Supporting Information

The kinase PERK and the transcription factor ATF4 play distinct and essential roles in autophagy resulting from tunicamycin-induced ER stress

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Running title: *PERK and ATF4 in ER stress-induced autophagy*

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List of Material included:

Supporting information included as separate files:

Table S3-S8. These tables contain the differential gene expression data that was used to perform the analysis in Fig 6C-D.

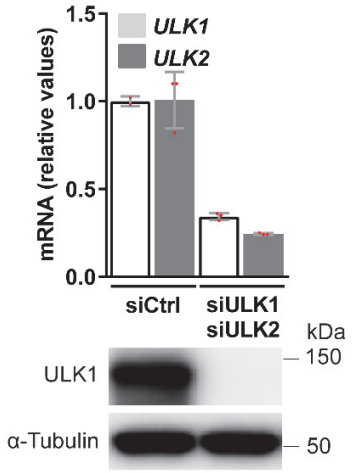

Figure S1. ULK1/2 knockdown confirmation (related to Figure 2B)

siULK1/siULK2-mediated knockdown of *ULK1* and *ULK2* mRNA (top) and ULK1 protein (bottom) levels in LNCaP cells transfected for 48 h, and treated with DMSO for 24 h, as indicated, was analyzed by real-time RT-PCR (mean±SD of triplicate measurements) and immunoblotting, respectively. Red dots represent individual data points.

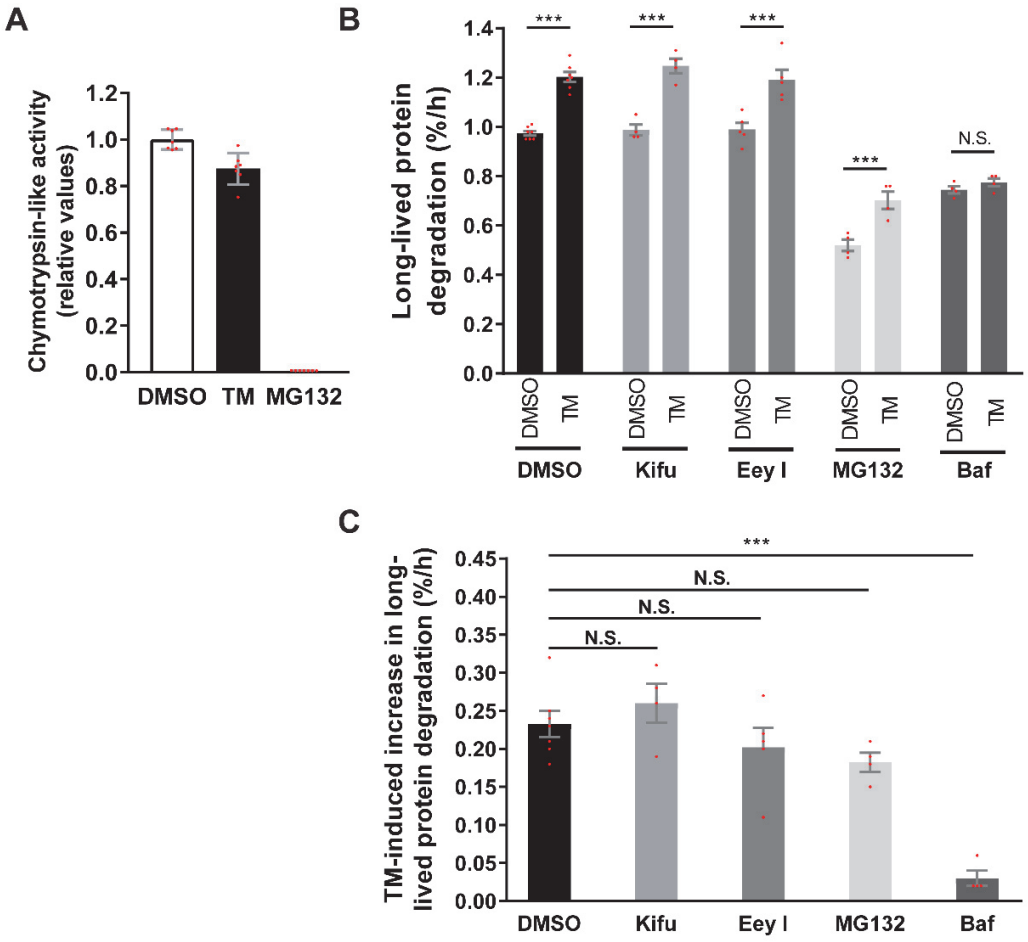

Figure S2. TM does not increase proteasomal (chymotrypsin-like) activity, and TM-induced degradation of long-lived proteins is unaffected by proteasomal- and ERAD-inhibitors (related to Figure 2B)

A. LNCaP cells were treated with DMSO (0.05%) or TM (2.5 µg/mL) for 24 h, or with MG132 (5 µM) for 3 h. Subsequently, proteasomal degradation (chymotrypsin-like activity) was measured using the Proteasome-Glo Chymotrypsin-like kit from Promega (WI, USA). One representative out of two independent experiments is shown (mean ± SD of 7 biological replicates). Red dots represent individual data points.

