Materials and Methods

Materials

Reagents were obtained from the following sources. Antibodies to LAMP2, NOP17, and HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; to PEX19 from Abcam; to NUFIP1 from Protein Tech; to ZNHIT3 from Bethyl Laboratories; to raptor from EMD Millipore; to S6K1 phospho-T389, S6K1, GSKb, LAMP1, LC3B, Histone H3, mTOR, RagA, VDAC, Calreticulin, Golgin-97, GAPDH, ZFYVE26, Catalase and the FLAG and HA epitopes from Cell Signaling Technology (CST); to SPG11 from Proteintech; to SNU13, NOP58, FBL, RPL7, RPS15A, RPL21, RPL23, and RPL26 from Bethyl Laboratories; and to RPS23 from Thermo. SAR405 from Selleck; RPMI and Flag-M2 affinity gel was obtained from Sigma Aldrich. DMEM from SAFC Biosciences; leucine-, arginine-, and lysine-free RPMI from US Biologicals; HBSS without glucose was used to maintain pH and osmotic balance; adenosine, guanosine, uridine, and inosine from Sigma; Giemsa stain from Sigma; XtremeGene9 and Complete Protease Cocktail from Roche; Alexa-488- and -568-conjugated secondary antibodies and inactivated fetal bovine serum (IFS) from Invitrogen; anti-HA magnetic beads, and ECL western blotting substrate from Thermo Fisher Scientific; Protein G Agarose beads from Pierce; and Actinomycin D from Tocris. Torin1 was generously provided by Dr. Nathanael Gray (DFCI).

Cell Culture

HEK-293T, MIA-PaCa, 8988T, and P53$^{-/-}$ MEFs and their derivatives were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% inactivated fetal calf serum, 2 mM glutamine, penicillin, and streptomycin. For experiments involving amino acid starvation, cells were incubated in RPMI base media lacking the indicated amino acid for 60 min or the indicated time points. For experiments involving analysis of ribosomal protein levels, experiments were preformed at no greater than 50% confluency to ensure active proliferation. As the abundance of ribosomes is extremely high, typically 2 ul of dilute lysate was analyzed via immunoblotting to ensure ribosome protein signal did not saturate and stayed witihin linear range. For all experiments involving lysosomal purifications, the media on the cells was changed to fresh RPMI base media 1 hr prior to the start of the experiment.

Cell lysis, immunoprecipitations, and cDNA transfections

Cells were rinsed with chilled PBS and lysed immediately on ice with a Triton X-100-based lysis buffer (1% Triton, 10 mM B-glycerol phosphate, 10 mM pyrophosphate, 40 mM HEPES pH 7.4, 2.5 mM $MgCl_2$) supplemented with 1 tablet of EDTA-free protease inhibitor (Roche) per 25 mL buffer. Lysates were kept at 4°C for 15 min and then clarified by centrifugation in a microcentrifuge at 13,000 rpm at 4°C for 10 min. For anti-FLAG immunoprecipitations, the FLAG-M2 affinity gel was washed with 1 mL lysis buffer three times and 30 uL of a 50% slurry of the affinity gel was then added to the clarified lysate and incubated with rotation at 4°C for 90 min. In order to reduce non-specific binding of ribosomes in anti-FLAG immunoprecipitations, low protein binding tubes were used to reduce non-specific binding of ribosomes. Further, the beads were washed for 15 min three times in lysis buffer containing 500 mM NaCl on rotation at 4°C. Immunoprecipitated proteins were denatured by the addition of 50 uL of sample buffer and boiling for 5 min.

For transfection-based experiments in HEK-293T cells, 2 million cells were plated in 10 cm culture plates. After twenty-four hours, cells were transfected using the polyethylenimine method using pRK5-based cDNA expression vectors as indicated (*45*). The total amount of transfected plasmid DNA in each transfection was normalized to 5 μg using the empty pRK5 plasmid. After thirty-six hours, cells were lysed and analyzed as described above.

Clonogenic Survival Assays

Indicated cells were seeded in 12-well plates in normal growth medium (RPMI). The following day, when cells reached ~50% confluence, RPMI was removed and replaced with HBSS or supplemented with 2 mM nucleosides for the indicated time points. Cells were then returned to normal media for 72 hours, fixed with cold methanol for 10 min, and stained with Giemsa.

