Supplemental material

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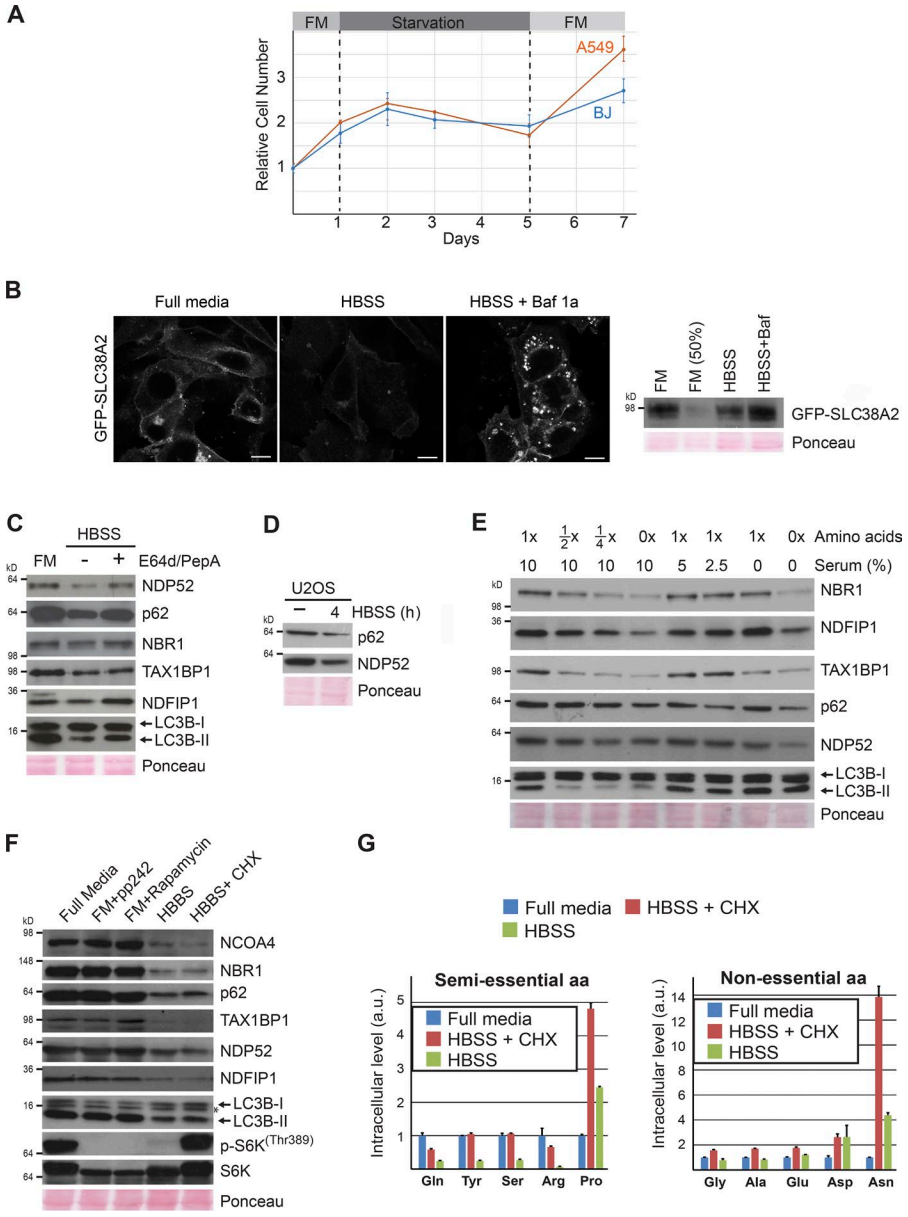