B. LNCaP cells were treated with DMSO (0.1%) or TM (2.5 µg/ml) for 24 h, with the additional presence of DMSO (0.1%), Kifunensine (Kifu; 10 µg/ml), Eeyarestatin I (Eey I; 8 µM), MG132 (5 µM), or Bafilomycin A1 (Baf; 100 nM) for the last 3 h of the treatment only. Long-lived protein degradation was measured in the 21-24 h time period (mean±SEM, n≥4). Red dots represent individual data points. Statistical significance was evaluated using regular 1-way ANOVA. ***P < 0.001. N.S., not significant.

C. From the experiments shown in B, the TM-induced increase in LLPD was calculated for each of the experimental treatment conditions (mean±SEM, n≥4). Red dots represent individual data points. Statistical significance was evaluated using regular 1-way ANOVA. ***P < 0.001. N.S., not significant.

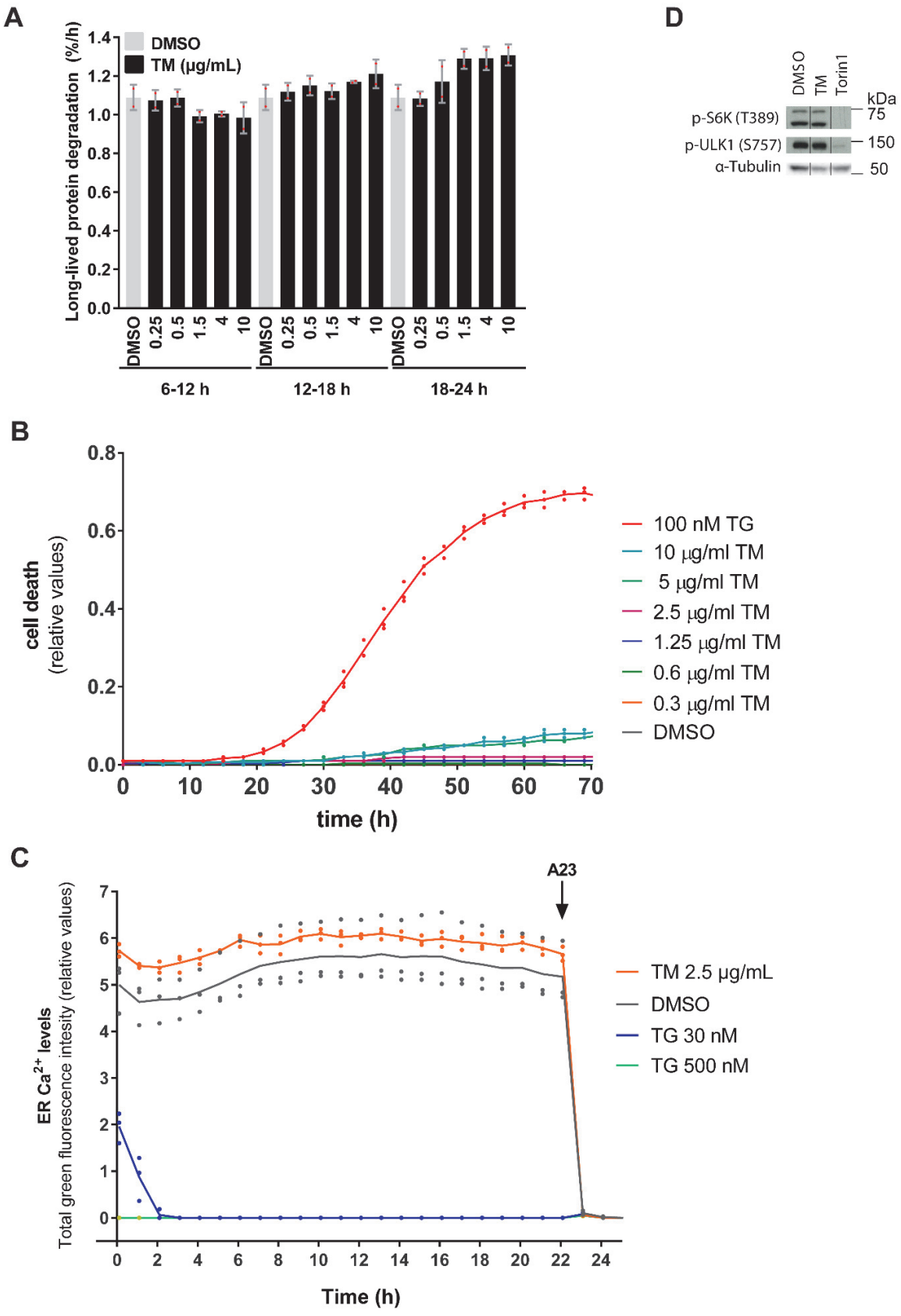
A
Long-lived protein degradation (%/h)
DMSO
TM (μg/mL)
DMSO 0.25 0.5 1.5 4 10 DMSO 0.25 0.5 1.5 4 10 DMSO 0.25 0.5 1.5 4 10
6-12 h
12-18 h
18-24 h
D
DMSO
TM
Torin1
kDa
p-S6K (T389)
p-ULK1 (S757)
α-Tubulin
75
150
50
B
cell death
(relative values)
time (h)
100 nM TG
10 μg/ml TM
5 μg/ml TM
2.5 μg/ml TM
1.25 μg/ml TM
0.6 μg/ml TM
0.3 μg/ml TM
DMSO
C
A23
ER Ca2+ levels
Total green fluorescence intesity (relative values)
Time (h)
TM 2.5 μg/mL
DMSO
TG 30 nM
TG 500 nM

Figure S3. TM increases the degradation of long-lived proteins without inducing cell death or altering ER Ca2+ levels and mTORC1 activity (related to Figure 2)

A. Long-lived protein degradation was measured in LNCaP cells treated with DMSO (0.2%), or the specified concentrations of TM for the indicated time periods (mean±absolute deviation of duplicate biological replicates). Red dots represent individual data points.

