Supporting Information

Supporting Materials and Methods

Antibodies and reagents. We obtained ubiquitin (sc-8017), p62/SQSTM1 (sc-28359), LAMP1 (sc-20011), COL1A1 (sc-293182), Pro-COL3A1 (sc-166316), PECAM-1 (sc-376764), MMP-2 (sc-53630), MMP-3 (sc-21732), MMP-9 (sc-393859), MMP-1/8 (sc-137044), LOX (sc-373995), MMP-13 (sc-515284), CTGF (sc-365970), TIMP-3 (sc-373839) and STING (sc-241046) antibodies from Santa Cruz Biotechnology, phospho-Ser403 p62 (MABC186) antibody from Millipore, STING (3337), phospho-Ser172 TBK1 (5483), TBK1 (3013), acetylated lysine (9441), cGAS (15102), LC3 (2775) and p62 (5114) antibodies from Cell Signaling Technology, Actin (JLA20) antibody from Developmental Studies Hybridoma Bank (DSHB), α-smooth muscle actin (ab5694) from Abcam, F4/80 (MF48000) from Invitrogen, Tubulin (T5168) antibody, fatty acid free and low endotoxin bovine serum albumin (BSA), PA, OA, SA, DHA, tunicamycin (Tm), Tg, cGAMP, verapamil and nicardipine from Sigma, BX795 from Selleckchem, amlexanox from Tocris Bioscience, TBBt from Calbiochem, lambda protein phosphatase (λPP) and calf intestine alkaline phosphatase (CIP) from New England Biolabs, and bafilomycin A1 from LC Labs.

Cell culture. HepG2 cells, obtained from ATCC (HB-8065), were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Sigma), 50 U/ml penicillin and 50 mg/ml streptomycin. All cultures were maintained in a 37°C incubator with 5% CO_2. The cells were authenticated by Short Tandem Repeat (STR) profiling at ATCC, tested negative for mycoplasma infection, and subcultured for

less than 6 months prior to initiation of the described experiments. Primary hepatocytes were isolated and cultured as previously described.[1] To induce lipotoxicity, de-lipidated low-endotoxin BSA was loaded with PA and other fatty acids, as formerly described,[1,2] and applied to cultured cells.

Mice and diets. Mice of C57BL/6 genetic background were used for the study. *Tbk1*$^{F/F}$ mice (L-*Tbk1*-WT), originally generated by the Takeda Pharmaceutical Company, were crossed with *Albumin-Cre* to generate *Albumin-Cre*/*Tbk1*$^{F/F}$ (L-*Tbk1*-KO) mice. These mice are all in C57BL/6 background, and littermates of L-*Tbk1*-WT and L-*Tbk1*-KO mice were used for the experiments. *Sting*-KO and *cGas*-KO mice are also congenic to the C57BL/6 background and were obtained from the Jackson Laboratory. Mice were maintained in filter-topped cages and given free access to autoclaved regular chow/low-fat diet (LFD, Lab Diet 5L0D), high-fat diet (HFD, Bio-Serv S3282 for the BX795 study and Research Diets D12451 for the L-*Tbk1*-KO study), methionine-restricted choline-deficient HFD (CD-HFD; Research Diets A06071302), methionine-supplemented choline-sufficient HFD (CS-HFD; Research Diets A06071306) and water. A06071302 and A06071306 are identical except the supplemented levels of methionine (0.8g in A06071302 and 5.1g in A0607130, in ~750g diet) and choline (0g in A06071302 and 2g in A0607130, in ~750g diet). In HFD, 45% (D12451) or 60% (S3282, A06071302 and A06071302) of calories come from fat, mostly in the form of lard. In LFD, only 13% (5L0D) of calories come from fat. For diet modulations, 2-month-old male mice raised on LFD were switched to HFD, CD-HFD or CS-HFD diets or kept on LFD for control. Mice were kept on the corresponding diets for two to four additional months. After the diet

modulations, the resulting 4- to 6-month-old mice were analyzed for experiments. When indicated, BX795 (25 mg/kg body weight) was administrated once daily to mice through intraperitoneal (i.p.) injection for the last 10 days before the experiments. Each experiment was done with 5-16 mice per group, and exact animal numbers are indicated in the corresponding figure legends. Exact information regarding mouse age, gender, diet duration, drug dose, route and frequency are also indicated in the corresponding figure legends. All animal studies were ethically approved and overseen by the University Committee on Use and Care of Animals (UCUCA) at the UM.

