Systematic analysis of ribophagy in human cells reveals bystander flux during selective autophagy

Heeseon An and J. Wade Harper

Ribosomes are abundant cellular machines that are regulated by assembly, supernumerary subunit turnover and nascent chain quality control mechanisms. Moreover, nitrogen starvation in yeast has been reported to promote selective ribosome delivery to the vacuole in an autophagy conjugation system dependent manner, a process called ‘ribophagy’. However, whether ribophagy in mammals is selective or regulated is unclear. Using Ribo–Keima flux reporters, we find that starvation or mTOR inhibition promotes VPS34-dependent ribophagic flux, which, unlike yeast, is largely independent of ATG8 conjugation and occurs concomitantly with other cytosolic protein autophagic flux reporters. Ribophagic flux was not induced upon inhibition of translational elongation or nascent chain uncoupling, but was induced in a comparatively selective manner under proteotoxic stress induced by arsenite or chromosome mis-segregation, dependent upon VPS34 and ATG8 conjugation. Unexpectedly, agents typically used to induce selective autophagy also promoted increased ribosome and cytosolic protein reporter flux, suggesting significant bulk or ‘bystander’ autophagy during what is often considered selective autophagy. These results emphasize the importance of monitoring non-specific cargo flux when assessing selective autophagy pathways.

Substantial cellular resources are used to produce the 10⁶ to 10⁷ ribosomes present in a typical mammalian cell (~10% of protein mass), raising the question of to what extent ribosome recycling into building blocks is regulated. Previous studies in Saccharomyces cerevisiae revealed approximately threefold faster delivery of ribosomes to the vacuole when compared with other cytosolic proteins under conditions of nitrogen starvation. Ribophagic flux in yeast required conjugation of the Atg8p Ub-like protein to phosphatidylethanolamine via Atg7p and the Atg1p–Atg17p kinase that promotes phagophore formation. In mammals, starvation-induced autophagy requires the VPS34-BECN1 system dependent manner, a process called ‘ribophagy’. To address these questions, we established a Ribo–Keima system in which chromosomal RPS3 (40S protein) or RPL28 (60S protein) in HCT116 and HEK293 cells was C-terminally tagged with Keima by gene editing in the heterozygous or homozygous form (Fig. 1b–d and Supplementary Fig. 1a). Keima itself is also stable to lysosomal proteases in mammals, and the appearance of a processed Keima protein therefore reveals lysosomal trafficking. Keima proteins formed ribosomes and polysomes (Fig. 1e,f and Supplementary Fig. 1b) and efficiently incorporated puromycin into nascent chains, indicating little effect on global translation (Supplementary Fig. 1c). Excess ribosomal subunits are degraded via the proteasome. Ribo–Keima cells treated with the proteasome inhibitor bortezomib (BTZ, 4 h) failed to accumulate free monомерic RPS3–Keima or RPL28–Keima protein (Fig. 1e,f and Supplementary Fig. 1b), indicating stoichiometric assembly into ribosomes. As with DsRed (ref. 18), heat-induced hydrolysis of the N-acyl imine in Keima during denaturation produced a ~9 kDa product (~20%) of Keima puncta were coincident with the eGFP–LC3B-positive puncta observed on addition of Tor1 (24 h) (Fig. 2h), indicating trafficking through the autophagosome. Control experiments in which the Keima signal was monitored by confocal microscopy at various times after washout of Tor1 (24 h treatment) revealed that Keima puncta are maintained for at least 8 h, and the number of puncta is not altered by treatment with lysosomal hydrolase inhibitors, indicating that Keima is very stable within...
the lysosome, as previously reported15 (Supplemental Fig. 1h,i). An estimate of ~10% of ribosomes trafficking to lysosomes over the 24 h period in Tor1-treated cells was obtained by comparing the Keima fluorescence of cells at pH 7.3 and 4.5 with that of Tor1-treated cells in the presence of BafA (see Methods). By contrast, <1% of ribosomes trafficked into lysosomes in complete growth media (based on flow cytometry; see Methods), and this level of ribophagy is generally undetectable by immunoblotting for processed Keima in Ribo–Keima reporter cells (Fig. 2c), indicating low basal ribophagic flux.

Ribophagy in yeast following nitrogen deprivation requires Atg8p conjugation, the Ubp3p deubiquitylating enzyme and...
mTOR inhibition promotes ribophagy flux in a VPS34-dependent manner.

**Fig. 2** | Nature Cell Biology, Vol 20 | February 2018 | 135–143 | www.nature.com/naturecellbiology

**a**. Ribo–Keima reporter cells were treated with BafA (50 nM, 1 h), Tor1 (150 nM, 24 h, Tor1), or a combination of the two, then analysed by flow cytometry. Frequency distributions of 561/488 nm ex. ratios are shown (n = 10,000 cells per condition).

**b**. Histograms of mean values of the biological triplicate experiments for 561/488 nm ex. ratios (from *a*). Error bars represent standard error of the mean (s.e.m.). ***P < 0.0001, **P < 0.01, *P < 0.05, two-way ANOVA.

**c**. Ribo–Keima reporter cell lines treated with Tor1 (150 nM, 24 h), SAR405 (an inhibitor of VPS34, 1 µM, 24 h), or a combination of the two were immunoblotted for Keima and LC3B. (Asterisk indicates Keima with different folding status, see Supplementary Fig. 1d.)

**d**. Confocal images of live HEK293 cells expressing RPS3–Keima after Tor1 (150 nM, 24 h) or Tor1 (150 nM, 24 h)/SAR405 (1 µM, 24 h) co-treatment. Scale bars, 20 µm.

**e**. Unbiased quantitation of the live-cell images in *d*, the numbers of red Keima puncta per cell are shown. Data are shown as mean ± s.e.m. (n = 52, 60 and 60 cells from three independent experiments).

**f**. HEK293 RPS3–Keima cells stably expressing LAMP1-eGFP were incubated in the presence or absence of Tor1 for 24 h before live-cell imaging. Scale bars, 20 µm.

**g**. HEK293 RPS3–Keima cells treated as in *f* were stained with LysoTracker Green before live-cell fluorescence microscopy. Scale bars, 20 µm.

**h**. HEK293 RPS3–Keima cells stably expressing eGFP–LC3 were treated as in *f*, then subjected to live-cell fluorescence microscopy. Scale bars, 20 µm. For *f–h* bottom left, co-occurrence (%) of red Keima puncta with LAMP1-eGFP (n = 1,800 puncta), LysoTracker Green (n = 2,000 puncta) and eGFP–LC3 (n = 110 puncta) were calculated then plotted as black bars. Random co-occurrence (%) is shown as white bars (see Methods). For *f–h* bottom right, fluorescence line scans along the white lines in the merged insets. Data are presented as mean ± s.e.m. ***P < 0.0001, **P < 0.001, *P < 0.01, two-way ANOVA. Statistical source data for *b* and *f–h* are provided in Supplementary Table 2.