Figure S1. **Amino acid starvation triggers a rapid lysosomal degradation response independently of mTOR. (A)** Growth curves for BJ and A549 cells cultured for 24 h in FM and then starved for 4 d in HBSS before returning to FM for another 2 d. Error bars represent SD of a technical triplicate. One representative experiment of two replicas is shown. **(B)** Fluorescence microscopy and Western blot analysis of GFP-SLC38A2 degradation after 4-h starvation in HBSS. Clones with doxycycline (Dox)-inducible GFP-tagged SLC38A2 were generated in MEF p62 KO background cell lines. Expression was induced for 24 h using 500 ng/ml doxycycline. This experiment was performed two independent times with similar results. **(C)** Western blot analyses comparing expression levels of indicated proteins in A549 cells grown in FM or starved in HBSS for 1 h with/without inhibitors of lysosomal proteases (10 µg/ml E64d and 10 µg/ml Pepstatin A). The experiment was performed two independent times with similar results. **(D)** Western blot analyses comparing expression levels of p62 and NDP52 in U2OS cells grown in FM or starved in HBSS for 4 h. This experiment was performed two independent times with similar results. **(E)** Western blot analyses assessing the effect of 4-h amino acid versus serum starvation in A549 cells. The experiment was performed three independent times with similar results. **(F)** Western blot analyses of expression levels of indicated proteins in BJ cells treated as indicated for 1 h. pp242 (250 nM) and rapamycin (250 nM) were used to inhibit mTOR. Cycloheximide (CHX, 50 µg/ml) was used to block protein synthesis. This experiment was performed three independent times with similar results. **(G)** Quantification of free pools of intracellular amino acids by HPLC-MS/MS in BJ cells starved for 1 h with/without CHX (50 µg/ml). This experiment was performed three independent times with similar results. Error bars represent SD of a technical triplicate.

A

BJ cells

Protein (gene name)	Stvd: Fed	Stvd+Baf: Stvd	Stvd:Fed in A549
NCOA4	-2,84	NaN	-2,99
CALCOCO2	-2,36	NaN	-2,04
TAX1BP1	-2,36	NaN	-1,98
SQSTM1	-2,30	1,23	-2,24
NBR1	-2,05	1,80	-1,30
TMEM200A	-2,00	NaN	NaN
AXL	-1,98	NaN	-0,31
CALCOCO1	-1,94	NaN	NaN
APLP2	-1,79	0,35	-1,00
TNIP1	-1,78	NaN	NaN
TMEM59	-1,69	1,34	NaN
GABARAPL2	-1,67	1,12	NaN
CRIM1	-1,67	NaN	NaN
SLC38A2	-1,58	1,37	-0,94
IL6ST	-1,58	NaN	NaN
MXRA8	-1,58	1,43	NaN
TNFRSF10B	-1,53	1,00	NaN
LRP10	-1,49	1,47	NaN
RNF149	-1,47	1,36	NaN
TNFRSF10D	-1,33	NaN	NaN
TPRG1L	-1,30	0,54	NaN
GJA1	-1,23	0,72	NaN
DCI;ECI1	-1,22	NaN	NaN
SERINC1	-1,12	1,20	-1,38
CASP3	-1,11	NaN	NaN
PIP4K2B	-1,08	NaN	NaN
PCDHGB7	-1,07	1,30	NaN
PSAP	-1,05	0,57	-1,58
SLC2A1	-1,02	NaN	-0,12
CYFIP2	-1,01	NaN	0,15
SLC7A1	-1,00	NaN	-0,57
JAK1	-1,00	NaN	-0,41
TM9SF4	-0,98	NaN	-0,10
TGFBR2	-0,96	NaN	-0,65
C1GALT1C1	-0,96	0,61	NaN
MAP1LC3B	-0,95	NaN	-1,27
DCBLD2	-0,93	NaN	-0,32
VPS53	-0,92	NaN	-0,07
FTH1	-0,89	0,76	-2,80
CDH11	-0,88	-0,46	-0,02
NTMT1	-0,88	0,03	NaN
SLC38A1	-0,83	0,63	-0,41
ATG9A	-0,80	NaN	0,15
CDK11A/B	-0,80	NaN	NaN
APIP	-0,79	NaN	NaN
ASL	-0,79	NaN	NaN
ZNF598	-0,78	NaN	NaN
PKN1	-0,77	-0,37	-0,31
TMF1	-0,76	NaN	-0,32
ATP8B1	-0,73	NaN	NaN

