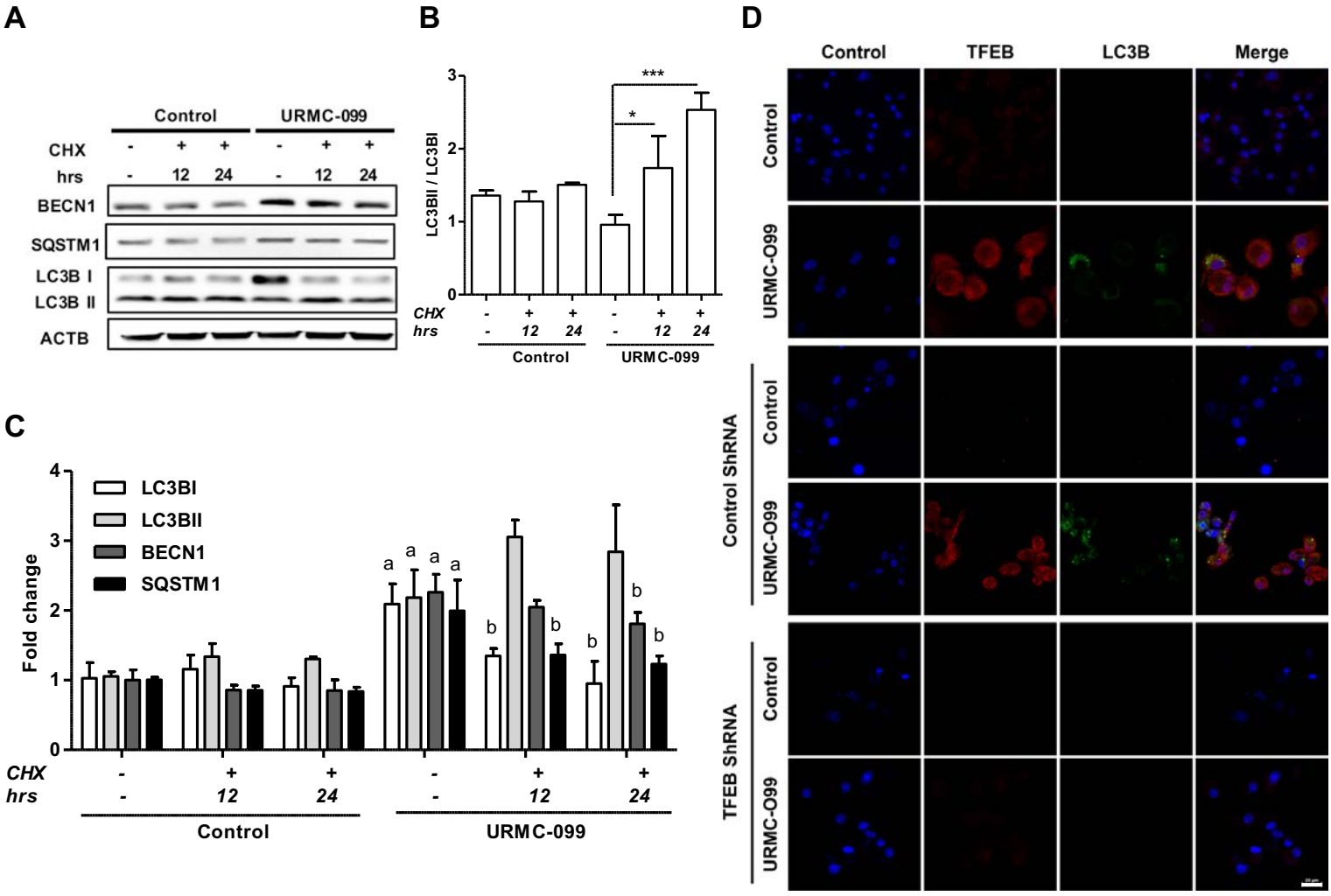
Supplemental Figure 4. URMC-099 modulates cellular trafficking and nanoATV release. Human MDMs were treated in the presence or absence of 400 ng/ml of URMC-099 for 12 days. Cells were transfected with LC3-GFP construct and allowed for 48 h and at day 14 stained for (A) Rab 7 or (B) Rab 11 and DAPI and imaged with confocal microscope. (C) URMC-099 regulates release of nanoATV. Human MDM were treated with 100 μM nanoATV for 16 h, washed with PBS and incubated with or without 400 ng/ml URMC-099. At different days, supernatant was collected, ATV concentration was quantified using HPLC and cumulative ATV release was plotted against time.