Lentiviral constructs. The lentiviral plasmids for sh-STING (TRCN0000160895), sh-TBK1 (TRCN0000003182 and TRCN0000003186), sh-cGAS (TRCN0000128706 and TRCN0000148694) and sh-p62 (TRCN0000007234 and TRCN0000007236) were purchased from Open Biosystems. Viruses were generated and amplified in the Vector Core facility at the University of Michigan (UM).

Subcellular Fractionation and Immunoblotting. Cells and tissues were lysed in radioimmunoprecipitation assay (RIPA) buffer or solubility fractionation buffer, and processed as formerly described.[(2)] In brief, cells and tissues were lysed in a lysis buffer [(2)] containing 1% Triton X-100. Cell and tissue lysates were centrifuged at 15,000 r.p.m. for 15 min at 4ºC. After centrifugation, pellets were separated from supernatants, resuspended in a lysis buffer containing 2% SDS and boiled in SDS sample buffer (insoluble fraction). Supernatants were also boiled in SDS sample buffer (soluble fraction), and both fractions were analyzed by immunoblotting. For Fig. 1D, cells were initially lysed in a lysis buffer containing 0.1% Triton X-100 so that the lysates were

separated into three fractions. Protein samples were separated by SDS-PAGE, transferred to PVDF membranes and probed with primary antibodies (1:200 for Santa Cruz antibodies, and 1:1,000 for all other antibodies). After incubation with secondary antibodies conjugated with HRP (1:2,000), chemiluminescence was detected using LAS4000 (GE) systems. For the samples analyzed in the same fraction, protein loading amount was normalized to the actin expression level. The degree of phosphorylation-induced gel shift was variable in different gels, and typically more pronounced in gels with extended gel running time. All protein samples were run with the precision plus protein standards (Bio-rad) for molecular weight estimation in the same gel. Exact or nearest positions of protein standards were indicated in all immunoblot images.

Immunocytochemistry. Cells grown on coverslips were fixed with 4% paraformaldehyde and incubated overnight with primary antibodies (1:50 for Santa Cruz antibodies, and 1:400 for all other antibodies). After washing, cells were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen, 1:250) for 1.5 hr, counterstained with DAPI (Invitrogen), and analyzed under a laser confocal microscope (Olympus).

Immunoprecipitation. For immunoprecipitation of endogenous p62, cell lysates were prepared in RIPA buffer plus protease inhibitor cocktail (Roche), and incubated overnight at 4°C with anti-p62 antibody (sc-28359) conjugated to a protein G/A bead (Calbiochem). The immunocomplexes were washed three times with the RIPA buffer and then three times with the RIPA buffer plus 0.5 M NaCl, and analyzed through immunoblotting.

Protein identification. Insoluble fraction from PA-treated (9 hr) HepG2 cells was prepared through the method described above. After separation by SDS-PAGE, major protein bands were visualized by Coomassie Blue staining. One of the most prominent bands, appearing around 54kD, was excised from the gel and subjected to the LC-MS/MS analysis at the University of Michigan Proteomics Core facility.