B. LNCaP cells were treated with DMSO, TM, or TG at the indicated concentrations, and monitored by automated phase-contrast and fluorescence microscopy (Incucyte Zoom from Essen Bioscience). Propidium iodide (PI; 2.5 µg/mL) was included in the medium to detect dead/dying cells. Cell death was plotted as PI-stained cell confluence relative to total confluence. One representative out of two independent experiments is shown (mean of 3 biological replicates). The differently colored dots represent individual data points for each experimental treatment condition. 100 nM TG was included as a positive control that efficiently induces cell death.

C. ER Ca^{2+} levels were monitored using the ER-targeted Ca^{2+} indicator G-CEPIA1er. G-CEPIA1er-expressing LNCaP cells were treated with DMSO, TG, or TM at the indicated concentrations. The calcium ionophore A23 (5 µM) was added to the cells after 23 h to demonstrate that calcium levels could be depleted also in DMSO- and TM-treated cells. The fluorescent signal obtained after A23 treatment defines the baseline. One representative out of two independent experiments is shown (mean of 3 biological replicates). The differently colored dots represent individual data points for each experimental treatment condition. TG (30 and 500 nM) was included as a positive control that efficiently depletes ER Ca^{2+}.

D. LNCaP cells were treated with DMSO (0.05%) or TM (2.5 µg/mL) for 24 h, or Torin1 (50 nM) for 3 h, and whole-cell lysates were immunoblotted for the indicated proteins. One representative out of two independent experiments is shown. The blots were spliced at the locations indicated by the black lines.

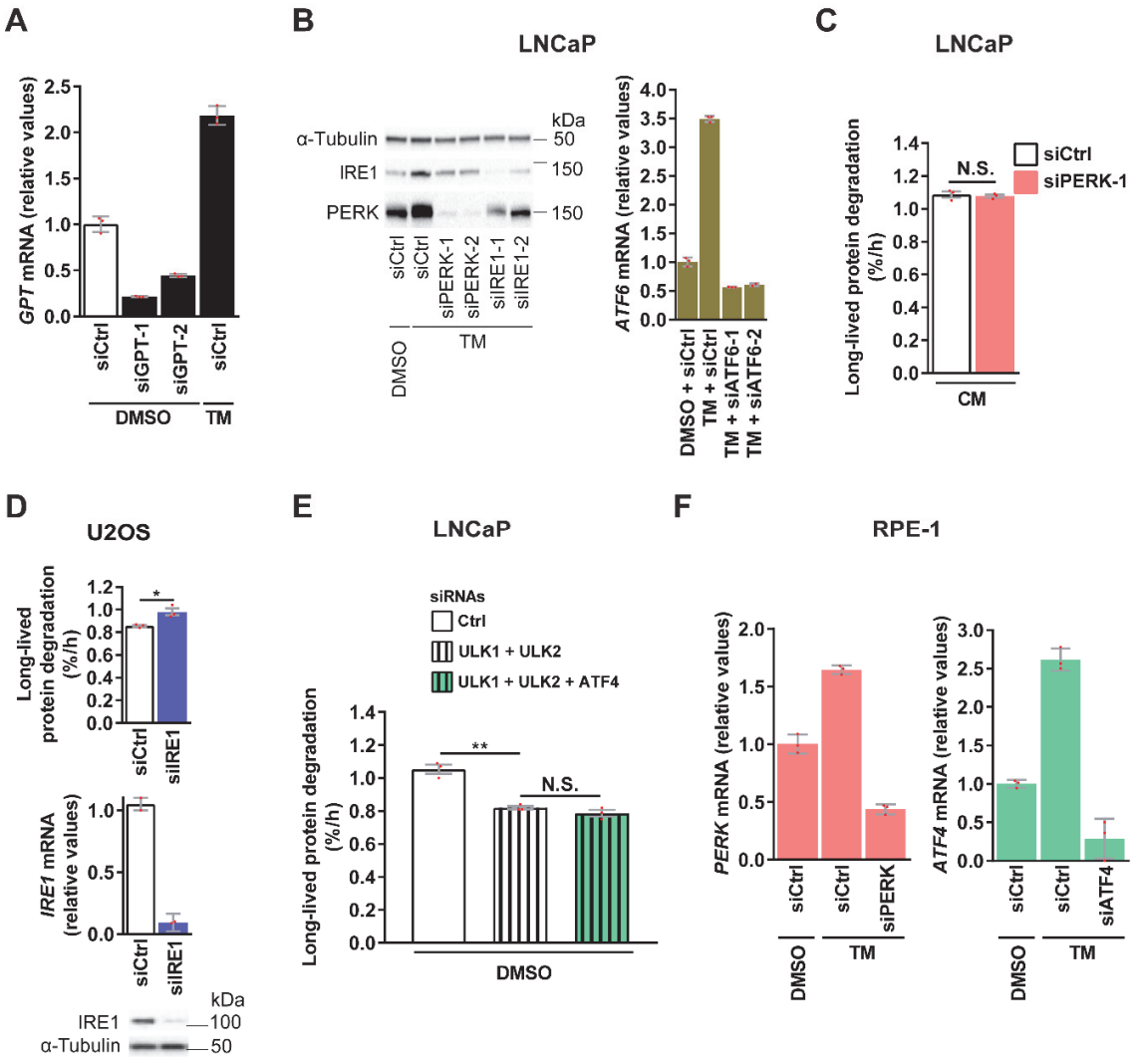

Figure S4. Knockdown confirmations, and effects of IRE1- or ATF4 depletion on basal autophagy (related to Figures 3 and 5)