A549 cells

Protein (gene name)	Stvd: Fed	Stvd:Fed in BJ
TMPRSS11D	-8,81	NaN
GINM1	-5,46	NaN
NCOA4	-2,99	-2,84
EXOSC6	-2,95	NaN
UBE2D2/3	-2,82	NaN
FTH1	-2,80	-0,89
NDFIP1	-2,73	NaN
HSPA6	-2,71	NaN
C3	-2,57	NaN
DDI2	-2,50	NaN
FTL	-2,45	-0,60
SQSTM1	-2,24	-2,30
CALCOCO2	-2,04	-2,36
IVNS1ABP	-2,02	NaN
TAX1BP1	-1,98	-2,36
TCEB2	-1,92	-0,18
ABLIM1	-1,88	NaN
RBP4	-1,84	4,53
MED20	-1,84	NaN
CD99L2	-1,77	NaN
CHMP1A	-1,69	NaN
LAPTM4A	-1,66	NaN
PSAP	-1,58	-1,05
C1orf85	-1,51	NaN
LARS2	-1,42	NaN
CEP164	-1,40	NaN
SERINC1	-1,38	-1,12
SLC39A14	-1,36	-0,20
ZNF622	-1,34	NaN
CTSD	-1,32	-0,18
GPX8	-1,32	NaN
NBR1	-1,30	-2,05
MAP1LC3B	-1,27	-0,95
HDDC2	-1,26	NaN
HS2ST1	-1,21	NaN
CHM;CHML	-1,21	NaN
SCD	-1,18	-0,23
LOXL2	-1,14	-0,21
VPS28	-1,12	NaN
SFSWAP	-1,10	NaN
TRIM32	-1,07	-0,64
CENPE	-1,07	NaN
TRUB1	-1,06	NaN
RHOF	-1,04	NaN
HMGCS1	-1,03	-0,66
MPHOSPH8	-1,03	NaN
CCDC37	-1,02	NaN
TRAFD1	-1,01	NaN
GGA3	-1,01	NaN
APLP2	-1,00	-1,79

Figure S2. **The top 50 most degraded proteins in BJ and A459 cells upon 4-h amino acid starvation based on SILAC experiments.** BJ cells (left) and A549 cells (right). For BJ cells, data from the starvation plus bafilomycin A1 treatment are included. NaN, not any number (below the detection range); Stvd, starved.