Figure S5


A
Wortmannin
RT activity (cpm/ml x 10^5)
Days
B
Bafilomycin A1
RT activity (cpm/ml x 10^5)
Days
Control
HIV
nanoATV-D1
nanoATV-D3
URMC 099 +nanoATV-1dpi
URMC 099 +nanoATV-3dpi
ab
ac
C
Dehydrogenase activity %
***
**
HIV-1
URMC-099
3-MA
Chloroquine
D
RT activity (cpm/ml x 10^5)
Days
HIV-1
nanoATV
Rapamycin
Rapamycin + nanoATV
ab
abc
E
3-MA
RT activity (cpm/ml x 10^5)
Days
F
Chloroquine
RT activity (cpm/ml x 10^5)
Days
Control
HIV-1
nanoATV
Rapamycin
Rapamycin + nanoATV
ab
abc

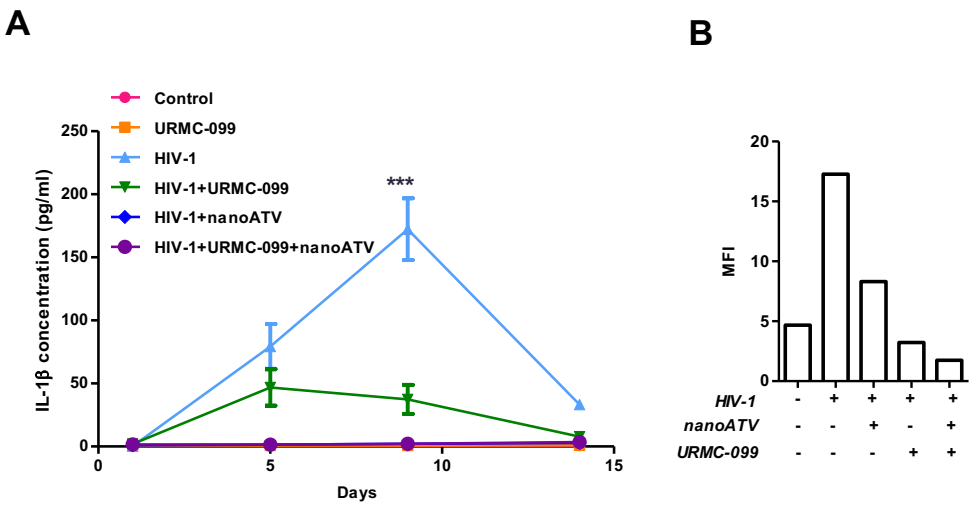
Supplemental Figure 5. URMC-099 modulates autophagy, HIV-1 infection and cytokine secretion. HIV-1_{ADA} infected human MDMs were treated with 1 μM nanoATV at day 1 (D1) or day 3 (D3) post infection and incubated with or without URMC-099 (400 ng/ml) and in the presence of autophagy inhibitors, (A) wortmannin (50 nM) or (B) bafilomycin A1 (20 nM). Same control plot was represented in A, B, E and F. Supernatants were collected at different days after infection and analyzed for HIV-1 reverse transcriptase activity (n=5). $p < 0.05$ compared to [a]HIV, [b]nanoATV-D1, or cnanoATV-D3 by two-way ANOVA. Control is HIV infected MDM without any treatment. (C) Human MDM were incubated with combinations of 400 ng/ml of URMC 099 or 100 μM of 3-MA or 10 μM of chloroquine with or without HIV-1_{ADA} infection for 14 days. MTT assay was performed to assess the mitochondrial activity (n=5). Values represent mean ± SD. **, $p \leq 0.01$, ***, $p \leq 0.001$, Students' t-test. (D – F) HIV-1_{ADA} infected human MDM were treated with 1 μM nanoATV at 1 day post infection and incubated with or without rapamycin (20 nM). (D) No autophagy inhibitors, $p < 0.05$ compared to [a]HIV-1 control [b]nanoATV, or [c]Rapamycin by two-way ANOVA., (E) 3-MA (100 μM) and (F) chloroquine (10 μM), $p < 0.05$ compared to [a]HIV-1 with inhibitor, [b]nanoATV with inhibitor, or [c]Rapamycin with inhibitor by two-way ANOVA. Control is HIV-1 infected MDM without any treatment. Supernatants were collected at different days after infection and analyzed for HIV-1 reverse transcriptase activity (n=5).