A. In parallel with the experiments shown in Fig 3, siRNA-transfected LNCaP cells were harvested for RNA isolation and real-time RT-PCR-mediated assessment of *GPT* mRNA levels, confirming GPT knockdown. Interestingly, *GPT* mRNA levels were increased by TM. One representative out of two independent experiments is shown (mean±SD of three measurements). Red dots represent individual data points.

B. LNCaP cells transfected with the indicated siRNAs were treated with DMSO (0.05%) or TM (2.5 µg/mL) for 24 h, as indicated, and harvested for western blotting of the specified proteins (left panel, representative of two independent experiments) or real-time RT-PCR of *ATF6* mRNA (left panel, mean±SD of three measurements). Red dots represent individual data points. Related to Figure 5B.

C. LNCaP cells were transfected with a non-targeting siRNA (siCtrl) or siPERK for 48 h, followed by measurement of long-lived protein degradation in a 6 h period (mean±SEM, n=3, paired samples t-test, N.S. not significant). Red dots represent individual data points.

D. Upper panel: U2OS cells were transfected with a non-targeting siRNA (siCtrl) or siIRE1 for 48 h, followed by measurement of long-lived protein degradation in a 6 h period (mean±SEM, n=3, paired samples t-test, *P< 0.05). In parallel, cells were harvested for RT-PCR (middle panel) and immunoblotting (bottom panel) to assess the knockdown of *IRE1* mRNA (mean±absolute deviation of duplicate measurements) and protein, respectively. Red dots represent individual data points.

E. LNCaP cells were transfected with the indicated siRNAs for 48 h, followed by measurement of long-lived protein degradation in a 6 h period (mean±SEM, n=3, repeated measures 1-way ANOVA, **P< 0.01. N.S. not significant). Red dots represent individual data points.

F. RPE-1 cells were transfected with the indicated siRNAs and treated with DMSO (0.05%) or TM (2.5 μg/mL) for 22 h. Subsequently, cells were harvested for RNA isolation and real-time RT-PCR determination of PERK and ATF4 mRNA levels (mean±SD of triplicate measurements). Red dots represent individual data points. Related to Figure 5F.