Co-Immunoprecipitation assay. For co-immunoprecipitation between endogenous TBK1 and p62, HepG2 cells were treated with 0.5 mM PA for 3 hr. Then the cells were lysed in 0.3% CHAPS buffer (a lysis buffer[3] containing 0.3% CHAPS) plus protease inhibitor cocktail (Roche), and subjected to immunoprecipitation with control IgG, anti-p62 antibody (sc-28359) or anti-TBK1 antibody (3013, Cell Signaling) conjugated to a protein G/A bead (Calbiochem) overnight at 4°C. The immunocomplexes were washed three times with 0.3% CHAPS buffer and two times with 0.3% CHAPS buffer plus 0.5 M NaCl, and analyzed by immunoblotting.

In vitro kinase assay. Cell lysates were prepared in 0.3% CHAPS buffer plus protease inhibitor cocktail (Roche), and incubated overnight at 4°C with anti-TBK1 antibody (3013, Cell Signaling) conjugated to a protein G/A bead (Calbiochem). After washing three times with 0.3% CHAPS buffer and two times with 0.3% CHAPS buffer plus 0.5 M NaCl, the immunocomplexes were suspended in 50 μl kinase reaction buffer (20 mM Tris-Cl (pH 7.4), 10 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, and 100 μM ATP) with 1 μg of GST-p62 (BML-UW1035, Enzo Life Sciences) as a substrate and then incubated for 1 hr at 25°C. The reaction was terminated by adding 10 μl 5X SDS sample buffer and incubating the mixture at 95°C for 5 min. The sample was analyzed by immunoblotting.

Quantitative RT-PCR. Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen), and cDNA was made using MMLV-RT (Invitrogen) and random hexamers (Invitrogen). Quantitative PCR was performed in a Real-Time PCR detection system (Applied Biosystems) with iQ^{TM} SYBR Green Supermix (Bio-rad) and relevant primers. Relative mRNA expression was calculated from the comparative threshold cycle (Ct) values relative to β-Actin. Following primer pairs were used for RT-PCR experiments (5'-3' sequence; F, forward; R, reverse):

α-SMA-F	GTCCCAGACATCAGGGAGTAA
α-SMA-R	TCGGATACTTCAGCGTCAGGA
TGFB1-F	CTCCCGTGGCTTCTAGTGC
TGFB1-R	GCCTTAGTTTGGACAGGATCTG
COL1A1-F	GCTCCTCTTAGGGGCCACT
COL1A1-R	CCACGTCTCACCATTGGGG
GFAP-F	CGGAGACGCATCACCTCTG
GFAP-R	AGGGAGTGGAGGAGTCATTCG
DESMIN-F	GTGGATGCAGCCACTCTAGC
DESMIN-R	TTAGCCGCGATGGTCTCATAC
PDGFB-F	CATCCGCTCCTTTGATGATCTT
PDGFB-R	GTGCTCGGGTCATGTTCAAGT

CTGF-F	GGGCCTCTTCTGCGATTTC
CTGF-R	ATCCAGGCAAGTGCATTGGTA
MMP-2-F	CAAGTTCCCCGGCGATGTC
MMP-2-R	TTCTGGTCAAGGTCACCTGTC
MMP-9-F	CTGGACAGCCAGACACTAAAG
MMP-9-R	CTCGCGGCAAGTCTTCAGAG
TIMP-2-F	TCAGAGCCAAAGCAGTGAGC
TIMP-2-R	GCCGTGTAGATAAACTCGATGTC
CCL2-F	TTAAAAACCTGGATCGGAACCAA
CCL2-R	GCATTAGCTTCAGATTTACGGGT
VEGFA-F	GCACATAGAGAGAATGAGCTTCC
VEGFA-R	CTCCGCTCTGAACAAGGCT
ANGPT1-F	CACATAGGGTGCAGCAACCA
ANGPT1-R	CGTCGTGTTCTGGAAGAATGA
TNF1-F	CCCTCACACTCAGATCATCTTCT
TNF1-R	GCTACGACGTGGGCTACAG
IL10-F	GCTCTTACTGACTGGCATGAG
IL10-R	CGCAGCTCTAGGAGCATGTG