Figure S6



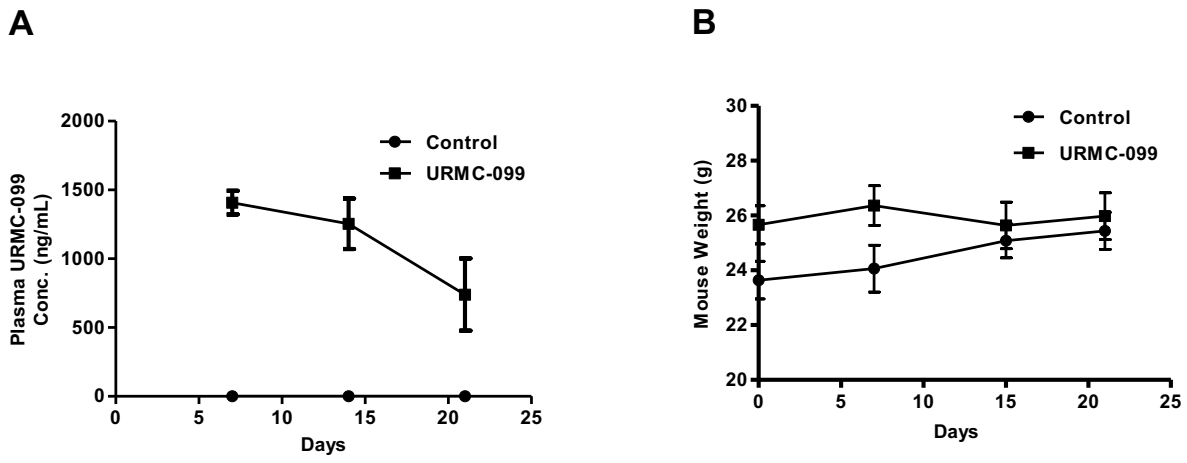
Supplemental Figure 6. URMC-099 induces autophagy through TFEB. (A) Human MDM were treated in the presence or absence of 400 ng/ml of URMC-099 for 7 days. 24 h or 12 h before harvesting, cells were treated with cycloheximide (10 μM) to inhibit translation. Total cell lysate was analyzed for different autophagy markers using Western blot and densitometry measurements are expressed as (B) LC3BII/LC3BI ratio, * $p \leq 0.05$ and *** $p \leq 0.001$ and (C) fold change, $p \leq 0.05$ when compared to respective protein from [a]no URMC-099/ no CHX control and [b]URMC-099/ noCHX control. $p < 0.05$ compared to the respective protein from [a]no URMC-099 and no CHX control and [b]URMC-099 and no CHX control. (D) Human MDM were treated with 400ng/ml URMC-099 and on day 3 and day 7 cells were transfected with TFEB shRNA. MDMs were transfected with LC3B tagged GFP construct (green), stained with TFEB (red), DAPI (blue), and analyzed by confocal microscopy. Scale bar = 20 μm