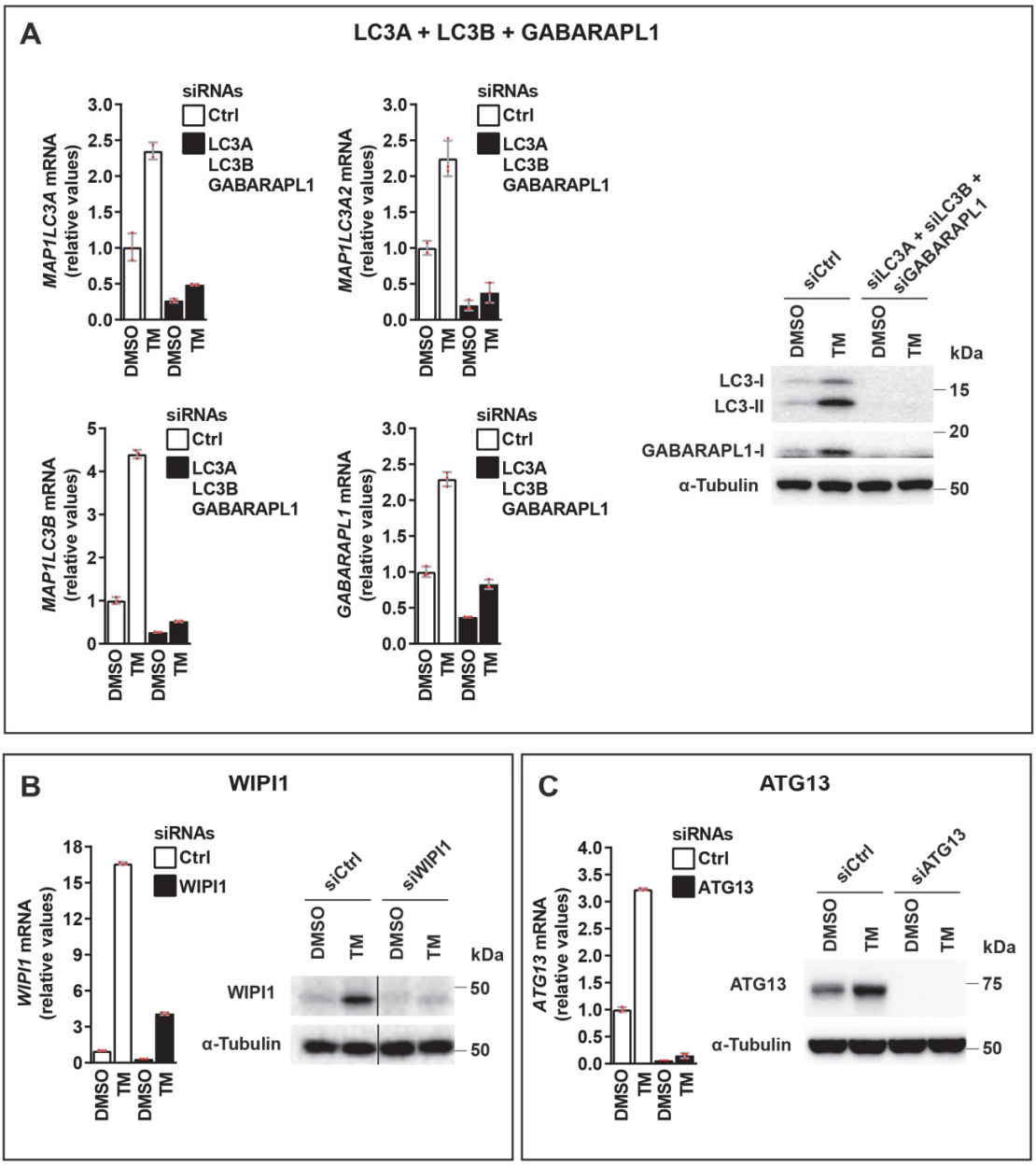

Figure S5. Knockdown confirmations of WIPI1, ATG13, LC3s, and GABARAPL1 (related to Figure 7A-B)

A-C. LNCaP cells were transfected with the indicated siRNAs and treated with DMSO (0.05%) or TM (2.5 μg/mL) for 24 h, as specified. Subsequently, RNA and protein was isolated to determine relative expression of LC3A, LC3B, and GABARAPL1 (A), WIPI1 (B), and ATG13 (C) by real-time RT-PCR (mean±SD of triplicate measurements for RT-PCR) and immunoblotting, respectively. Note that the siLC3A targets both MAP1LC3A and MAP1LC3A2, and that the siLC3B targets both MAP1LC3B and MAP1LC3B2. The expression of the latter is extremely low in LNCaP cells (Fig. 6D and Table S1), and was undetectable by real-time RT-PCR in this as well as in a previous study (1). The LC3 antibody detects both the LC3A and LC3B isoform. Red dots represent individual data points. The blots in (B) were spliced at the locations indicated by the black lines.

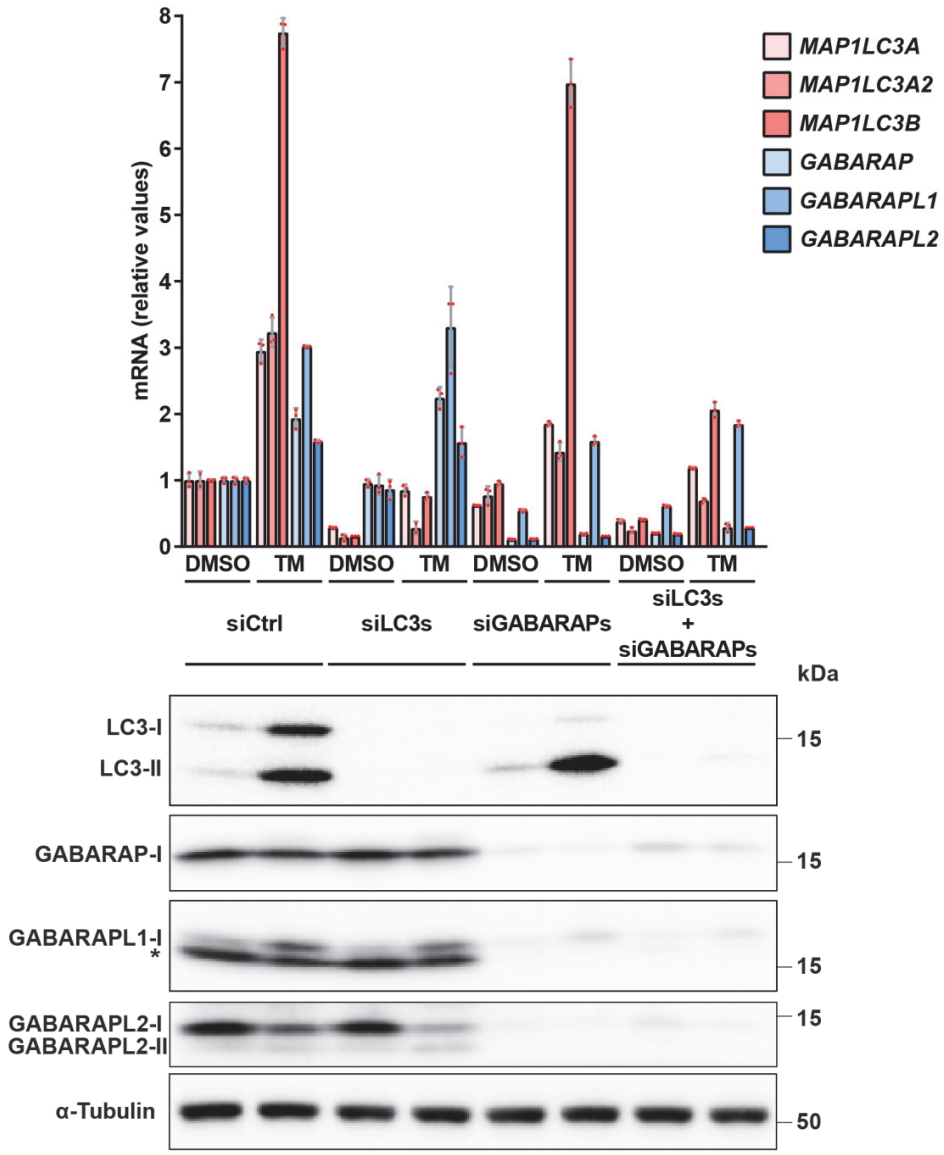

Figure S6. Knockdown confirmations of the LC3s and GABARAPs (related to Figure 7E)