All experiments were performed three times with similar results. Unprocessed original scans of blots are provided in Supplementary Fig. 6.
Cdc48p ATPase\(^\text{a}\). Surprisingly, ATG5 deletion in HEK293 Ribo–Keima cells had no effect on ribophagic flux in response to Tor1 or starvation (Fig. 3a–g and Supplementary Fig. 2a,c,e), while, as expected based on VPS34 inhibition, deletion of the VPS34 regulatory subunit BECN1 (ref. \text{a}) greatly reduced ribophagic flux measured by flow cytometry, confocal imaging or immunoblotting for processed Keima (Fig. 3a–d and Supplemental Fig. 2a–d). Moreover, ribophagic flux in HEK293 RPS3–Keima;ATG5\(^{-/}\) cells induced by Tor1 (24 h) was reversed by treatment with SAR405 (Fig. 3c,d and Supplementary Fig. 2c), indicating that ATG5-independent flux nevertheless requires VPS34 (refs \text{a,}20). HEK293 RPS3–Keima;ATG5\(^{-/}\) cells also generated lysosomally processed Keima at rates similar to those seen in ATG5-expressing cells (Supplementary Fig. 2c). Ribophagic flux in HEK293 RPS3–Keima cells was initially detectable 8 h post-starvation and increased slightly at 16 h (Fig. 3e–g). However, no discernable kinetic differences in Keima puncta per cell were found to depend upon ATG5 status (Fig. 3e–g).

In HCT116 RPL28–Keima cells, starvation promoted a time-dependent increase in Keima puncta per cell, which was 24–44% lower in parallel ATG5\(^{-/}\) cells (Fig. 3h and Supplementary Fig. 2f–i). The reduced autophagic flux was rescued upon stable expression of ATG5 in ATG5\(^{-/}\) cells, as found by immunoblotting for lysosomally processed Keima (Supplementary Fig. 2i). Additional evidence that ribophagy in HEK293 cells upon mTOR inhibition is independent of the conjugation system includes (1) the presence of ribosomes in autophagosomal structures of ATG5\(^{-/}\) cells by electron microscopy (Fig. 3i), (2) co-localization of the Keima signal (561 nm excitation (ex.)) with LAMPI–eGFP-labelled lysosomes (Supplementary Fig. 2j), (3) no effect on Keima puncta number or intensity in ATG5\(^{-/}\) cells treated with lysosomal hydrolyse inhibitors for 14 h during mTOR inhibition, indicating that the Keima turnover rate in the lysosome is similar in ATG5\(^{+/}\) and ATG5\(^{-/}\) cells (Supplementary Fig. 2k), and (4) absence of eGFP–LC3 puncta associated with 561 nm excitation puncta (Supplementary Fig. 2l). Thus, neither Tor1 nor starvation-induced ribophagy absolutely requires ATG8 conjugation, but both require the VPS34 pathway. Similar results were obtained using HEK293 cells expressing levels of free Keima protein comparable to that in cells expressing RPS3–Keima (Supplementary Fig. 3a), consistent with the absence of selectivity in ribophagy flux observed following Tor1 treatment or starvation. Interestingly, Keima–LC3 protein expressed in ATG5\(^{-/}\) cells showed lysosomal flux comparable to ribosomes, further suggesting ATG8 conjugation-independent bulk autophagy.

We examined the involvement of USP10 (Ubp3p in yeast) and p97/VCP (Cdc48p in yeast) in mammalian ribosome\(^\text{a}\). The p97 inhibitor CB5083 (ref. \text{a}) had no effect on Ribo–Keima processing during mTOR inhibition (Supplementary Fig. 3b), indicating that p97 is not required for this form of ribophagy. The USP10 inhibitor spautin\(^\text{a}\) reduced ribophagy of both 40S and 60S subunits (Supplementary Fig. 3c,d); however, this probably reflects the role of USP10 in maintaining BECN1–VPS34 activity in mammals\(^\text{a}\) rather than being directly involved in ribophagy. Consistent with recent work\(^\text{a}\), we did not observe a proposed role for the trans-Golgi as a source of membranes for ATG8 conjugation-independent autophagy, as brefeldin A had no effect on Tor1-induced ribophagy in Ribo–Keima ATG5\(^{-/}\) cells (Supplementary Fig. 3e). Moreover, we did not observe a correlation of Keima-positive lysosome localization with the Golgi network but did observe association with the endoplasmic reticulum (ER), a source of autophagic membranes\(^\text{a}\) (Supplementary Fig. 3f,g).

Maintaining healthy ribosomes is thought to be critical for cellular homeostasis, and agents that induce ribosomal stress could promote a need for the re-establishment of ribosome pools\(^\text{a}\). We therefore screened translation inhibitors and stress agents for increased ribophagic flux. Addition of the nascent chain terminator puromycin, the elongation inhibitor cycloheximide (CHX) and the p97 inhibitor CB5083 (ref. \text{a}), which blocks extraction of ubiquitylated nascent chains from the ribosome, had no appreciable effect on ribophagic flux (Fig. 4a,b). Similarly, proteasome inhibitor BTZ or ER-stress agent tunicamycin (TM) did not promote ribophagic flux (Fig. 4a,b). By contrast, ribophagic flux similar to or greater than that seen with mTOR inhibition was induced by revesine, an MP51 inhibitor that creates proteome imbalance through chromosome mis-segregation\(^\text{1,25,26}\) and by sodium arsenite (NaAsO\(_2\), AS), which induces stress granules\(^\text{2}^\text{a,}20\) and translational inhibition (Supplementary Fig. 4a). Oxidative stress by H\(_2\)O\(_2\) tended toward an increase in ribophagic flux (P < 0.1) (Fig. 4a,b).

Stress granules are thought to undergo ATG8- and p97-dependent selective autophagy\(^\text{27,28}\). Consistent with previous reports\(^\text{2,}29\), AS induces stress granules marked with eIF4E–eGFP and 40S ribosomal protein RPS3–Keima, but not with RPL28–Keima (Supplementary Fig. 4b,c). However, stress granule formation was dependent on AS concentration (Supplementary Fig. 4d). At 20 μM AS, processed Keima in HEK293 RPS3–Keima cells was detected by ~3 h post AS treatment and was blocked by SAR405 up to 18 h post treatment without obvious stress granule or ribosome aggregate formation (Supplementary Fig. 4e,f). We found that AS-induced ribophagy as measured in HEK293 RPS3–Keima cells was largely dependent upon ATG5 and its ATG12 conjugate, and was reversed by BafA\(_1\) (Fig. 4c–f and Supplementary Fig. 4g). Consistent with flow cytometry (Fig. 4a,b), AS produced more processed Keima than Tor1 treatment, and also led to inhibition of pT389 S6K (Supplementary Fig. 4h), in agreement with reduced mTOR activity\(^\text{a}\). Unexpectedly, however, ribophagic flux was also increased by AS in HEK293 RPL28–Keima cells and was reduced by BafA\(_1\) (Fig. 4c,e), indicating that the 60S subunit also undergoes autophagy. Ribophagic flux for both HEK293 RPS3–Keima and RPL28–Keima was reduced upon addition of CB5083 (Supplementary Fig. 4i). Thus, although both Tor1 and AS inhibit S6K phosphorylation and induce ribophagic flux, AS-induced ribophagy largely requires ATG5 and p97 while Tor1 does not.