Figure S7



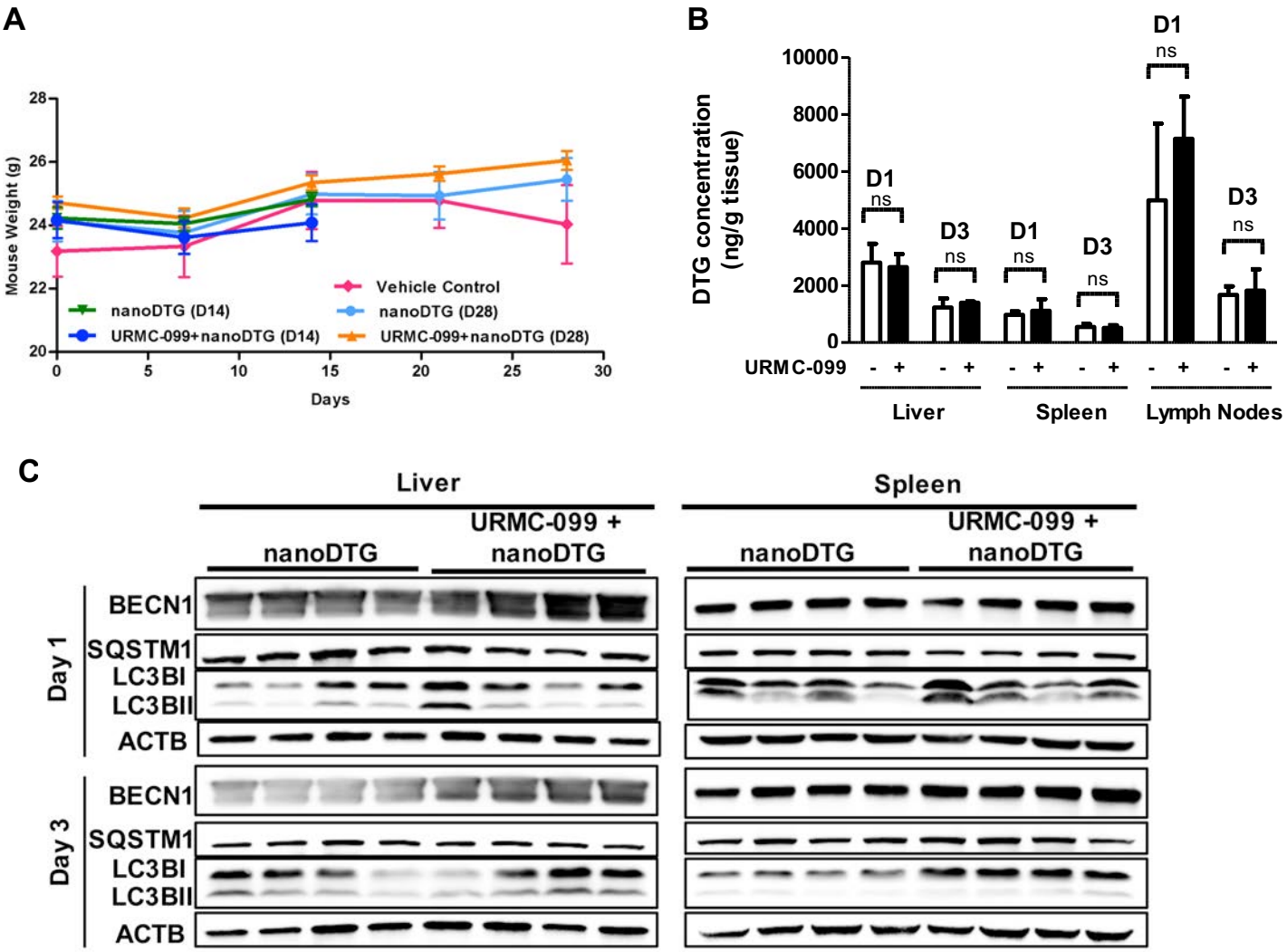
Supplemental Figure 7. URMC-099 reduces HIV-1 induced IL-1β secretion. (A and B) Human MDMs were pretreated with 100 μM nanoATV for 16 h, infected with HIV-1 and then incubated with or without 400 ng/ml of URMC-099 for 14 days. (A) At different days IL-1β concentration was quantified in culture supernatant using ELISA (n=4). Values represents mean ± SD. Students' t-test was performed between HIV-1 and HIV-1+URMC-099 at day 9. ***, $p \leq 0.001$. (B) At day 9, IL-1β level was quantified using cytokine bead array and mean fluorescence index (MFI) was calculated.