Lysosome immunopurification (LysoIP)

LysoIP was performed largely as previously described (*5*). Briefly, ~35 million cells were used for each replicate. Cells were rinsed twice with pre-chilled PBS and then scraped in one mL of PBS containing protease and phosphatase inhibitors and pelleted at 1000 x g for 2 min at 4°C. Cells were then resuspended in 950 µL of the same buffer, and 25 µL (equivalent to 2.5% of the total number cells) was reserved for further processing to generate the whole-cell sample. The remaining cells were gently homogenized with 20 strokes of a 2 ml dounce-type homogenizer. The homogenate was then centrifuged at 1000 x g for 2 min at 4°C to pellet the cell debris and intact cells while cellular organelles including lysosomes remained in the supernatant which was incubated with 150 µL of anti-HA magnetic beads prewashed with PBS on a rotator shaker for 3 min. Immunoprecipitates were then gently washed three times with PBS on a DynaMag Spin Magnet. Beads with bound lysosomes were resuspended in 100 µL ice-chilled 1% Triton X lysis buffer to extract proteins. After 10 min incubation on ice the beads were removed with the magnet. For proteomics experiments all the steps were performed using low protein binding tubes (LoBind, Eppendorf) to minimize variability between samples and maximize the recovery of proteins.

MS data acquisition

Sample preparation for mass spectrometry (MS)

Samples were solubilized by the addition of 20% (w/v) SDS to a final concentration of 2% followed by sonication in a Bioruptor Plus (Diagenode) (5 cycles: 1 min on, 30 sec off, 20°C) at the highest settings. Samples were spun down at 20,800x *g* for 1 min and the supernatant transferred to fresh tubes. Reduction was performed with 2.9 µL DTT (200 mM) for 15 min at 45°C before alkylation with 200 mM IAA (5 µL, 30 minutes, room temperature, in the dark). Proteins were then precipitated with 4 volumes ice cold acetone to 1 volume sample and left overnight at -20°C. The samples were then centrifuged at 20,800x *g* for 30 min at 4°C. After removal of the supernatant, the precipitates were washed twice with 500 µL 80% (v/v) acetone (ice cold). After each wash step, the samples were vortexed, then centrifuged again for 2 min at 4°C. The pellets were then allowed to air-dry before being dissolved in digestion buffer (50 µL, 3M urea in 0.1 M HEPES, pH 8) containing 0.1 µg of LysC (Wako), and incubated for 4 h at 37°C with shaking at 600 rpm. Then the samples were diluted 1:1 with milliQ water (to reach 1.5 M urea) and incubated with 0.1 µg trypsin (Promega) for 16 h at 37 °C. The digests were then acidified with 10% trifluoroacetic acid and then desalted with Waters Oasis® HLB µElution Plate 30µm in the presence of a slow vacuum. In this process, the columns were conditioned with 3x100 µL solvent B (80% (v/v) acetonitrile; 0.05% (v/v)

formic acid) and equilibrated with 3x 100 µL solvent A (0.05% (v/v) formic acid in milliQ water). The samples were loaded, washed 3 times with 100 µL solvent A, and then eluted into PCR tubes with 50 µL solvent B. The samples were then dried in a Speed-Vac and resuspended in 10 µL reconstitution buffer (5% (v/v) acetonitrile, 0.1% (v/v) TFA in water) prior to MS analysis.