Reversine-induced chromosome mis-segregation produces supernumerary protein subunits, protein complex imbalance, proteotoxic stress and activation of autophagy to presumably facilitate removal of misfolded or supernumerary proteins\(^\text{1}\). To examine reversine-induced ribophagic flux, we employed HCT116 Ribo–Keima cells, which have a stable and near diploid karyotype. We observed increased ribophagic flux with both RPS3– and RPL28–Keima upon reversine treatment, but the 60S subunit RPL28–Keima reporter showed approximately twofold higher Keima ratio change responsive to BafA\(_1\) (Fig. 4g). In RPL28–Keima cells, processed Keima was detected as early as 24 h post reversine treatment (Fig. 4h,i). Reversine-dependent ribophagic flux was reversed by BafA\(_1\) and SAR405 (Fig. 4g–i) and was strikingly ATG5-dependent (Fig. 4j–m).

The distinct ATG5 dependence observed between Tor1-induced versus AS or reversine-induced ribophagic flux led us to examine the relative selectivity of ribophagy in comparison to additional Keima flux reporters, including autophagosomes themselves (Keima–LC3), cytosol (LDHB–Keima), cytoskeleton (Keima–ACTB–), proteasome (PSMD12–Keima) or mitochondria (TOMM20–Keima). Stable Keima reporter cells were sorted to achieve comparable levels of expression, though lower than that for the Ribo–Keima reporters (Fig. 5a–d and Supplementary Fig. 5a). To assess relative cargo flux, HEK293 reporter cell lines were treated with either Tor1, AS or SAR405 to block basal autophagy, and extracts were examined by immunoblotting for processed Keima (Fig. 5b) with signals then normalized to Tor1-treated cells (see Methods) (Fig. 5c). AS induces lysosomal delivery of Keima–LC3 to a level comparable to that induced by Tor1, indicating elevated overall autophagy flux. Similarly, non-ribosomal Keima reporter proteins displayed a similar level of increase in autophagic flux after...
Fig. 3 | Ribophagy in response to mTOR inhibition in HEK293 cells is ATG5-independent but BECN1-dependent. a, Frequency distributions of 561/488 nm ex. ratios measured on HEK293 RPS3-Keima cells lacking ATG5 or BECN1 were compared after Torin1 or Torin1/BafA treatment using flow cytometry (n = 10,000 cells per condition). b, Average 561/488 nm ex. ratios calculated from the biological triplicate experiments from a (orange, blue, and grey dotted lines). Data are presented as mean ± s.e.m. ***P < 0.0001, **P < 0.01, two-way ANOVA. c, Confocal images of live HEK293 RPS3-Keima cells lacking ATG5 after Torin1 (150 nM, 24 h) or Torin1 (150 nM, 24 h)/SAR405 (1 μM, 24 h) co-treatment. Scale bars, 20 μm. d, Number of red Keima puncta per cell measured from live-cell images of HEK293 RPS3-Keima WT, ATG5−/− or BECN1−/− cells taken after Torin1 (150 nM, 24 h) or Torin1 (150 nM, 24 h)/SAR405 (1 μM, 24 h) co-treatment. Data are presented as mean ± s.e.m. The total numbers of cells from three biologically independent samples are indicated in the graph. e, f, HEK293 RPS3-Keima cells (with or without ATG5) were incubated with HBSS in the presence of lysosomal hydrolase inhibitors (LHI, E64d and pepstatin, 30 μM each) for the indicated times prior to live-cell imaging. Scale bars, 20 μm. g, h, Unbiased quantification of the live-cell images obtained as shown in e and f. h, Unbiased quantification of red Keima puncta obtained from live HCT116 RPL28-Keima cells (with and without ATG5) treated with HBSS/LHI, as shown in Supplementary Fig. 2g,h. In g and h, total numbers of cells from three biologically independent samples are indicated in the graph. Data are presented as mean ± s.e.m. i, Electron microscopy images of HEK293 RPS3-Keima WT, ATG5−/− and BECN1−/− cells 4.5 h after HBSS treatment in the presence of BafA (50 nM). Red arrow: ribosomes in autophagosomes or autophagolysosomes; yellow arrow: ribosomes bound to ER in cytosol. Scale bars, 500 μm. Data represent two independent experiments. The statistical source data for b is provided in Supplementary Table 2. All experiments were repeated at least three times unless otherwise indicated.
**Fig. 4** | A screen of ribosome stress agents identifies sodium arsenite and reversine as ribophagy inducers. a, HEK293 RPS3–Keima cells were exposed to Tor1, 150 nM, 24 h; reversine, 0.5 μM, 48 h; AS, 10 μM, 24 h; H2O2, 250 μM, 24 h; cycloheximide, 10 μM, 5 h; bortezomib, 250 nM, 5 h; puromycin, 2 μM, 5 h; p97 inhibitor CB5083, 100 nM, 24 h; or tunicamycin, 0.6 μM, 24 h, and the 561/488 ratio was measured (n = 10,000 cells per condition). b, HEK293 RPS3–Keima:WT, ATG5−/−, and BECN1−/− cells treated as in a (DB, diazaborine, 200 μM, 24 h). Mean 561/488 nm ex. ratios are plotted (n = 3 independent experiments, Supplementary Table 2). c, Frequency distributions of 561/488 nm ex. ratios measured in HEK293 RPS3–Keima:ATG5−/−, ATG5−/− cells treated with AS (n = 4200 cells per condition) ± BafA. d, The average 561/488 nm ex. ratios from triplicate experiments as in c. e, HEK293 RPS3–Keima:ATG5−/− cells transduced with a lentivirus expressing either ATG5WT or conjugation defective ATG5K130R mutant were treated with AS (10 μM, 24 h) ± BafA. The average 561/488 ratios of biological triplicate experiments are shown. Mean of two independent experiments is shown for ATG51230 cells. f, Immunoblotting of indicated cells treated with AS (20 μM, 20 h) ± SAR405. g, Average 561/488 nm ex. ratios of the indicated cell lines treated with reversine (0.5 μM, 48 h) ± BafA (50 nM, 1 h) (n = 3 independent experiments). h, Frequency distributions of 561/488 nm ex. ratios for HCT116 RPL28–Keima cells with or without reversine treatment ± BafA (n = 10,000 cells per condition). i, Immunoblot of HCT116 RPL28–Keima cells treated with reversine ± SAR405. j, Imaging of HCT116 RPL28–Keima:ATG5−/−, ATG5−/− cells ± reversine (0.5 μM, 48 h). Scale bars, 20 μm. k, Quantification Keima puncta number per cell of the images in j. Mean ± s.e.m. Total numbers of cells are indicated as n. l, Average 561/488 nm ex. ratios of cells treated with reversine (0.5 μM, 48 h) ± BafA (n = 3 independent experiments). m, Immunoblots of indicated cell lines treated as in l. Mean ± s.e.m. is shown in b, d, e, g and l. ****P < 0.0001, ***P < 0.001, **P < 0.01. In b, one-way ANOVA. In d, e, g and l, two-way ANOVA. All experiments were repeated three times unless otherwise indicated. Statistical source data are provided in Supplementary Table 2. Unprocessed original scans are provided in Supplementary Fig. 6.
Fig. 5 | Quantitative western blot analyses of various Keima reporter cell lines reveal selective capture of ribosomes during AS and revorine treatment and the relative quantity of bystander autophagy during selective autophagy. a, Confocal images of live HEK293 cells expressing seven different Keima reporter proteins show proper intracellular localization. b, Extracts from the indicated cell lines treated with SAR405 (1 μM, 20 h), AS (20 μM, 20 h), or Tor1 (150 nM, 20 h) were subjected to immunoblotting. The representative western blot images were developed using HRP-conjugated secondary antibodies, whereas the relative quantity of bystander autophagy during selective autophagy. Quantitative western blot analyses of various Keima reporter cell lines reveal selective capture of ribosomes during AS and revorine treatment and the relative quantity of bystander autophagy during selective autophagy. a, Confocal images of live HEK293 cells expressing seven different Keima reporter proteins show proper intracellular localization. b, Extracts from the indicated cell lines treated with SAR405 (1 μM, 20 h), AS (20 μM, 20 h), or Tor1 (150 nM, 20 h) were subjected to immunoblotting. The representative western blot images were developed using HRP-conjugated secondary antibodies, whereas the relative quantity of bystander autophagy during selective autophagy. Quantitative western blot analyses of various Keima reporter cell lines reveal selective capture of ribosomes during AS and revorine treatment and the relative quantity of bystander autophagy during selective autophagy. a, Confocal images of live HEK293 cells expressing seven different Keima reporter proteins show proper intracellular localization. b, Extracts from the indicated cell lines treated with SAR405 (1 μM, 20 h), AS (20 μM, 20 h), or Tor1 (150 nM, 20 h) were subjected to immunoblotting. The representative western blot images were developed using HRP-conjugated secondary antibodies, whereas the relative quantity of bystander autophagy during selective autophagy. Quantitative western blot analyses of various Keima reporter cell lines reveal selective capture of ribosomes during AS and revorine treatment and the relative quantity of bystander autophagy during selective autophagy. a, Confocal images of live HEK293 cells expressing seven different Keima reporter proteins show proper intracellular localization.
AS and Tor1 treatment (Fig. 5b,c). Moreover, in contrast to other reporter proteins, ribophagic flux measured with both RP53–Keima and RPL28–Keima was three- to fourfold higher than with Tor1, indicating a level of selectivity for this cargo. The increased selectivity for ribophagic flux with AS reflects a much lower rate of basal flux when compared to the other cargo and a larger induced flux relative to Tor1 treatment (Fig. 5b and Supplementary Fig. 5b,c). Analogous experiments were performed with HCT116 cells in the context of reversine treatment, again revealing selectivity in reversine-dependent turnover of RPL28–Keima when compared with Tor1 treatment, while with the other reporters, the increased flux was again comparable to that seen with Tor1 treatment (Fig. 5c,d,l). Unlike AS, however, reversine did not inhibit S6K phosphorylation (Fig. 5b,d) and did not promote selective turnover of RP53–Keima (Fig. 5f), suggesting distinct flux parameters for large and small ribosome subunits in this setting.