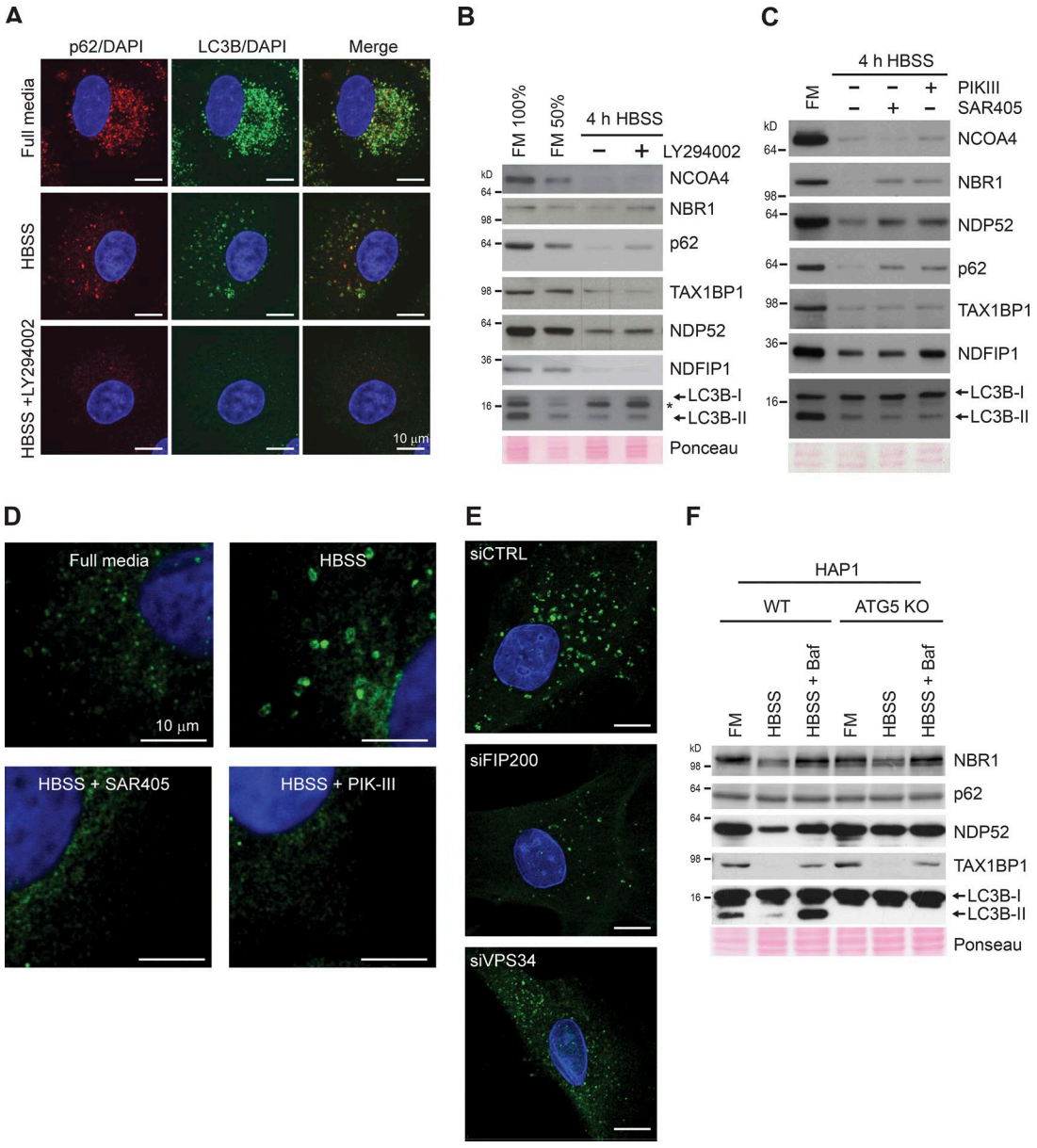

Figure S3. **Macroautophagy does not play a major role in the immediate autophagic response to amino acid starvation. (A)** Representative images showing p62 and LC3B in A549 cells stained by immunofluorescence after 1-h starvation in the presence/absence of the PI3K-inhibitor LY294002 (25 μM). **(B)** Western blot analyses of expression levels of indicated proteins in A549 cells grown in FM or starved for 4 h in the presence or absence of the PI3K-inhibitor LY294002 (25 μM). **(C)** Western blot analyses of expression levels of indicated proteins in BJ cells grown in FM or starved for 4 h in the presence or absence of the PI3K-inhibitors SAR405 (5 μM) or PIK-III (2.5 μM). **(D)** Representative images showing LC3B in BJ cells stained by immunofluorescence after 1-h starvation in the presence/absence of the PI3K-inhibitors SAR405 (5 μM) or PIK-III (2.5 μM). **(E)** Representative immunofluorescence images of the cellular distribution of endogenous LC3B in BJ cells treated with indicated siRNA and then starved for 1 h in HBSS. These analyses were done in parallel to the Western analysis shown in Fig. 5 D. **(F)** Western blot of expression levels of NBR1, p62, and TAX1BP1 in WT HAP1 and ATG5 KO HAP1 cells after 4 h of starvation. Bafilomycin A1 (Baf) was used at 200 nM to inhibit lysosomal protein degradation.