IL6-F	TAGTCCTTCCTACCCCAATTTCC
IL6-R	TTGGTCCTTAGCCACTCCTTC
F4/80-F	CCCCAGTGTCCTTACAGAGTG
F4/80-R	GTGCCCAGAGTGGATGTCT
β-Actin-F	CAAAAGCCACCCCCACTCCTAAGA
β-Actin-R	GCCCTGGCTGCCTCAACACCTC
SRX-F	GCCCAGGGAGGTGACTACTT
SRX-R	GTGGATGCTCCCAGGTACAC
NQO1-F	CGCAGACCTTGTGATATTCCAG
NQO1-R	CGTTTCTTCCATCCTTCCAGG
GSTA1-F	AGCCCAAGCTCCACTACTTCAAT
GSTA1-R	CTTCAAACTCTACTCCAGCTGCAG
SQSTM1-F	ATCGGAGGATCCGAGTGT
SQSTM1-R	TGGCTGTGAGCTGCTCTT
β-Actin (human)-F	CACGAAACTACCTTCAACTCCATC
β-Actin (human)-R	AATGATCTTGATCTTCATTGTGCT

Histology. Liver tissues were fixed in 10% buffered formalin, embedded in paraffin and subjected to immunohistochemical staining, as previously described.[1] In brief, paraffin-embedded liver sections were incubated with primary antibody (Santa Cruz

Biotechnology sc-28359 for p62 staining; 1:100, Invitrogen MF48000 for F4/80; 1:100), followed by incubation with biotin-conjugated secondary antibodies (Vector Lab, BA-9200 for p62; 1:200 or BA-9401 for F4/80; 1:200) and horseradish peroxidase (HRP)-conjugated streptavidin (BD Biosciences, 554066; 1:300). The HRP activity was visualized with diaminobenzidine staining. Haematoxylin counterstaining was applied to visualize nuclei. For α-SMA staining, liver sections were incubated with anti-α-SMA antibody (Abcam ab5694), followed by incubation with Alexa Flour 594-conjugated secondary antibody (Invitrogen). Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay was performed using In Situ Cell Death Detection Kit-TMR-Red (Roche). Dihydroethidium (DHE) staining was performed using freshly frozen liver sections and DHE (Thermo Fisher Scientific, D11347) as formerly described.[4] α-SMA-, TUNEL- and DHE-stained samples were examined under a fluorescence microscope (Meiji). To visualize collagen fibers, liver sections were stained with saturated picric acid containing 0.5% Sirius Red (Sigma). The sections were washed with distilled water, dehydrated, mounted, and examined under a light microscope (Meiji). For Oil Red O staining, OCT-embedded frozen liver sections were allowed to air dry and rinsed with 60% isopropanol, followed by staining with fresh 0.5% Oil Red O solution for 15 min. After staining, the slides were rinsed with 60% isopropanol, washed with distilled water, mounted and analyzed under a light microscope (Meiji).

Serum ALT assay. Serum ALT levels were measured with an ALT (SGPT) activity assay kit (Pointe Scientific, Inc.) according to the manufacturer's instructions.