Figure S8



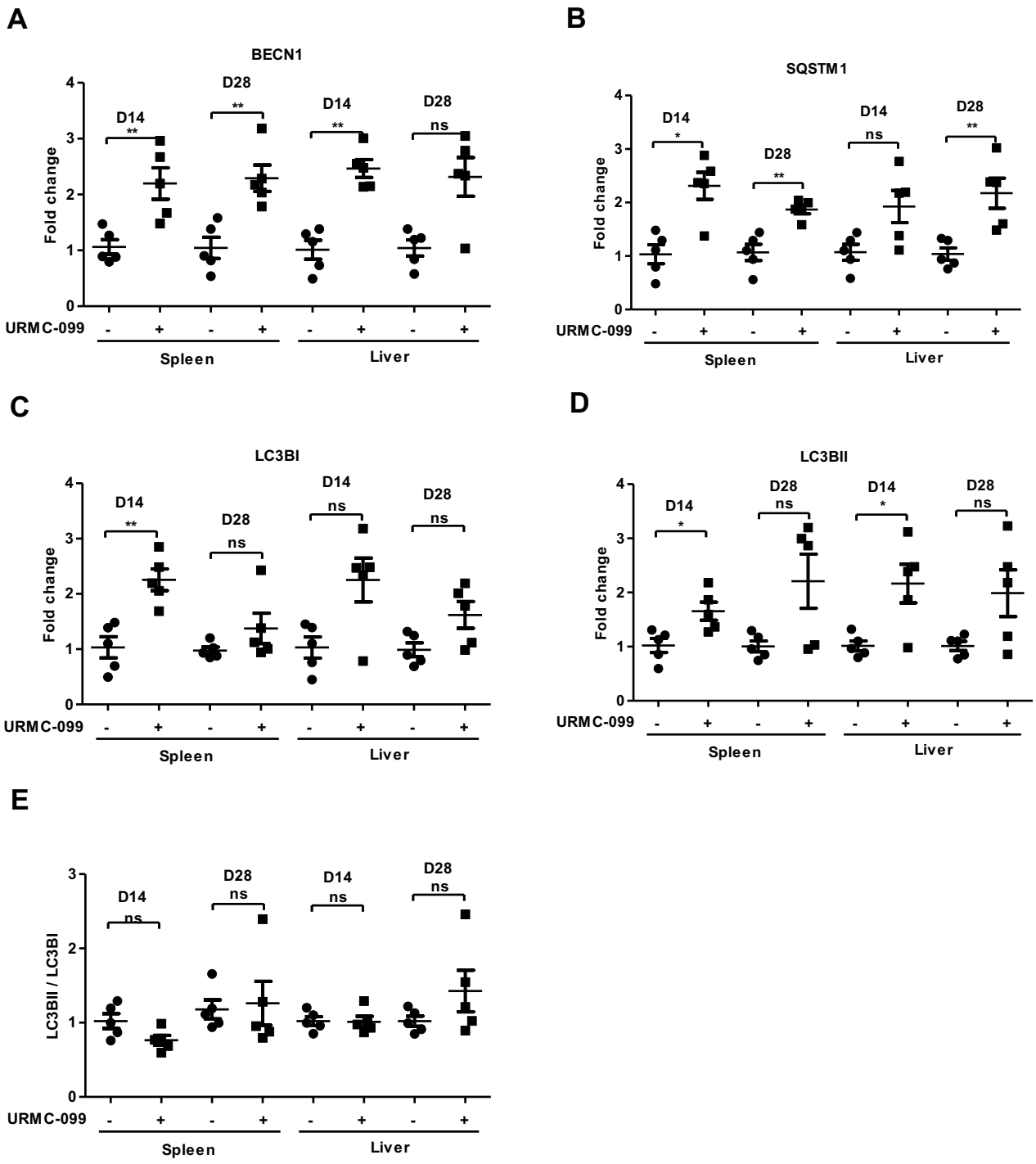
Supplemental Figure 8. URMC-099 treatment in mice. (A and B) Mice were given twice daily IP injection of buffer (control) or URMC-099 (10 mg/kg) for 21 days. (A) At different days blood plasma was collected and quantified for URMC-099 by UPLC-MS/MS. Blood was collected 5 h after URMC-099 injection on days 7 and 14 after initiation of treatment, and 16 h after URMC-099 injection at day 21. (B) Mice weight were recorded every week. 5 mice in control group and 4 mice in URMC-099 group.

Figure S9



Supplemental Figure 9. URMC-099 and nanoDTG treatment in mice. Mice were injected with single dose of 45 mg/kg nanoformulated dolutegravir (nanoDTG) intramuscularly and treated with or without daily URMC-099 (10 mg/kg) IP injections. (A) Every week mice weights were recorded for day 14 group mice (D14) and day 28 group mice (D28). D14 mice were sacrificed on day 14 and D28 on day 28 for further analysis. 6 mice per group. (B) Drug (DTG) measurements in liver, spleen and lymph nodes in mice treated with or without URMC-099 (10 mg/kg/daily) sacrificed at day 1 and day 3 after nanoDTG administration (single dose of 45 mg/kg). 4 mice per group. (C) Liver and spleen from mice from B were analyzed for autophagy proteins by Western blot. Each column represents one mouse.