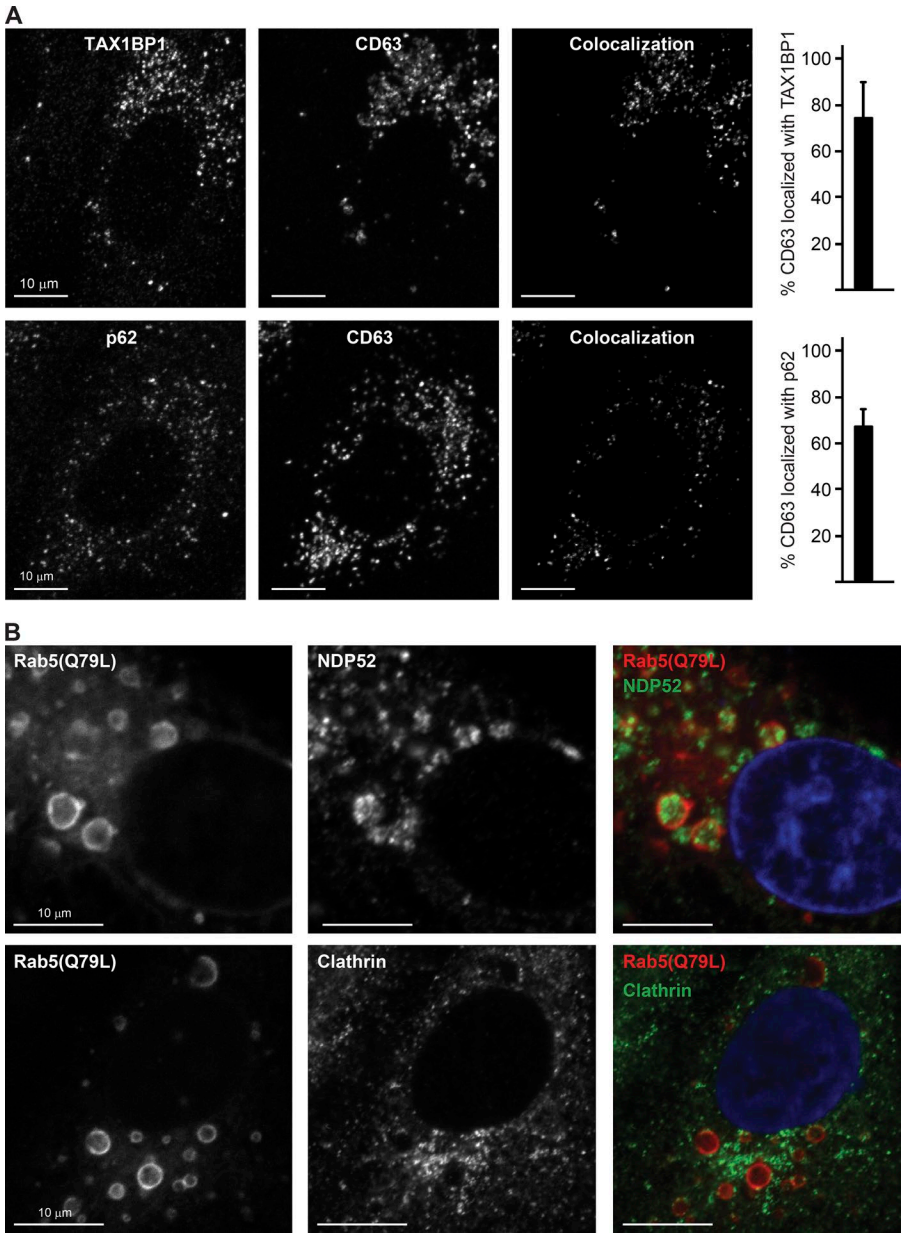

Figure S4. **Substrates of the immediate autophagic response to starvation are associated with the endosomal network. (A)** Left: Representative immunofluorescence microscopy images of endogenous CD63, p62, and TAX1BP1 in A549 cells. Colocalization of TAX1BP1/p62 with CD63 was determined and visualized using Volocity. Right: Manders correlation coefficients showing percentage of CD63 signal colocalizing with TAX1BP1 and p62, respectively. Error bars represent SD. **(B)** Representative immunofluorescence images of the cellular distribution of NDP52 and clathrin heavy chain in A549 cells transfected with mCherry-RAB5(Q79L).