Supporting Information Figure Legends

Fig. S1. SFA induces p62 phosphorylation and TBK1 activation. (A) KRT8 is one of the major components of palmitic acid (PA)-induced protein inclusion. Insoluble fractions from BSA- and PA-treated HepG2 were analyzed by SDS-PAGE. The band in red box was analyzed by MS/MS and identified as KRT8. (B-F) HepG2 cells (B,D-F) or mouse primary hepatocytes (C) were treated with BSA (Con), PA (500 μM), thapsigargin (Tg, 1μM), bafilomycin A1 (Baf, 100 nM), tunicamycin (Tm, 5 μg/ml) or tetrabromobenzotriazole (TBBt, 50 μM) for 9 h. (B,D,E) Cell lysates were treated with lambda protein phosphatase and calf intestinal phosphatase treatment (λPP+CIP; B) or CIP only (D,E) for 1 hr at 37°C, followed by immunoblotting (B,D) and band quantification (E). Cell lysates were analyzed by immunoblotting (C,F). Quantification data are shown as mean ± s.e.m. $*P < 0.05$, $***P < 0.001$ (Student's t-test). (G-I) HepG2 cells were treated with PA (500 μM) for indicated hours. The cells were then subjected to subcellular fractionation (G,H) or anti-TBK1 immunoprecipitation (IP) (I). Insoluble fraction (G) or indicated fractions (H) were analyzed by immunoblotting. Phosphotransferase activity of immunopurified TBK1 (IP-TBK1) was assayed using recombinant GST-p62 protein and p-p62 antibody (I). (J) PA-treated HepG2 cells (3 hr) were subjected to co-IP assay with normal IgG (-), anti-TBK1 or anti-p62 antibodies. IP complexes were analyzed by immunoblotting. Arrows indicate positions of shifted (black) and unshifted (grey) p62 bands. Arrowheads indicate the exact or nearest position of the protein molecular weight markers (kD).

Fig. S2. STING silencing suppresses SFA-induced protein inclusion formation. At 48 hr after infection with shRNA lentiviruses for luciferase (control) or STING (sh-STING), HepG2 cells were treated with BSA (Con) or PA (500 μM) for 9 hr. Then the cells were subjected to immunostaining. Boxed areas in fluorescence images (A,C) are magnified in rightmost panels. Immunostained images of PA-treated control cells were analyzed by line-scan evaluation of each signal across protein inclusions (B,D). Scale bars, 5 μm.

Fig. S3. cGAS silencing suppresses SFA-induced protein inclusion formation. At 48 hr after infection with shRNA lentiviruses for luciferase (control) or cGAS (sh-cGAS #1), HepG2 cells were treated with BSA (Con) or PA (500 μM) for 9 hr. Then the cells were subjected to immunostaining. Boxed areas in fluorescence images (A,C) are magnified in rightmost panels. Immunostained images of PA-treated control cells were analyzed by line-scan evaluation of each signal across protein inclusions (B,D). Scale bars, 5 μm.

Fig. S4. Extracellular cGAMP treatment activates TBK1 but does not induce p62 phosphorylation. (A,B) HepG2 cells were treated with BSA, PA (500 μM), bafilomycin A1 (Baf, 100 nM) and/or cGAMP (10-20 μM) for 9 h. Then the cells were subjected to subcellular fractionation and immunoblotting. Arrowheads indicate the exact or nearest position of the protein molecular weight markers (kD).

Fig. S5. cGAS-STING pathway mediates lipotoxic activation of TBK1 and subsequent phosphorylation of p62. Primary hepatocytes were isolated and cultured from two-month-old WT, *Sting*$^{-/-}$ and *cGas*$^{-/-}$ mice (n=6, 3 and 3, respectively). The cells were treated with BSA (Con) or PA (500 μM) for 9 hr. Then the cells were subjected to immunostaining

(A,B), subcellular fractionation and immunoblotting (C,D), and immunoblot quantification (E,F). Boxed areas in fluorescence images (A,B) are magnified in rightmost panels. Scale bars, 5 μm (A,B). All data are shown as mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). Arrowheads indicate the exact or nearest position of the protein molecular weight markers (kD).

Fig. S6. SFA induces expression of Nrf2 target genes through the TBK1 signaling**.** (A-D) At 48 hr after infection with shRNA lentiviruses for indicated genes, HepG2 cells were treated with BSA or PA (500 μM) for 9 hr. Then mRNA expression of indicated genes was examined through quantitative RT-PCR. Quantification data are shown as mean ± s.e.m. $^{***}P < 0.001$ between PA-treated sh-Luc and indicated groups (Student's *t*-test). (E) At 48 hr after infection with shRNA lentiviruses for luciferase (sh-Con) or p62 (sh-p62 #1 and #2), HepG2 cells were treated with BSA or PA (500 μM) for 9 hr. Then the cells were subjected to immunoblotting. Arrowheads indicate the exact or nearest position of the protein molecular weight markers (kD).