Peptides were spiked with retention time HRM kit (Biognosys AG), and analyed using the nanoAcquity UPLC system (Waters) fitted with a trapping (nanoAcquity Symmetry C18, 5 µm, 180 µm x 20 mm) and an analytical column (nanoAcquity BEH C18, 2.5 µm, 75 µm x 250 mm). The outlet of the analytical column was coupled directly to an Orbitrap Fusion Lumos (Thermo Fisher Scientific) using the Proxeon nanospray source. Solvent A was water, 0.1% (v/v) formic acid and solvent B was acetonitrile, 0.1% (v/v) formic acid. Approx. 1 µg of peptides were loaded for each sample with a constant flow of solvent A at 5 µL/min, onto the trapping column. Trapping time was 6 min. Peptides were eluted via the analytical column at a constant flow of 0.3 µL/min, at 40°C, via a non-linear gradient from 0% to 40% in 90 min. Total runtime was 115 minutes, including clean-up and column re-equilibration. The RF lens was set to 30%. For spectral library generation, 4 pooled samples were generated by mixing equal portions of each sample belonging to a biological condition, and each pool was injected in triplicate (12 runs in total), and measured in Data Dependent Acquisition (DDA) mode. The conditions for DDA data acquisition were as follows: full scan MS spectra with mass range 350-1650 *m/z* were acquired in profile mode in the Orbitrap with resolution of 60000. The filling time was set at maximum of 50 ms with limitation of 2×10^5 ions. The “Top Speed” method was employed to take the maximum number of precursor ions (with an intensity threshold of 5×10^4) from the full scan MS for fragmentation (using HCD collision energy, 30%) and quadrupole isolation (1.4 Da window) and measurement in the Orbitrap (resolution 15000, fixed first mass 120 *m/z*), with a cycle time of 3 seconds. The MIPS (monoisotopic precursor selection) peptide algorithm was employed but with relaxed restrictions when too few precursors meeting the criteria were found. The fragmentation was performed after accumulation of 2×10^5 ions or after filling time of 22 ms for each precursor ion (whichever occurred first). MS/MS data were acquired in centroid mode. Only multiply charged (2^+ - 7^+) precursor ions were selected for MS/MS. Dynamic exclusion was employed with maximum retention period of 15s and relative mass window of 10 ppm. Isotopes were excluded. For data acquisition and processing of the raw data Xcalibur 4.0 (Thermo Scientific) and Tune version 2.1 were employed.

For the Data Independent Acquisition (DIA) data the same gradient conditions were applied to the LC as for the DDA and the MS conditions were varied as follows: full scan MS spectra with mass range 350-1650 *m/z* were acquired in profile mode in the Orbitrap with resolution of 120000. The filling time was set at maximum of 20ms with limitation of 5×10^5 ions. DIA scans were acquired with 34 mass window segments of differing widths across the MS1 mass range. HCD fragmentation (30% collision energy) was applied and MS/MS spectra were acquired in the Orbitrap with a resolution of 30000 over the mass range 200-2000 *m/z* after accumulation of 2×10^5 ions or after filling time of 70 ms (whichever occurred first). Ions were injected for all available parallelizable time. Data were acquired in profile mode.

MS data analysis

For library creation, the DDA data was searched using the Pulsar search engine (version 1.0.15764.0, Biognosys AG). The data were searched against a human database (Swiss-Prot entries of the Uniprot KB database release 2016_01, 20198 entries). The

data were searched with the Biognosys default settings with the following modifications: Carbamidomethyl (C) and Oxidation (M)/ Acetyl (Protein N-term) (Variable). A maximum of 2 missed cleavages were allowed. The identifications were filtered to satisfy FDR of 1 % on peptide and protein level. The library contained 70796 precursors, corresponding to 5401 protein groups using Spectronaut protein inference. DIA data were then searched against this library. Precursor matching, protein inference and quantification was performed in Spectronaut (version 11) using default settings(*46*). Peptide and protein level FDR for DIA data were controlled to 1% (*47*). Differential protein expression was evaluated using a pairwise t-test performed at the precursor level followed by multiple testing correction according to (*48*). The data (candidate table, Supplementary Tables 1 and 2) was exported from Spectronaut and used for further data analyses.

Generation of cells lacking NUFIP1, ATG7, or ATG5

To generate HEK-293T cells lacking NUFIP1, sgRNAs targeting the first exon of NUFIP1 were designed and cloned into the px459 CRISPR vector using the following olignucleotides.

Sense: AGGGGAGACTGGGCGTCGAA
Antisense: TTCGACGCCCAGTCTCCCCT

To generate HEK-293T cells lacking ATG5, sgRNAs targeting ATG5 were designed and cloned into the px459 CRISPR vector using the following olignucleotides.

Sense: GATCACAAGCAACTCTGGAT
Antisense: ATCCAGAGTTGCTTGTGATC

sgNUFIP1 Mia-PaCa cell lines were made using the pLenticrispr system utilizing the same sgRNAs as described above. sgNUFIP1 $P53^{-/-}$ MEFs were generated using the pLenticrispr system utilizing sgRNA sequences targeting the first exon of murine NUFIP1. HEK-293T cells lacking ATG7 were described previously (*5*).