LNCaP cells were transfected with the indicated siRNAs and treated with DMSO (0.05%) or TM (2.5 µg/mL) for 24 h, as specified. Subsequently, RNA and protein was isolated to determine relative expression of the indicated gene transcripts and proteins by real-time RT-PCR (upper panel) and immunoblotting (lower panel), respectively (mean±SD of triplicate measurements for RT-PCR). Note that the siLC3A targets both MAP1LC3A and MAP1LC3A2, and that the siLC3B targets both MAP1LC3B and MAP1LC3B2. The expression of the latter is extremely low in LNCaP cells (Fig 6D and Table S1), and was undetectable by real-time RT-PCR in this as well as in a previous study (1). The LC3 antibody detects both the LC3A and LC3B isoform. The GABARAPL1 antibody recognizes GABARAPL1-I (upper band) and GABARAP-I (lower band, denoted by*) (1). Red dots represent individual data points.

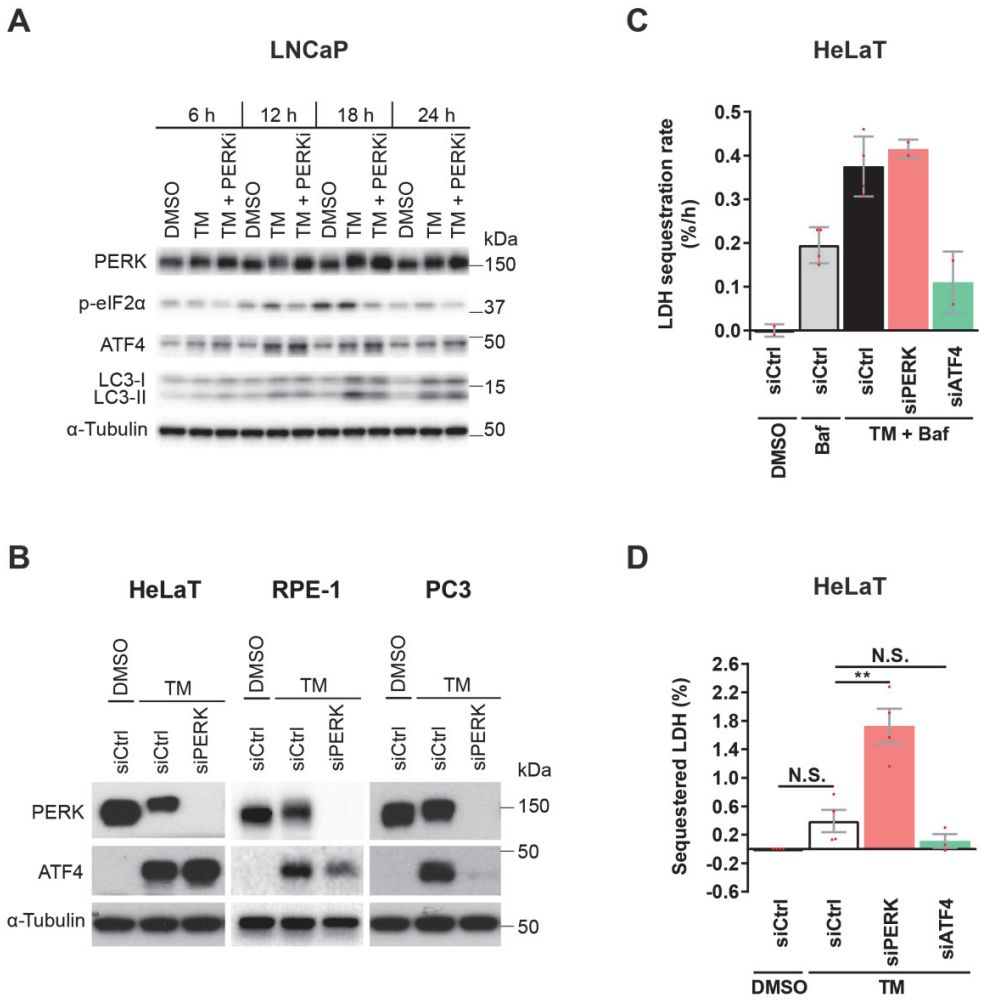

Figure S7. PERK and ATF4 are uncoupled in LNCaP and HeLa cells but not in RPE-1 and PC3 cells (related to Figure 8)

A. LNCaP cells were treated with DMSO (0.05%) or TM (2.5 µg/mL) in the absence or presence of PERKi (100 nM) for the indicated time points, and whole-cell lysates were immunoblotted for the specified proteins. One representative out of two independent experiments is shown.