Increased flux for various cytosolic cargo (for example, LDHB) in response to AS treatment, which ostensibly promotes selective targeting of stress granules for autophagy10, led us to ask the extent to which conditions that are thought to promote selective forms of autophagy induce bystander autophagy21,22. The extent to which cytosolic proteins are captured during what would otherwise be considered selective cargo capture has not been examined systematically. Relative to basal conditions, PARKIN-dependent mitophagy via mitochondrial depolarization with antimycin A and oligomycin A (AO) induced autophagic flux for all reporters tested, to an extent similar to that seen with Tor1, with LDHB–Keima, for example, increasing two- to fourfold above basal conditions (Fig. 5g). Similar results were obtained for cells treated with pexophagy-inducing clofibrate (Fig. 5b) and lysophagy-inducing LLOMe (Fig. 5i). Thus, agents used for the induction of selective autophagy of cytosolic membrane-bound organelles also promoted capture of additional cytosolic proteins with diverse subcellular localization and assembly patterns.

Here, we set out to systematically assess ribophagic flux in mammalian cells. Under basal conditions, ribophagic flux is extremely low (<1% over an ~24h period) in HEK293 cells. As with yeast, nutrient deprivation (as well as mTOR inhibition) increases ribophagic flux (to ~10% of ribosomes over a 24h period in human cells), but in contrast with yeast, neither ATG8 conjugation nor the p97 ATPase is required. Moreover, ribophagic flux was comparable to free Keima flux, indicating that ribophagy under these conditions is not selective, as classically defined60. Surprisingly, agents that induce defects in ribosomal quality control did not promote robust ribophagy, suggesting that autophagy is not generally used for turnover of translationally impaired ribosomes. However, we found that proteotoxic stress agents such as AS and reversine promote ATG5-dependent ribophagic flux and, in the case of AS, flux depended substantially on p97. The comparative selectivity of ribophagic flux relative to other cytosolic cargo primarily reflected a lower basal rate of ribophagic flux. Interestingly, autophagic flux of the cytosolic reporter LDHB–Keima in response to either AS or reversine was largely dependent upon ATG5−/− (Supplementary Fig. 5c,d). In contrast, Tor1-dependent turnover of cytosolic forms of Keima is independent of ATG5, but with similar overall flux rates as observed with ribosomes (Supplementary Fig. 3a). This suggests that bulk autophagy induced by mTOR inhibition is mechanistically distinct from that induced by some, but not all, forms of proteotoxic stress. It remains to be determined whether the key parameters for determining the dependency on ATG8 conjugation involve either the types of cargo being produced or, alternatively, the overall abundance of the cargo needing to be degraded through autophagy.