Figure S10



Supplemental Figure 10. URMC-099 induces autophagy in nanoDTG injected mice. (A-E) Quantification of western blots performed for (A) BECN1 (B) SQSTM1 (C) LC3BI and (D) LC3BII with liver tissue lysates from mice injected with single dose of 45 mg/kg nanoDTG intramuscularly and treated with or without daily URMC-099 (10 mg/kg) IP injections and (E) represents LC3BII/LC3BI ratios from different groups. N=5 mice per group and each dot represents a mouse. ns, not significant, *, $p \leq 0.05$ **, $p \leq 0.01$, Mann–Whitney U test.

Supplemental Experimental Procedures

Reagents

Sources of chemicals were as follows: atazanavir (ATV) and drug free-based with triethylamine (Gyma Laboratories of America Inc., Westbury, NY); poloxamer 407 (P407, Sigma-Aldrich, St. Louis, MO); CF633-succinimidyl ester (CF633, Biotium, Hayward, CA). Antibodies included transcription factor EB (TFEB, ab96834, Abcam, Danvers, MA), mTOR (ab25880, Abcam), mTOR-phospho-S2448 (ab109268 Abcam), histone H3 (ab1791, Abcam), LC3B (NB100, Novus Biologicals, Minneapolis, MN), b-actin (A2228, Sigma-Aldrich), SQSTM1/P62 (PM045, MBL International, Woburn, MA), BECN1 (H300, Santa-Cruz Biotechnology, Dallas, Texas), CD68 (ab955, Abcam), ATG13 (SAB4200100, Sigma-Aldrich), and HIV-1p24 (Dako, Carpinteria, CA); horse radish peroxidase (HRP) -conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Santa Cruz Biotechnology); alexa fluor 594 goat anti-rabbit IgG (ThermoFisher Scientific, Waltham, MA). Silencing RNA (siRNA) for tfeb and atg13 was from Ambion, Waltham, MA and Lipofectamine RNAiMAX reagent from Invitrogen. Macrophage colony stimulating factor (MCSF) was prepared from culture fluids of 5/9m alpha3-18 cells (ATCC®, CRL-10154TM) (Clark, 1989). Dolutegravir (DTG) was a generous gift from ViiV Healthcare (Brentford, Middlesex, United Kingdom). URMC-099 (M.W: 421.54 g/mol) was synthesized by Califla Bio Inc. (San Diego, CA).

Preparation of nanoformulated antiretroviral drugs

Nanoformulations of ATV and DTG were prepared by high-pressure homogenization (Avestin EmulsiFlex-C3, Avestin Inc., Ottawa, ON, Canada).. For preparation of nanoATV, free-base ATV (1% w/v) and P407 (0.2%, w/v) were suspended in 10 mM HEPES, pH 7.8, and mixed overnight. CF633 labeled nanoATV was prepared by mixing P407 (0.4%, w/v), CF633-P407 (0.1%, w/v) and free-base ATV (1%, w/v) suspended in 10 mM HEPES, pH 7.8, overnight. For nanoDTG preparations, DTG (5% w/v) and P407 (0.3% w/v) were combined in HPLC-grade water at a ratio of 100:6. The suspensions were homogenized at 20,000 psi until the desired particle size (300-400 nm) was

achieved. The crude nanosuspension was purified by centrifugation at 5,000 × g for 5 minutes. Supernatant was collected and again centrifuged at 10,000 × g for 20 minutes to separate nanosuspensions from free drug. The nanosuspension pellet was suspended in phosphate-buffered saline (PBS), and centrifuged at 200 × g for 3 minutes. Particle size, polydispersity and surface charge (zeta potential) were determined by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments Inc., Westborough, MA). Particle size, zeta potential and polydispersity index (PDI) were 344 ± 30, -0.7 ± 0.40 and 0.20 ± 0.08 for ATV, and 315 ± 30, -13 ± 0.10 and 0.21 ± 0.02 for DTG nanoformulations. ATV loading of the nanoparticles was determined by UPLC by both UV/Vis and tandem mass spectrometry (MS/MS) detection methods. Nanoparticle ATV loading was determined by HPLC with UV/Vis detection as previously described. DTG loading was determined by HPLC using a Phenomenex Kinetix C18 column (5 µm; 150 x 4.6 mm) with a mobile phase of 65% 50 mM KH2PO4, pH 3.2 / 35% acetonitrile at a flow rate of 1.0 ml/min and 254 nm detection. ATV and DTG were quantitated by comparison of peak areas to those of known standards (0.05-50 µg/ml in methanol).