B. HeLaT, RPE-1, and PC3 cells were transfected with the indicated siRNAs and treated with DMSO (0.05%) or TM (1 µg/mL for HeLaT and PC3, 2.5 µg/mL for RPE-1) for 18 h. Subsequently, whole-cell lysates were immunoblotted for the indicated proteins.

C. HeLaT cells were siRNA transfected, followed by treatment with DMSO (0.01%) or TM (1 µg/mL) for 24 h, as indicated, and with Baf (100 nM) included the last 3 h only. LDH sequestration was determined at 21-24 h (mean±SD, n≥2). Red dots represent individual data points. One representative experiment out of two.

D. HeLaT cells were siRNA transfected, and total LDH sequestration was determined after 24 h treatment with DMSO (0.01%) or TM (1 µg/mL) (mean±SEM, n≥3). Statistical significance was determined using regular 1-way ANOVA. **P < 0.01. N.S., not significant. Red dots represent individual data points.

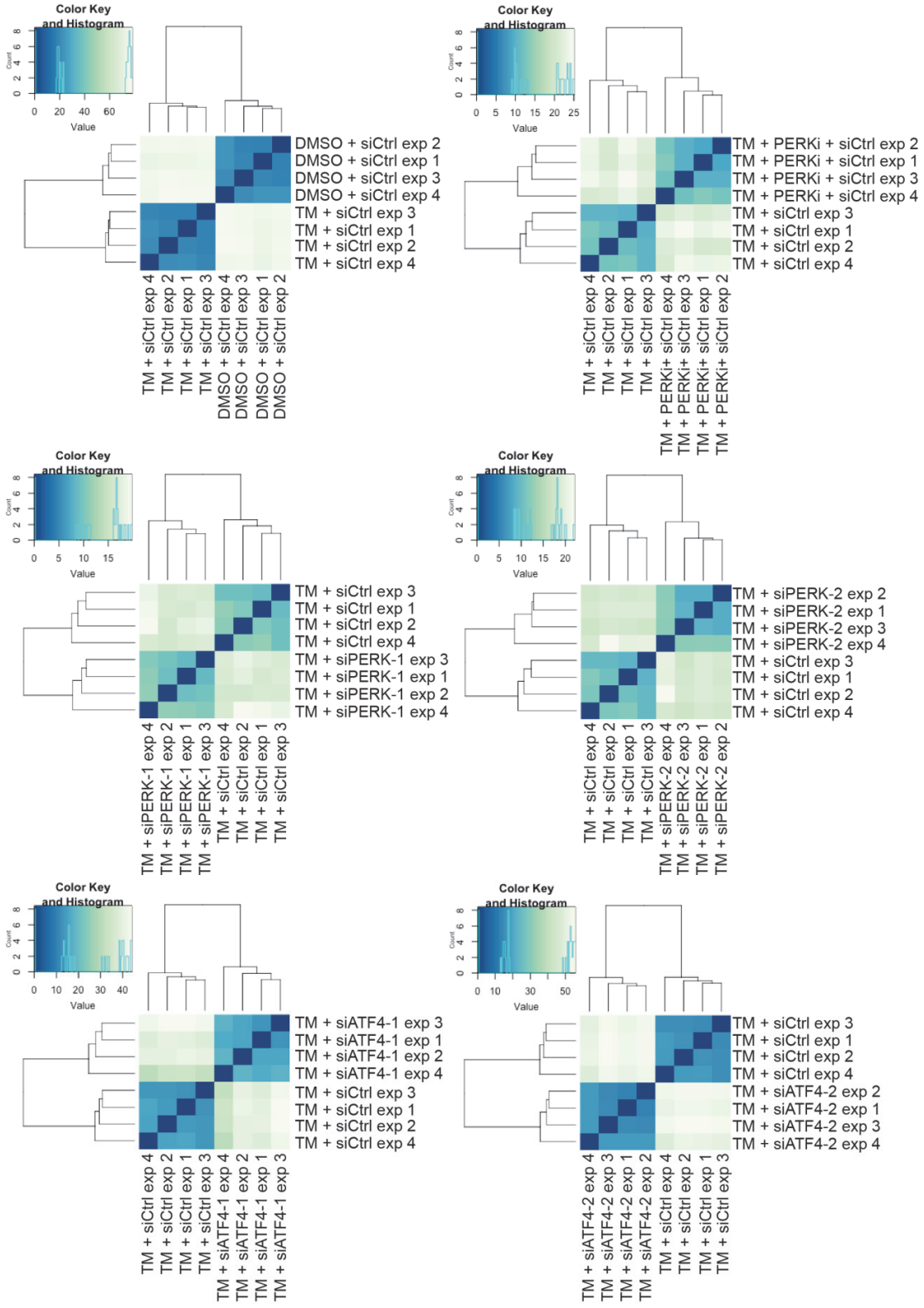
Color Key and Histogram
Count
Value
DMSO + siCtrl exp 2
DMSO + siCtrl exp 1
DMSO + siCtrl exp 3
DMSO + siCtrl exp 4
TM + siCtrl exp 3
TM + siCtrl exp 1
TM + siCtrl exp 2
TM + siCtrl exp 4
TM + PERKi + siCtrl exp 2
TM + PERKi + siCtrl exp 1
TM + PERKi + siCtrl exp 3
TM + PERKi + siCtrl exp 4
TM + siPERK-1 exp 3
TM + siPERK-1 exp 1
TM + siPERK-1 exp 2
TM + siPERK-1 exp 4
TM + siPERK-2 exp 2
TM + siPERK-2 exp 1
TM + siPERK-2 exp 3
TM + siPERK-2 exp 4
TM + siATF4-1 exp 3
TM + siATF4-1 exp 1
TM + siATF4-1 exp 2
TM + siATF4-1 exp 4
TM + siATF4-2 exp 2
TM + siATF4-2 exp 1
TM + siATF4-2 exp 3
TM + siATF4-2 exp 4