Selective autophagy often involves the Ub system60. Whether and how Ub supports ribophagy in mammals is unknown, although 40S ribosomal subunits are ubiquitylated upon stress61. We found that the Ub activating enzyme UBA1 inhibitor MLN7243 reduces AS-induced ribophagic flux by three- to fourfold and LDHB flux to a lesser degree (a factor of 1.6; Supplementary Fig. 5e–h), but did not affect Tor1 or reversine-induced ribophagic flux (Supplementary Fig. 5e–i). AS promotes phosphorylation of the Ub-binding autophagy receptor p62 (also called SQSTM1), stimulating its ability to bind and inhibit the NRF2 Ub ligase KEAP1 as well as promoting p62 recruitment to autophagic cargo62. However, p62 deletion in Ribo–Keima reporter cells had no effect on AS or reversine-induced ribophagic flux (Supplementary Fig. 5j–l), indicating that p62 is not essential for ribophagic flux under these conditions. Further studies are necessary to understand the mechanism of Ub usage in cargo selection during stress-induced autophagy. Finally, we found that diverse cytosolic proteins are captured under conditions ostensibly used to promote selective forms of organelar autophagy at levels comparable to that seen upon induction of bulk autophagy with mTOR inhibition. Although cytosolic proteins may be captured within autophagosomes during organelar capture, referred to as bystander autophagy23,24, the magnitude of non-selective cargo capture observed here was often comparable to that seen upon induction of bulk autophagy by Tor1. This raises the possibility that some agents used to promote selective organelar autophagy may also induce proteotoxic stress, which then simultaneously promotes bulk autophagy. In this regard, formation of ectopic autophagosomes unlinked to mitochondrial cargo has been observed upon treatment with depolarization agents63. Whether or not stress granules are selectively degraded as opposed to being cleared via stress-induced bulk autophagy that is ATG8-dependent remains unclear, but is suggested by our finding that levels of AS that do not generate obvious stress granules nevertheless induce widespread autophagic degradation of ribosomes and LDHB (Supplementary Fig. 5c). This work suggests the importance of testing alternative cargo when examining the selectivity of cargo capture and degradation, emphasizing the emerging theme64 that some forms of induced autophagy need not be ATG8-conjugation-dependent, and provides a framework and tools for analysis of selective autophagy.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-017-0007-x.

Received: 8 April 2017; Accepted: 14 November 2017;
Published online: 11 December 2017

References

1. Warner, J. R. The economics of ribosome biosynthesis in yeast. Trends Biochem. Sci. 24, 437–440 (1999).
2. Zhang, T., Shen, S., Qu, J. & Ghaemmaghami, S. Global analysis of cellular protein flux quantifies the selectivity of basal autophagy. Cell Rep. 14, 2426–2439 (2016).
3. Shao, S. & Hegde, R. S. Target selection during protein quality control. Trends Biochem. Sci. 41, 124–137 (2016).
4. Sung, M. K., Reitsma, J. M., Sweredski, M. J., Hess, S. & Deshaies, R. J. Mature ribosomes produced in excess are degraded by the ubiquitin–proteasome system. Mol. Biol. Cell 27, 2642–2652 (2016).
5. Sung, M. K. et al. A conserved quality-control pathway that mediates degradation of unassembled ribosomal proteins. eLife 5, e19105 (2016).
6. Kraft, C., Deplazes, A., Sohrmann, M. & Peter, M. Ribosomal proteins are selectively degraded upon starvation by an autophagy pathway requiring the Ub3p/Bre5p ubiquitin protease. Nat. Cell Biol. 10, 602–610 (2008).
7. Ossareh-Nazari, B. et al. Cdc48 and Ufd3, new partners of the ubiquitin–proteasome system. Mol. Biol. Cell 27, 2642–2652 (2016).
8. Nishida, Y. et al. Discovery of Atg5/Atg7-independent alternative macroautophagy. Nature 461, 654–658 (2009).
9. Tsukeyama, K. et al. The ATG conjugation systems are important for degradation of the inner autophagosomal membrane. Science 354, 1036–1041 (2016).
10. Anderson, P. & Kedersha, N. RNA granules. J. Cell Biol. 172, 803–808 (2006).
11. Santaguida, S., Vasile, E., White, E. & Amon, A. Aneuploidy-induced cellular stress limits autophagic degradation. Gene. Dev. 29, 2010–2021 (2015).
12. Galluzzi, L. et al. Molecular definitions of autophagy and related processes. EMBO J. 36, 1811–1836 (2017).
13. Anding, A. L. & Baehrecke, E. H. Cleaning house: selective autophagy of organelles. Dev. Cell 41, 10–22 (2017).
14. Kishi-Itakura, C., Koyama-Honda, I., Itakura, E. & Mizushima, N. Ultrastructural analysis of autophagosome organization using mammalian autophagy-deficient cells. J. Cell Sci. 127, 4089–4102 (2014).
15. Katayama, H., Koyama-Honda, I., Yoshimori, T. & Mizushima, N. Ultrastructural analysis of autophagosome organization using mammalian autophagy-deficient cells. J. Cell Sci. 127, 4089–4102 (2014).
16. Mizushima, N., Yoshimori, T. & Levine, B. Methods in mammalian autophagy research. Cell 140, 313–326 (2010).
17. Ni, H. M. et al. Dissecting the dynamic turnover of GFP-LC3 in the autolysosome. Autophagy 7, 186–204 (2011).
18. Gross, L. A., Baird, G. S., Hoffman, R. C., Baldridge, K. K. & Tsien, R. Y. The structure of the chromophore within DsRed, a red fluorescent protein from coral. Proc. Natl Acad. Sci. USA 97, 11990–11995 (2000).
19. Chan, E. Y., Kir, S. & Tooze, S. A. siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy. J. Biol. Chem. 282, 25464–25474 (2007).
20. Hurley, J. H. & Young, L. N. Mechanisms of autophagy initiation. Annu. Rev. Biochem. 86, 225–244 (2017).
21. Ktistakis, N. T. & Tooze, S. A. Digesting the expanding mechanisms of autophagy. Trends Cell Biol. 26, 624–635 (2016).
22. Anderson, D. J. et al. Targeting the AAA ATPase p97 as an approach to treat cancer through disruption of protein homeostasis. Cancer Cell 28, 653–665 (2015).
23. Liu, J. et al. Reclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 147, 223–234 (2011).
24. Harper, J. W. & Bennett, E. J. Proteome complexity and the forces that drive proteome imbalance. Nature 537, 328–338 (2016).
25. Hewitt, L. et al. Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex. J. Cell Biol. 190, 25–34 (2010).
26. Santaguida, S., Tighe, A., D’Alise, A. M., Taylor, S. S. & Musacchio, A. Dissecting the role of MPS1 in chromosome biorientation and the spindle checkpoint through the small molecule inhibitor reversine. J. Cell Biol. 190, 73–87 (2010).
27. Panas, M. D., Ivanov, P. & Anderson, P. Mechanistic insights into mammalian stress granule dynamics. J. Cell Biol. 215, 313–323 (2016).
28. Buchan, J. R., Kolatis, R. M., Taylor, J. P. & Parker, R. Eukaryotic stress granules are cleared by autophagy and Cdc48/VCP function. Cell 153, 1461–1474 (2013).
29. Ichimura, Y. et al. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. Mol. Cell 51, 618–631 (2013).
30. Stolz, A., Ernst, A. & Dikic, I. Cargo recognition and trafficking in selective autophagy. Nat. Cell Biol. 16, 495–501 (2014).
31. Higgins, R. et al. The unfolded protein response triggers site-specific regulatory ubiquitylation of 40S ribosomal proteins. Mol. Cell 59, 35–49 (2015).
32. Yoshii, S. R., Kishi, C., Ishihara, N. & Mizushima, N. Parkin mediates proteosome-dependent protein degradation and rupture of the outer mitochondrial membrane. J. Biol. Chem. 286, 19630–19640 (2011).