Fig. S7. TBK1 inhibitors alleviate SFA-induced autophagic arrest. HepG2 cells were treated with BSA (Con), PA (500 μM), PA + BX795 (20 μM), PA + Amlexanox (Amx, 100 μM) for 9 h. Then the cells were subjected to immunostaining with LC3 (green, an autophagosome marker) and LAMP1 (red a lysosome marker) antibodies (A). DNA was stained with DAPI (blue). Co-localization between LAMP1 and LC3 (B), as well as LC3-covered fluorescent area (C), were quantified. Boxed areas in fluorescence images are magnified in right-most panels (A). Scale bars, 5 μm. Data are shown as mean ± s.e.m. $^{*}P < 0.05$ (Student's t test).

Fig. S8. Effects of TBK1 inhibition on body weight. Mouse cohorts described in Fig. 5 were analyzed for body weight (A,C,D) and food consumption (B). (A,B) During daily administration of PBS and BX795, body weight (A) and daily food consumption (B) of the mice described in Fig. 5A,B were examined. (C,D) Body weights of the mice described in Fig. 5C,D were examined before (C; 2-month-old mice) and after (D; 5-month-old mice) HFD feeding. Data are shown as mean ± s.e.m.

Fig. S9. CD-HFD induces strong p62 phosphorylation and accumulation of ubiquitin-p62 inclusions. (A-D) 2-month-old C57BL/6 male mice kept on LFD were kept on LFD or switched to methionine-restricted choline-deficient diet (CD-HFD) and methionine-supplemented choline-sufficient diet (CS-HFD) for an additional 2 months. The resulting 4-month-old mice were analyzed. (A) Liver sections were subjected to Sirius Red staining, hematoxylin and eosin (H&E) staining, and Oil Red O (ORO) staining. (B) Body weight was monitored throughout the diet modulation. (C) Serum ALT levels were quantified. (D) Livers were subjected to solubility fractionation. 1% Triton X-100-insoluble fractions were analyzed by immunoblotting. (E-G) Mouse cohorts described in Fig. 6-7 were analyzed for body weight (E) throughout the course of experiment. Vehicle and BX795 were only administered in the last 10 days of the experiment. (F) ORO staining area and TUNEL fluorescence intensities were quantified. (G) Relative mRNA expressions were quantified through RT-PCR. All data are shown as mean ± s.e.m. NS, not statistically significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test). Arrowheads indicate the exact or nearest position of the protein molecular weight markers (kD).

Fig. S10. Involvement of STING in HFD-induced p62 phosphorylation and aggregation. (A-E) Six-month-old WT and *Sting*$^{-/-}$ male mice (n=5) of C57BL/6 background, kept on HFD for 4 months, were analyzed. Livers were subjected to solubility fractionation. 1% Triton X-100-soluble and -insoluble fractions were analyzed by immunoblotting (A) and immunoblot quantification (B,C; mean ± s.e.m.). Body weights of the mice were examined before (D; 2-month-old mice) and after (E; 6-month old mice) HFD feeding. (F-H) Four-month-old WT (n=7) and *Sting*$^{-/-}$ (n=8) male mice of C57BL/6 background, kept on CD-HFD for 2 months, were analyzed. Livers were subjected to solubility fractionation, and 1% Triton X-100-insoluble fractions were analyzed by immunoblotting (F) and immunoblot quantification (G; mean ± s.e.m.). Mouse cohorts were analyzed for body weight (H; mean ± s.e.m.) throughout the course of experiment. **P* < 0.05, ***P* < 0.01 (Student's *t*-test).

Supporting Information References

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