Generation of cells stably expressing cDNAs

The following lentiviral expression plasmids were used: pLJM1-FLAG-metap2, pLJM60-RAP2A, pLJM60-FLAG-NUFIP1 and subsequent mutants. For lysosomal purifications, pLJC5-3XHA-TMEM192 and pLJC5-2XFLAG-TMEM192 or pLJC6-3XHA-TMEM192 and pLJC6-2XFLAG-TMEM192 were used to tag the lysosome. Lentiviruses were produced by transfecting HEK-293T cells with the plasmids indicated above in combination with the VSV-G and CMV ΔVPR packaging plasmids. Twelve hours post transfection, the media was changed to DMEM supplemented with 30% IFS. Thirty-six hours later, the virus-containing supernatant was collected and frozen at -80°C for 30 min. Cells to be infected were plated in 12-well plates containing DMEM supplemented with 10% IFS with 8 μg/ml polybrene and infected with the virus containing medium. Twenty-four hours later, the cell culture medium was changed to media containing puromycin or blasticidin for selection.

Immunofluorescence assays

HEK-293T cells were plated on fibronectin-coated glass coverslips in 6-well cell culture dishes at 300,000 cells/well. After 12 hr, the coverslips were washed once in PBS and subsequently fixed and permeabilized in a single step using 1 mL of ice-cold methanol at -20°C for 15 min. The coverslips were washed twice in 1 mL PBS and then incubated with primary antibody (FLAG (CST) 1:300 dilution, LC3B (CST) 1:200 dilution, LAMP2 (SCBT) 1:400 dilution) in 5% normal donkey serum for 1 hr at room temperature. After

incubation with the primary antibody, the cover slips were rinsed 4 times in PBS and incubated with secondary antibodies (1:400 dilution in 5% normal donkey serum) for 45 min at room temperature in the dark. The coverslips were then washed 4 times with PBS and once in dH_2O. Coverslips were mounted on slides using Vectashield containing DAPI (Vector Laboratories) and imaged on a spinning disc confocal microscopy system (Perkin Elmer).

In vitro binding of NUFIP1-ZNHIT3 to LC3B

In brief, 4 million HEK-293T cells were plated in 15 cm culture dishes. For proteins produced via transient expression, after 48 hr cells were transfected with following amounts of cDNAs in the pRK5 expression vector using the PEI method (*45*): 5 μg HA-GST-Rap2a; 10 μg HA-GST-GABARAP; 10 μg HA-GST-LC3B. For isolation of heterodimeric NUFIP1-ZNHIT3: 4 μg FLAG-NUFIP1; 10 μg HA-ZNHIT3.

Thirty-six hours post transfection, cells were lysed as indicated above. After clearing of cell lysates, 200 μL of 50% slurry of immobilized glutathione affinity resin equilibrated in lysis buffer was added to lysates expressing GST-tagged proteins. Recombinant proteins were incubated with the affinity resin for 2 hr at 4°C with rotation. Each sample was washed 3 times in binding buffer consisting of 0.1% TX-100, 2.5 mM $MgCl_2$, 20 mM HEPES pH 7.4, and 150 mM NaCl.

When transiently expressed FLAG-NUFIP1-HA-ZNHIT3 was purified from Torin1 treated HEK-293T cells, cells were first treated with 250 nM Torin1 for 1 hour prior to the purification. For experiments involving in vitro binding to purified ribosomes, low protein binding tubes were used to reduce background signal from ribosomes. Similarly, each sample was washed 3 times for 15 mins each in binding buffer supplemented 500 mM NaCl. In order to reduce non-specific background signal, great care must be taken to wash the walls of the tube.

Cell fractionations

Isolation of nuclear and post-nuclear supernatant fractions was preformed as in (*49*) with the following modifications. Confluent 15 cm plates were deprived of amino acids or treated with 250 nM Torin1 prior to isolation. Cells were lysed in PNS buffer containing 0.01% TritionX-100, 10 mM B-glycerol phosphate, 10 mM pyrophosphate, 40 mM HEPES pH 7.4, and 2.5 mM $MgCl_2$. After 15 min the lysate was clarified by centrifugation at top speed. The supernatant represented the cytosolic fraction containing lysosomes. The pellet (nuclear fraction) was washed twice in PNS buffer containing 500 mM NaCl and lysed for 1 hour with DNase I.