Acknowledgements
This work was supported by the National Institutes of Health (grants R37NS083524 and RO1GM095567 to J.W.H.). The authors acknowledge the Nikon Imaging Center and the Imaging and Data Analysis Core (Harvard Medical School) for imaging assistance.

Author contributions
J.W.H. and H.A. conceived the study. H.A. performed all experiments. H.A. and J.W.H. analysed the data and wrote the paper.

Competing Interests
The authors declare no competing financial interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41556-017-0007-x.
Reprints and permissions information is available at www.nature.com/reprints.
Correspondence and requests for materials should be addressed to J.W.H.
Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Methods

Antibodies and chemicals. The following antibodies and reagents were used in this study: Keima (M126-3M, MBL), RPS3 (9538s, Cell Signal Technology), RPL28 (ab138123, Abcam), LCB27 (2773s, Cell Signal Technology), ATG5 (12994s, Cell Signal Technology), ATG12 (14180s, Cell Signal Technology), BECN1 (3495, Cell Signal Technology), p70 S6K (2708T, Cell Signal Technology), p70 S6K phospho-T389 (9234S, Cell Signal Technology), actin (sc-69789, Santa Cruz), tubulin (ab7291, Abcam), PCNA (SC56, Santacruz), KEAP1 (10503-2, AP-ProteinTech), p62 (H000887-001, Novus Biologicals), Flag (M8823, Sigma), puromycin antibody (MABE343, EMD Millipore), p2-pS531 (AP20696b, Abgent), ubiquitin (r045801-2, Dako), anti-mouse IgG (H+L) HRP conjugate (w4021, Promega), Western Lightning Plus-ECL (NEL51001, Perkin Elmer), anti-rabbit IgG (H+L), HRP conjugate (w4011, Promega), bortezomib (A2614, APELSBio), IRDye 800CW goat anti-mouse IgG-H+L (925-22210, LI-COR), Torin1 (14379, Cell Signal Technology), SAR405 (A8883, APExBio), Bafilomycin A (R1857, Sigma), chloroquine diphosphate salt (C6628, Sigma), LysoTracker Green (L7526, ThermoFisher Scientific), ER-Tracker Green (E4325, ThermoFisher Scientific), CellLight Golgi-GFP (C10592, ThermoFisher Scientific), HBSS (14025092, Invitrogen), E64d (A1903, APEBIO), pepstatin A (P5318, Sigma), reversine (R3904, Sigma), sodium (meta) arsenite (S7400, Sigma), H2O2 (25088, Sigma), brefeldin A (9972, Cell Signal Technology), CB5083 (CB-5083, Selleckchem), tunicamycin (T7756, Sigma), poly-l-lysine solution (P4832, Sigma), lipofectamine 3000 (L30000080, Invitrogen), FluoroBrite DMEM (A1896701, ThermoFisher Scientific), benzimidazole (Sigma), HC (71205, EMDmillipore), the Surveyor mutation detection kit (706025, Integrated DNA Technologies), GeneArt precision gRNA synthesis kit (A29377, ThermoFisher Scientific), RNAsin (N2111, Promega) and the Neo transfection system (MPX1025, ThermoFisher Scientific). Dilutions and clone numbers of all antibodies used in this study are provided in Supplementary Table 3.

Cell culture. HEK293, HCT116 and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and maintained in a 5% CO2 incubator at 37 °C.

Generation of Keima knock-in cell lines using CRISPR-Cas9 gene editing. gRNAs targeting the C-terminus region of human RPS3 and RPL28 genes were designed using the CHOPCHOP website (http://chopchop.chubio.unio). The guide sequence for the RPS3 (5’-GACATACTCTTATATGCTTG-3’) and RPL28 (5’-CTTCTTACACCGGTGCA-3’) guide genes were assembled into pX330 plasmid33. The targeting region was amplified from wild-type HEK293T cell cDNA using Primer5. gRNA targeting the C-terminus region of human ATG5 was carried out using standard site-directed mutagenesis. The destination pHAGE vectors containing N-terminal or C-terminal eGFP and Keima tags were prepared by Gibson assembly. Using the Gateway cloning system, lentiviral-based expression vectors containing N-terminal tandem eGFP-tagged LC3, C-terminal tandem eGFP-tagged Lamp1 and eIF4E, and N- or C-terminal tandem Keima-tagged constructs were generated. In the case of the Lamp1–eGFP construct, the linker sequence between Lamp1 and the eGFP was replaced with GCT (AAC) sequence by Gibson assembly to prevent possible mis-localization of the protein because the linker immediately follows the XXXI coding motif on the C-terminal site of Lamp1.

Stable cell line generation. The 293T cells were transfected with the corresponding lentiviral expression vectors using lipofectamine 3000 (Invitrogen). Virus containing supernatant was collected 48 h post-transfection, and HEK293 and HCT116 cell lines were infected with the virus to create stable cell lines. In the case of eGFP-LC3 and Lamp1–eGFP expressing cell lines, eGFP-positive cells were single cell sorted by flow cytometry to generate clonal cell lines. Free Keima–LC3, mito–Keima, LDHB–Keima, TOMM20–Keima, ACTB–Keima, PSMD12–Keima and PEX3–Keima expressing cell lines were first selected with puromycin (Invitrogen) for at least one week, then sorted by flow cytometry (MoFlo Astrios EQ, Beckman Coulter) twice, with an interval of two weeks, to equalize the Keima expression levels. The Keima signal level of RP53–Keima expressing cells was used as the standard for sorting cells with similar Keima expression level.