Figure S8. Biclustering of experimental conditions in the RNA sequencing experiments

Heatmap view of hierarchical clustering based on sample distances to illustrate similarities and dissimilarities between samples; similar samples cluster together due to similarities in their profiles. The histograms illustrate the frequency of the Euclidean distances between the samples. The lowest Euclidean distances (closely related samples) are shaded dark blue, whilst the highest Euclidean distances (distantly related samples) are shaded a very pale green. The peaks in the histogram indicate where most Euclidean distances lie. The order of samples is obtained by biclustering the expression matrix, using the DESeq2 v1.10.1 Bioconductor package (2).

Gene ID	Average reads count DMSO + siCtrl	Average reads count TM + siCtrl	Log2 FC TM + siCtrl VS DMSO + siCtrl	padj
WIPI1	215	1434	2.731	6.70E-280
MAP1LC3B	573	3335	2.535	0.00E+00
MAP1LC3B2	9	49	2.270	9.52E-14
ATG13	1338	3313	1.304	4.53E-101
MAP1LC3A	70	175	1.298	5.20E-17
GABARAPL1	739	1743	1.233	2.22E-83
ATG4A	392	705	0.839	2.41E-21
ATG101	562	996	0.823	1.78E-29
ATG2B	812	1179	0.535	3.55E-17
RB1CC1	1200	1672	0.477	9.70E-12
ATG16L1	358	459	0.354	4.05E-05
GABARAP	5380	6754	0.327	1.15E-12
GABARAPL2	1292	1602	0.310	8.38E-07
ATG4B	680	822	0.273	7.42E-05
ATG12	589	689	0.224	1.93E-03
ATG5	641	739	0.205	3.73E-03
ATG3	1099	1230	0.163	1.44E-02
BECN1	1138	1274	0.161	1.11E-02
ATG9A	1512	1333	-0.180	1.52E-02
SNX4	1211	1019	-0.249	5.37E-05
ATG16L2	78	52	-0.569	2.89E-03
ATG9B	25	13	-0.876	1.18E-02

Table S1. Autophagy-related genes altered by TM

The effect of TM on the expression of autophagy-related genes was analyzed using the RNA sequencing data set and the HGNC-defined list of human ATGs. Shown are the averaged reads count from the four biological replicates in DMSO+siCtrl and TM+siCtrl, $\log_2$ FC values, and padj values of the autophagy-related genes that were significantly altered by TM (padj< 0.05).

Sample name	Raw reads count	Alignment rate (%)
DMSO + siCtrl Exp 1	20129684	92.9
DMSO + siCtrl Exp 2	18860575	91.7
DMSO + siCtrl Exp 3	18262718	89.9
DMSO + siCtrl Exp 4	20853157	92.7
TM + siCtrl Exp 1	20472971	92.7
TM + siCtrl Exp 2	20791539	92.4
TM + siCtrl Exp 3	16877483	92.4
TM + siCtrl Exp 4	18282646	92.3
TM + PERKi + siCtrl Exp 1	13856862	91.9
TM + PERKi + siCtrl Exp 2	14918859	92.3
TM + PERKi + siCtrl Exp 3	18914702	91.8
TM + PERKi + siCtrl Exp 4	16640156	92.9
TM + siPERK-1 Exp 1	20193824	92.9
TM + siPERK-1 Exp 2	18579050	88.5
TM + siPERK-1 Exp 3	17086764	91.2
TM + siPERK-1 Exp 4	16069867	91.4
TM + siPERK-2 Exp 1	14697366	92.2
TM + siPERK-2 Exp 2	14624785	92.2
TM + siPERK-2 Exp 3	15126148	91.2
TM + siPERK-2 Exp 4	15127610	91.0
TM + siATF4-1 + Exp 1	14286346	93.0
TM + siATF4-1 + Exp 2	16290886	92.4
TM + siATF4-1 + Exp 3	15748217	91.5
TM + siATF4-1 + Exp 4	14638600	92.9
TM + siATF4-2 + Exp 1	12924234	92.6
TM + siATF4-2 + Exp 2	16365921	91.9
TM + siATF4-2 + Exp 3	13091753	89.8
TM + siATF4-2 + Exp 4	14387130	91.1

Table S2. Raw reads count and alignment rates in the RNA sequencing experiments

Overview of RNA seq libraries generated for this study. For each library, number of reads and percentage of reads aligned to human genome (hg19) are shown.

References

1. Szalai, P., Hagen, L. K., Saetre, F., Luhr, M., Sponheim, M., Overbye, A., Mills, I. G., Seglen, P. O., and Engedal, N. (2015) Autophagic bulk sequestration of cytosolic cargo is independent of LC3, but requires GABARAPs. *Experimental cell research* **333**, 21-38
2. Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* **15**, 550