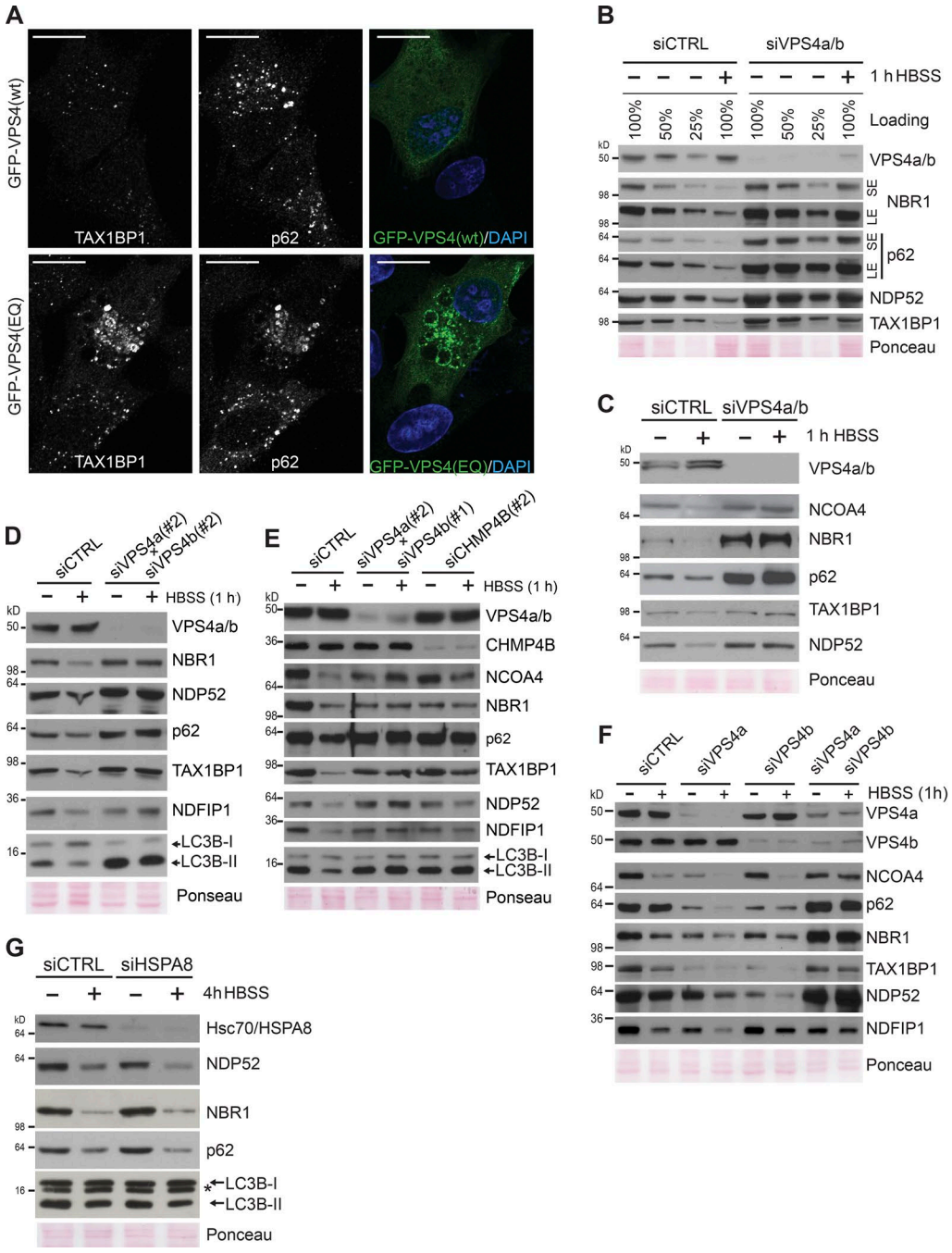

Figure S5. **The immediate autophagic response to amino acid starvation depends on VPS4 but not HSPA8. (A)** BJ cells were transfected with either WT GFP-VPS4 or ATPase-defective GFP-VPS4(EQ). After 48 h, cells were fixed and stained for p62 and TAX1BP1. Bars, 20 µm. The experiment was performed two independent times with similar results. **(B)** Western blot analyses assessing the immediate autophagic response to 1-h starvation in VPS4a/b siRNA-depleted BJ cells. LE, long exposure; SE, short exposure. **(C)** Western blot analyses assessing the immediate autophagic response to 1-h starvation in VPS4a/b-depleted A549 cells. In both A and B, cells were starved 72 h after transfection. **(D)** Western blot analyses assessing the immediate autophagic response to 1-h starvation in BJ cells depleted of VPS4 using alternative siRNAs targeting VPS4a and VPS4b. **(E)** Western blot analyses assessing the immediate autophagic response to 1-h starvation in BJ cells depleted of CHMP4b using alternative siRNAs. **(F)** Western blot analyses assessing the immediate autophagic response to 1-h starvation in BJ cells depleted of VPS4a, VPS4b, and both VPS4a and VPS4b. **(G)** Western blot analysis of the response to 4-h starvation in HSPA8-depleted BJ cells. Starvation was induced 48 h after siRNA transfection. This experiment was performed three independent times with similar results.

Data S1 is a separate Excel file showing the relative quantification of proteins in BJ cells grown in full media versus starved for 4 h.

Data S2 is a separate Excel file showing the relative quantification of proteins in A549 cells grown in full media versus starved for 4 h.

Data S3 is a separate Excel file showing the relative quantification of proteins in BJ cells starved for 4 h in the absence or presence of bafilomycin A1.