Cell lysis and immunoblotting assay. Cells were cultured in the presence of the corresponding chemicals to ~50–60% confluency in a six-well plate, then trypsinized, pelleted by centrifugation, washed with Dulbecco’s Phosphate-Buffered Saline (DPBS) and pelleted again. The cell pellet was lysed by radioimmunoprecipitation assay buffer (RIPA buffer) containing mammalian protease inhibitor cocktail (Sigma) and 20 units/ml benzamidine (Millipore), followed by centrifugation (13,000 r.p.m.). The concentration of the supernatant was measured by Bradford assay and the whole-cell lysate was denatured by the addition of NPAGE LDS (Lithium dodecyl sulfate) Sample Buffer (Thermo Fisher Scientific) supplemented with 100 mM dithiothreitol, followed by boiling at 75 °C for 5 min. Each lysate (40 μg) was loaded onto the 4–20% Tris-Gly gel (Thermo Fisher Scientific) or 4–12% NuPAGE Bis-Tris gel (Thermo Fisher Scientific), followed by SDS–PAGE with Tris-Glycin SDS running buffer (Thermo Fisher Scientific) or NPAGE MES (2-(N-morpholino)ethanesulfonic acid) SDS running buffer (Thermo Fisher Scientific), respectively. The proteins were electrophoresed to polyvinylidene difluoride (PVDF) membranes (0.45 μm, Millipore) and then immunoblotted using antibodies as indicated. For quantitative immunoblotting of Keima reporter cells, 80–100 μg of the lysate was loaded onto SDS–PAGE gel due to the low expression level of the Keima construct and small amount of processed Keima, except for Keima–LC3 (35 μg). A 4–20% Tris-Gly gel was used to resolve the proteins. After transfer, the membrane was blocked with 5% non-fat milk, incubated with anti-Keima mouse antibody (4°C, overnight), washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST) (total 30 min) and further incubated with fluorescent IRDye 800CW goat anti-mouse IgG H+L secondary antibody (1:20,000) for 2 h. After a severe wash with TBST for 30 min, the processed Keima band intensity (25 kDa) was measured using OdysseyCLx imager and divided by the normalized signal of intact Keima protein. The processed Keima protein to intact Keima protein ratio was calculated within the gel and, where quantitations obtained from independent immunoblots were compared, the Torin1 treatment condition was used as internal control. In the case of Keima–LC3 cell lines, tubulin was used to normalize the processed Keima signal because LC3 is an autophagy machinery rather than a cargo. Thus, the accumulated processed Keima level normalized by tubulin level reflects total autophagy flux.

Sucrose density gradient fractionation. Cells were grown to 60% confluency in 15 cm dishes with or without bortezomib for 4 h. The cells were then treated with cycloheximide (100 μg/ml) for 10 min and collected after trypsin treatment. In vitro translation reactions. The in vitro translation reactions were performed in the rabbit reticulocyte lysate system (200 μl mixture: 100 μl HEPES, 300 mM NaCl, 50 mM MgCl2, 100 μg/ml cycloheximide, 0.5% Triton-X, protease inhibitor tablet (Roche), 50 μl/ml RNasin) was added to resuspend the cells and the suspension was passed through a 25G needle over 10 times. After centrifugation for 5 min at 13,000 r.p.m. the supernatant was loaded on top of the 5–30% sucrose gradient column, prepared in house. Using an SW40 motor (Beckman Coulter), the sucrose gradient was centrifuged for 2.5 h at 35,000 r.p.m. (4 °C), followed by 1 h of no-break deceleration. A small hole was made at the bottom of the tube and the sample was fractionated into 1.5 ml tubes (750 μl each). RNA level was measured by Nano-drop (260 nm ultraviolet (UV) absorption) and each fraction was immunoblotted using the corresponding antibodies.

In vitro translation reactions. Cells were grown to 60% confluency in six-well plates. The cells were treated with puromycin (1 μg/ml) for 15 min, then collected after trypsin treatment. After cell lysis using RIPA buffer, an immunoblotting assay was performed with anti-puromycin antibody.
Flow cytometry analysis. Cells were grown in the presence of the corresponding chemicals to 60% confluency in six-well plates, then collected after trypsin treatment. After resuspending the cells in FACS buffer (1 x DPBS, 1 mM EDTA, 1% FBS, 25 mM HEPES, final pH 7.3–7.5), the cells were analysed by flow cytometry (MoFlo Astrios EQ, Beckman Coulter). Data were processed with FlowJo software. The 561/488 nm ratio distribution graph was processed in Prism software after exporting the 488 and 561 nm ex./em. intensities of individual cells (10,000 cells) were exported. An example of the gating strategy used for flow cytometry analysis is shown in Supplementary Fig. 3.

Calculation of approximate percentage of ribosomes undergoing ribophagy before and after Torin1 treatment. HEK293 RP53-Keima cells were collected in pH 7.3 or 4.5 buffer and incubated for 30 min. The 561/488 nm excitation fold increase from cells in pH 7.3 buffer to pH 4.5 buffer was calculated to be 2.020 (Value_a). This condition mimics the situation of nearly 100% ribosomes undergoing ribophagy, because all the cytosolic ribosomes are at pH 4.5. The 561/488 nm excitation fold increases of control cells and Torin1 (24 h) treated cells responsive to bafloymycin A were 0.027 and 0.194, respectively (Value_b and Value_c, respectively). These values represent the lysosomal population of Ribosome–Keima before and after 24 h of Torin1 treatment. The per cent ratio of (Value_b) to (Value_a) is 1.3% and the ratio of (Value_c) to (Value_a) is 10.4%, which represents the percentage of ribosomes undergoing ribophagy before and after Torin1 treatment compared to the total ribosomes.

Live-cell confocal microscopy. Cells were plated onto a 33-mm glass-bottomed dish (no. 1.5, glass diameter of 14 mm, MatTek) pretreated with poly-L-lysine, then incubated in phenol-red free medium (FluoroBrite DMEM, Thermo Fisher) containing 1-glutamine (2 mM) and sodium pyruvate (1 mM) for 48 h. Followed by treatment with corresponding chemicals or HBSS, the cells were stained with 2.5 μg/mL Hoechst 33342 (Thermo Fisher Scientific) for 10 min. The medium was replaced with fresh FluoroBrite DMEM (37°C) and cells were imaged using a Yokogawa CSU-X1 spinning disk confocal microscope with a Spectral Applied Research Aurora Borealis modification on a Nikon Ti motorized microscope equipped with a Nikon Plan Apo 60X/1.40 NA objective lens. Paired of images for ratiometric analysis of mKeima fluorescence were collected sequentially using 100 mW/488 nm and 100 mW/561 nm solid-state lasers attenuated and controlled with an AOTF (Spectral Applied Research LMM-5) and emission (em.) collected with a 620/60 nm filter (Chroma Technologies). Wide-field fluorescence images of Hoescht were collected using a Lumencor SOLA light source, 395/35 nm ex. and 480/40 nm em. filters (Chroma Technologies). Both confocal and wide-field images were acquired with the same Hamamatsu ORCA-ER cooled charge-coupled device camera and MetaMorph software. Seven z-series optical sections were collected with a step size of 0.7 μm, using the Nikon Ti internal focus motor. The z series are displayed as maximum z projections, and gamma, brightness and contrast were adjusted equally for each image using Fiji software. For the live-cell imaging of cells expressing Golgi-mEGFP, HEK293 RP53-Keima cells were transiently transfected with CellLight Golgi–GFP (C10592, ThermoFisher Scientific) for 24 h. Cells were then incubated with Torin (150 nM) or the combination of Torin and SAR405 (150 nM and 1 μM, respectively) for 24 h before imaging. Confocal images of mEGFP fluorescence were collected using a 100 mW/488 nm solid-state laser and a 525/50 nm em. filter (Chroma Technologies).