Reverse transcriptase (RT) activity

RT activity was measured in 10 µl aliquots of culture fluids from infected cells mixed with 10 µl of 100 mM Tris-HCl (pH 7.9), 300 mM KCl, 10 mM DTT and 0.1% nonyl phenoxylpolyethoxyl-ethanol- 40 (NP-40) in a 96-well plate, and incubated at 37°C for 15 minutes. Then 25 µl of a solution containing 50 mM Tris-HCl (pH 7.9), 150 mM KCl, 5 mM DTT, 15 mM MgCl2, 0.05% NP-40, 10 µg/ml poly(A), 0.250 U/ml oligo d(T),) and 10 µCi/ml 3H-TTP was added to each well, and incubated at 37°C for 18 h. After incubation, 50 µl of ice-cold 10% trichloroacetic acid was added to each well. The well contents were harvested onto glass fiber filters, and assayed for 3H-TTP incorporation by β-scintillation spectroscopy.

Autophagosome isolation

Cells treated with different conditions were washed thrice with ice-cold PBS, and collected into homogenization buffer (10 mM HEPES-KOH, pH 7.2, 250 mM sucrose, 1 mM EDTA and 1 mM $Mg(OAc)_2$). Cells were disrupted by 15 strokes in a Dounce homogenizer, and these homogenates were centrifuged at 400 × g for 10 min at 4°C to remove the cell debris, fractured cells and nuclei. LC3B antibody was conjugated to protein A/G paramagnetic beads (ThermoFisher Scientific) as per the manufacturer's instruction, and 20 µl of beads was incubated with cell homogenate for 24 h at 4°C. LC3B-positive compartments were washed with PBS, and collected by magnetic separation for 1 h at 4°C.

Macrophage nanoATV retention and drug release

MDMs were treated with 100 µM nanoATV for 16 h, washed with PBS and then treated with 400 ng/ml URMC-099. The cells were collected at different days, and quantified using a TC-20 automated cell counter (Bio-Rad) with 0.4% trypan blue (Bio-Rad). Cells were lysed in methanol, and ATV concentration was quantified and normalized to the cell number. The medium was collected at different days, and drug was extracted. The drug extract was vacuum dried, and resuspended in 100% methanol. ATV concentration in the cell and medium extracts was quantified by HPLC.

Plasma URMC-099 quantification

For URMC-099 quantification, 25 µL of plasma was mixed with 10 µL of 50% methanol and 65 µL of internal standard (IS) (200 ng/ml indinavir in methanol). Standards were prepared by mixing 25 µL of blank plasma from control mice, 65 µL of IS and 10 µL of URMC-099 for a final concentration range of 0.2-1000 ng/ml. All samples were vortexed, and then centrifuged at 17,000 x g for 10 min. Next, 80 µL of supernatant was mixed with 40 µL of 10% methanol for analysis. A Waters ACQUITY H-class UPLC system (Waters, Milford, MA, USA) connected to a Waters Xevo TQS-micro mass spectrometer with electrospray ionization (ESI) source was used for drug quantification. Chromatographic separation of 10 µL of sample was achieved on an ACQUITY UPLC

BEH Shield RP18 column (1.7 µm, 2.1 mm x 100 mm) using an 8- min gradient of mobile phase A (7.5 mM ammonium formate in Optima grade water, adjusted to pH 3 with formic acid) and mobile phase B (100% Optima grade methanol) at a flow rate of 0.25 ml/min. The initial mobile phase condition of 45% A was held for 6 min, decreased to 5% A over 0.25 min, held at 5% A for 0.5 min, then increased to 45% A over 0.25 min, and held for 1 min. URMC-099 was detected in the ESI- positive mode with a cone voltage of 50 V and collision energy of 20 V. Multiple reactions monitoring (MRM) transition for URMC-099 was 421.97 > 322.00. The IS was detected in the ESI -positive mode with a cone voltage of 36 V, and collision energy of 22 V with a MRM transition of 614.40 > 421.20. Spectra were analyzed and quantified by MassLynx software version 4.1. In all cases, ratios of analyte to IS peak areas were quantified.