Isolation of ribosomes using a sucrose cushion

Two 15 cm plates of confluent HEK-293T cells were lysed in freshly prepared lysis buffer as described above with the addition of RNAsin (Promega) and 300mM NaCl in DEPC treated water. After a 30 min lysis at 4° C, the lysate was clarified at maximum speed in a microcentrifuge for 10 min to remove debris, nuclei, and mitochondria. The supernatant was collected and loaded onto a 50% sucrose cushion prepared in Buffer A consisting of 40 mM HEPES pH 7.4, 2.5 mM $MgCl_2$, and 150 mM KCl in DEPC treated water and ultracentrifuged for 16-18 hr at 100,000g to obtain the ribosome-containing pellet. While loading the lysate on top of the cushion, great care must be taken to not disturb the sucrose by the very slow addition of the lysate onto the 50% sucrose. A translucent pellet at the bottom of the ultracentrifuge tube represents the ribosomes. Fractions were collected and homogenized gently using a dounce homogenizer,

precipitated, adjusted to 0.5% SDS, and heated in boiling water for 5 min prior to SDS-PAGE followed by immunoblotting.

When HEK-293T cells were first deprived of nutrients or treated with Torin1, two 15 cm plates of HEK-293T cells were deprived of amino acids or treated with 250 nM Torin1 for 1 hour prior to lysis. For experiments involving in vitro binding of NUFIP1-ZNHIT3 to purified ribosomes, the resulting ribosome pellet is washed once in Buffer A. prior to use.

Ribosome analysis using a sucrose gradient

Prior to lysis, cell were treated with 100 μg/ml cycloheximide for 5 minutes, washed in ice-cold PBS with 100 μg/ml cycloheximide, and then lysed as described above in freshly prepared lysis buffer with the addition of RNAsin (Promega) in DEPC treated water. After a 30 min lysis at 4°C, the lysate was clarified at maximum speed in microcentrifuge for 10 min to remove debris, nuclei, and mitochondria. When EDTA was used, 50 mM EDTA was added during the preparation of the sucrose gradient and to lysis buffer. Lysates were normalized by protein content using the Bradford reagent (Bio-rad) and layered onto an 11 mL 10-45% sucrose gradient made in 40 mM HEPES, 7.5 mM $MgCl_2$, 100 mM KCl, 2 mM DTT, 100 μg/ml cycloheximide, RNAsin. Lysates were ultracentrifuged at 40,000 RPM using a SW-41 Ti rotor at 4°C for 3 hr and 1 mL fractions were collected, precipitated, adjusted to 0.5% SDS, and heated for 5 min in boiling water prior to SDS-PAGE followed by immunoblotting.

LC/MS-based metabolomics and quantification of metabolite abundances

LC/MS-based metabolomics were performed and analyzed as previously described using 500 nM isotope-labeled internal standards(*5*). Briefly, a 80% methanol extraction buffer containing 500 nM isotope-labeled internal standards was used for whole cell metabolite extractions. Samples were briefly vortexed and dried by vacuum centrifugation. Samples were stored at -80°C until analysis. Upon analysis, samples were resuspended in 100 μL of LC/MS grade water and the insoluble fraction was cleared by centrifugation at 15,000 rpm in a microcentrifuge. The supernatant was then analyzed as previously described by LC/MS (*5*).

Electron Microscopy

In brief, NUFIP1-null HEK-293T cells expressing wild-type NUFIP1 or the W40A mutant were grown to 60% confluency in 10 cm plates and incubated with Torin1 (250 nM) and Concanamycin A (500 nM) for 4 hr. The cells were fixed with 2% gluteraldehyde + 3% paraformaldehyde + 5% sucrose in Sodium Cacodylate (pH 7.4) followed by osmication and uranyl acetate staining, ethanol dehydration, and the samples were then embedded in Epoxy resin. Sections were cut on formvar-coated grids, stained once more with uranyl acetate and lead citrate and imaged with a FEI Tecnai Spirit Transmission Electron Microscope. Autophagosomal sections in which ribosomes could be clearly enumerated were analzyed to determine the number of ribosomes per autophagosome. The number of ribosomes inside autophagosomes were determined by counting and normalized to the area of the autophagosomal section (# of ribosomes/autophagosomal area) as measured using ImageJ.