Quantitative image analysis. Serial z-stack image sections taken with a ×60 objective lens were combined using the maximum intensity projection function in Fiji software. Without any cutting, the original images were processed as in the following sections.

Step 1: Cell segmentation. The Hoescht channel showing stained nuclei was processed as follows: (1) background subtraction, (2) autothreshold, make binary, fill holes, dilate and water shed, (3) connected components labelling using ‘find connected regions’ plug-in to make ‘marker’. The 442 nm ex./620 nm em. Keima channel was processed as follows: (1) Gaussian blur, (2) subtract background, (3) autothreshold, make binary and dilate. Using the marker controlled segmentation function in MorphoLab plug-in, the two processed image channels were combined to generate ‘mask’.

Step 2: Track-mate. The 442 nm ex./620 nm em. and 561 nm ex./620 nm em. channels were stacked, and Keima puncta co-localized with in 3 pixel distance were analysed using TreackMate function. This step generated a xml file containing co-localization and puncta intensity information of the whole image plain.

Step 3. Matlab coding. Using Matlab software, the xml file was processed in a way that any puncta with a 561/448 intensity ratio over 0.5 were assigned to the corresponding cells segmented in Step 1. This resulted in two new images showing segmented cells and numerically assigned puncta in each cell. Co-localization of Lamp1–eGFP, eGFP–LC3 and Lysotracker Green with red Keima puncta was performed by Fiji software. Any puncta with a pixel size of 7 and intensity of 7 (arbitrary number) were found in both 488 nm ex./620 nm em. and 561 nm ex./620 nm em. channels. Among the puncta that passed the intensity and size thresholds, the total puncta number that appeared in both channels within a pixel distance of 5 was divided by the total number of puncta analysed in the 488 nm ex./620 nm em. channel image. For the random co-occurrence measurement, the 488 nm ex./561 nm em. channel image was shifted 10 μm in a random direction. Co-localization analysis between the two channels was performed following the same procedure.

Electron microscopy. HEK293; wt, ATG5−/−, BECN1−/− cells were grown to 60% confluency in a six-well plate and incubated with HBSS + BafA (50 nM) for 4.5 h. The cells were then fixed with 1.25% paraformaldehyde, 2.5% glutaraldehyde and 0.03% picric acid, followed by osmication and uranyl acetate staining, dehydration in alcohols, and then embedded in Taab 812 Resin (Marivac). Sections were cut with a Leica ultracut microtome and picked up on formvar/carbon-coated copper slot grids. Immediately before imaging, the grids were stained with 2% uranyl acetate for 5 min to increase ribosome contrast, followed by 0.2% lead citrate staining. The samples were imaged under a Phillips Tecnai BioTwin Spirit transmission electron microscope.

Statistics and reproducibility. All statistical data were calculated using GraphPad Prism 7. Comparisons of data in Fig. 4b were performed by one-way analysis of variance (ANOVA) with Sidak’s multiple comparisons test. Comparisons of all other data were performed by two-way ANOVA with Tukey’s multiple comparisons test. P values <0.01 were considered significant. All experiments were repeated at least three times unless otherwise indicated.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Code availability. The Matlab code used for unbiased image quantitation in this study is available from the corresponding author upon reasonable request.

Data availability. The source data for statistical analyses of Figs. 2b, f–h, 3b, 4b,d, e,g,l and 5e–f and Supplementary Figs. 2j, 3a and 5c,h are provided in Supplementary Table 2. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

References
33. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308 (2013).
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

> Experimental design

1. Sample size
   - Describe how sample size was determined.
   - **No method was used to determine sample size.**

2. Data exclusions
   - Describe any data exclusions.
   - **No data was excluded.**

3. Replication
   - Describe whether the experimental findings were reliably reproduced.
   - **The experimental findings were reliably reproduced.**

4. Randomization
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - **The experiments were not randomized.**

5. Blinding
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - **Blinding was not used for the experiments.**
   
   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   | n/a | Confirmed |
   |-----|-----------|
   | ☑ | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
   | ☑ | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
   | ☑ | A statement indicating how many times each experiment was replicated |
   | ☑ | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
   | ☑ | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
   | ☑ | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
   | ☑ | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
   | ☑ | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.
Software

Describe the software used to analyze the data in this study.

GraphPad Prism7 software was used for the data analysis.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

8. Materials availability

There are no restrictions on availability of materials used in this study.

9. Antibodies

We provide catalog/clone numbers for all antibodies in the methods section.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Cells were purchased from ATCC.

b. Describe the method of cell line authentication used.

Karyotyping (GTG-banded karyotype) of HCT116, and 293T cells (from ATCC) was performed by Brigham and Women’s Hospital Cytogenomics Core Laboratory.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines were found to be free of mycoplasma using Mycoplasma Plus PCR assay kit (Agilent).

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No ICLAC samples were used.

Animals and human research participants

11. Description of research animals

Animals and animal-derived materials are not used in this study.

12. Description of human research participants

This study does not involve human research participants.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.

1. Keima reporter cells were grown in 6-well plates under the described stress conditions. 2. 45 min prior to the flow-cytometry analysis, ~ 600000 cells were trypsinized, collected into a 1.5 ml eppendorf tube using 1ml DMEM media, and spin-down at 1000 rpm for 3 min, 4C. 3. The media was removed, and the cell pellet was reconstituted with 250ul of cold FACS buffer (1x PBS, 1mM EDTA, 25 mM HEPES, 1% Fetal Bovine Serum, pH 7.2, 0.2 um filter sterilized) containing 1ug/ml of DAPI. 4. The reconstituted cells were filtered through the cell strainer into the 5ml test tube (Corning, 352235).

6. Identify the instrument used for data collection.

MoFlo Astrios EQ, Beckman Coulter

7. Describe the software used to collect and analyze the flow cytometry data.

FlowJo-2 vX.0.7. The 561/488 nm ratio distribution graph was processed in Prism software after exporting the 488 and 561 nm ex/em intensity of individual 10000 cells were exported.

8. Describe the abundance of the relevant cell populations within post-sort fractions.

Once sorted, the cells were expanded for 1-2 weeks and analyzed again with flow-cytometry to insure the homogenous distribution of the cell population.

9. Describe the gating strategy used.

1. singlet cells were gated by SSC1 hight/FSC1 hight followed by SSC1 hight/SSC1-width. 2. Live cells were sorted by 405-448 (DAPI positive)/ SSC1 hight. 3. Keima signal was measured by 488ex/620em and 561ex/614em.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.