Plasma and tissue dolutegravir quantification

DTG in plasma and tissues was determined by UPLC-MS/MS for which DTG from plasma (25 µL) and tissues (100 mg) was extracted using 1 ml acetonitrile. Then 10 µl Internal standard (IS; 1000 ng/ml SN-38) was added to each sample, and the samples were vortexed, and then centrifuged at 17,000 x g for 10 min. Supernatants were dried using a Savant Speed Vacuum (ThermoFisher Scientific), and reconstituted in 100 µl of 50% acetonitrile in Optima grade water. Standard solutions of DTG (0.2-2000 ng/ml) were prepared by extracting 25 µl of blank plasma from control mice into 1 ml of acetonitrile, and adding 10 µL of IS and various concentrations of DTG. Chromatographic separation of 10 µL of sample was achieved on an ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm x 100 mm) using a 7- min gradient of mobile phase A (7.5 mM ammonium formate in water, adjusted to pH 3 with formic acid) and mobile phase B (100% acetonitrile) at a flow rate of 0.25 ml/min. The initial mobile phase conditions of 65% A were held for 3.5 min, decreased to 5% A over 1.5 min, held at 5% A for 1.5 min, then increased to 65% A over 0.5 min, and held for 1 min. Drug was detected in the ESI positive mode with a cone voltage of 10 V, and a collision energy of 25 V. The MRM transition used for DTG was 420.08 > 277.12., and for SN-38, was 393.07 > 264.10. Spectra were analyzed and quantified by MassLynx software version 4.1. In all cases, ratios of analyte to IS peak areas were quantified.

Cell viability detection

Mitochondrial dehydrogenase activity in MDMs was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in MDMs. MDMs were pretreated with 100 μM nanoATV for 16 h, and then infected with HIV-1_{ADA} for 4 h, washed with PBS, and incubated with or without 400 ng/ml URMC-099. At day 3, 7, 10 and 14, cells were washed, and incubated with MTT dye (5 mg/ml) for 4 h at 37°C. Depending on the mitochondrial dehydrogenase activity of the cells, MTT is reduced to insoluble formazan crystals, which were subsequently dissolved by DMSO into a purple-colored solution. The percentage dehydrogenase activity relative to untreated cells was determined by spectrophotometry at 540 nm (SynergyMX, multimode spectrophotometer, BioTek). Trypan blue exclusion assay was performed to determine the cell viability (Strober, 2001). Briefly, 10 μl of treated cells were mixed with 10 μl of 0.4% trypan blue (Bio-Rad), and incubated for 2 min at room temperature. The number of dye-excluding cells was counted by using automated cell counter (TC20™, Bio-Rad).

Western blot assays

Treated MDMs were lysed in cell lysis reagent – (CellLytic; Sigma-Aldrich) with 1X protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). For cell fractionation studies, cytoplasmic and nuclear fractions were separated using a NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific) as per the manufacturer's instructions. Protein concentrations were determined using the Micro BCA Protein Assay kit (ThermoFisher Scientific). Proteins were separated by gel electrophoresis on a 4-15% gradient or 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA), and immunoblotted onto an activated PVDF membrane (Bio-Rad)(Duan et al., 2013). The membrane was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween 20 (TTBS), and probed with primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Chemiluminescent signals were detected, and analyzed using a FluorChemM system (Proteinsimple, Santa Clara, CA).

Supplemental Table 1

Primer/Probes for qRT-PCR

Gene	Host	Dye	Amplicon Length	Probe ID
tfeb	Human	FAM	78	Hs00292981_m1
	Mouse	FAM	65	Mm00448968_m1
map1lc3b	Human	FAM	93	Hs00797944_s1
	Mouse	FAM	141	Mm00782868_sH
becn1	Human	FAM	107	Hs00186838_m1
	Mouse	FAM	90	Mm01265461_m1
sqstm1	Human	FAM	115	Hs01061917_g1
	Mouse	FAM	116	Mm00448091_m1