Autophagy variation within a cell population determines cell fate through selective degradation of Fap-1

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Autophagy regulates cell death both positively and negatively, but the molecular basis for this paradox remains inadequately characterized. We demonstrate here that transient cell-to-cell variations in autophagy can promote either cell death or survival depending on the stimulus and cell type. By separating cells with high and low basal autophagy using flow cytometry, we demonstrate that autophagy determines which cells live or die in response to death receptor activation. We have determined that selective autophagic degradation of the phosphatase Fap-1 promotes Fas apoptosis in Type I cells, which do not require mitochondrial permeabilization for efficient apoptosis. Conversely, autophagy inhibits apoptosis in Type II cells (which require mitochondrial involvement) or on treatment with TRAIL in either Type I or II cells. These data illustrate that differences in autophagy in a cell population determine cell fate in a stimulus- and cell-type-specific manner. This example of selective autophagy of an apoptosis regulator may represent a general mechanism for context-specific regulation of cell fate by autophagy.

Macropathology (hereafter autophagy) is a catabolic process that facilitates cell survival in response to stress by providing nutrients and biosynthetic monomers, and by mitigating cellular damage\(^1,2\). Several studies have suggested that autophagy is capable of regulating apoptosis but, surprisingly, autophagy can both promote or inhibit cell death in different cellular contexts\(^3,4\). The molecular underpinnings of this duality remain poorly defined despite the fact that they have important implications in human disease\(^5-7\). Despite many links between specific proteins of the autophagy and apoptosis pathways, surprisingly little is known about how the overall process of autophagy determines whether cells live or die in response to cell death stimuli\(^8-11\). Apoptosis is known to control autophagy (both positively and negatively) through molecular mechanisms that have been described\(^12-14\) and many autophagy regulators also control the apoptotic apparatus\(^15-18\). However, mechanisms responsible for the regulation of apoptosis by the overall process of autophagy are less clear\(^19-21\). Except in the case of salivary gland cell death in \textit{Drosophila}\(^22\) and the autophagic degradation of catalase\(^23\), precise mechanisms responsible for direct promotion of cell death by autophagy are unknown.

In populations of cells treated with apoptotic stimuli, some cells will escape death for reasons that have only recently been addressed but which have important clinical consequences, particularly in cancer therapy. Non-genetic heterogeneity, stochastic state differences and variation in levels of apoptotic proteins between cells have recently received attention as determinants of cell fate that govern which cells live and which die in a population\(^24-26\), but underlying cellular processes that alter or regulate these activities have not been identified. We reasoned that basal variability in autophagy could determine cell fate by altering levels of critical apoptosis regulators. Here, we reveal high steady-state variability in basal autophagy in a cell population, which acts as a non-genetic determinant of cell fate through the selective autophagic degradation of a key apoptosis regulatory protein. This provides an example of how variation in autophagy can regulate cell fate and identifies a specific mechanism by which autophagy can promote apoptosis in a cell-type- and stimulus-specific manner.

RESULTS

Quantitative cell-to-cell differences in basal autophagy in a homogeneous cell population

Differences in basal autophagy have been associated with certain oncogenes but the role of basal autophagy in cancer cell death has not been examined\(^27,28\). Stochastic variability in critical apoptotic proteins has been identified as a determinant of cell fate\(^24,26\). Therefore, variability in a cellular process capable of altering the levels of apoptotic proteins would also be predicted to determine cell fate. We sought to

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Figure 1  Log-phase proliferating cells in optimal growth media exhibit significant steady-state differences in autophagic flux. (a,b) BJAB lymphoma cells stably expressing mCherry–GFP–LC3 were serially cultured at log phase followed by fluorescence-activated cell sorting for cells with high and low autophagic flux using the ratio of mCherry/GFP (a). The high and low 20% were sorted (a), re-plated and treated with lysosomal protease inhibitors pepstatin and E-64d for 1 h; lysates were then immunoblotted for the indicated proteins (b). (c) Densitometry of LC3-II and p62 western blots (normalized to actin and hour 0, mean ± s.e.m., n = 3 blots from 2 independent experiments, P = 0.051, **P = 0.0091). (d,e) HeLa mCherry–GFP–LC3 cells were sorted as in a, cytopun on to slides, fixed and visualized by confocal microscopy (d); autophagic LC3 puncta were assessed by quantitative microscopy (e; punctate area per cell, mean ± s.e.m., n = 50 fields, *P = 0.010, **P = 0.053). (f) Electron micrographs of HeLa mCherry–GFP–LC3 cells sorted for autophagic flux as in a. Yellow arrows denote autophagosomes; red arrows indicate autolysosomes. (g) Quantification of autophagosomes and lysosomes from electron micrographs (mean ± s.e.m., n = 50 fields). Uncropped images of blots are shown in Supplementary Fig. 6.

quantify stochastic differences in basal autophagy in a cell population and determine the role of these differences in basal autophagy on cell death in response to specific apoptotic stimuli. To accomplish this, we used flow cytometry to sort cells on the basis of their relative levels of autophagic flux using mCherry–EGFP–LC3 as a reporter (Supplementary Fig. 1a). This reporter for autophagic flux takes advantage of the higher sensitivity of EGFP fluorescence to the acidic environment of the autolysosome relative to mCherry (ref. 30): cells with higher flux are less green owing to autophagosome fusion with lysosomes, thereby increasing the mCherry/EGFP ratio (Fig. 1a and Supplementary Fig. 1a,b). This method to measure flux has been extensively validated and accurately quantifies autophagic flux induction by multiple stimuli and chemical and genetic inhibition of autophagy (Fig. 1 and Supplementary Figs 1 and 2). To examine differences between high- and low-autophagic-flux cells under basal conditions, BJAB B-cell lymphoma cells were maintained near log phase in growth medium, collected and flow sorted into low- and high-flux populations (Fig. 1a). Immunoblots for LC3 and p62 autophagy proteins confirmed the relative levels of flux present in the sorted cells (Fig. 1b,c) and quantification of autophagosomes and lysosomes by saponin extraction11 followed by flow cytometry revealed substantial differences in the lysosome and autolysosome number between the high- and low-flux subpopulations (Supplementary Fig. 1d–f). Furthermore, fluorescent and electron microscopy further substantiated that the cells differ in their levels of autophagic flux (Fig. 1d–g and Supplementary Fig. 2a,b), and that these differences in flux were independent of cell size, cell cycle or spontaneous apoptosis (sub-G1 events; Supplementary Fig. 2c–f). Importantly, we found that differences in basal flux were transient, persisting for at least 4 h after sorting but returning back to steady state by 24 h (Fig. 2a–c); this rapid reversion of the sorted cells indicates that genetic heterogeneity in the starting population was not responsible for differences in flux. Together, these data show that substantial differences in autophagic flux exist within cell populations, even under optimal growth conditions, probably due to transient stochastic fluctuations in the level or activity of key autophagy regulators.

Differences in basal autophagic flux dictate the apoptotic response

To establish whether cell-to-cell differences in basal autophagic flux control cell fate, we treated flow-sorted high- and low-autophagic-flux populations with the death receptor agonists Fas ligand and TRAIL. BJAB cells with higher basal autophagic flux were more sensitive to Fas-ligand-induced apoptosis than low-flux cells (Fig. 2d,e and Supplementary Fig. 3a). Surprisingly, this was not found with TRAIL treatment—high- and low-autophagy cells (sorted at the same time from the same population) tended towards the opposite in their sensitivity to TRAIL (Fig. 2d,e and Supplementary...
Differences in basal autophagic flux are transient but determine cell viability. These data indicate that transient differences in the level of basal autophagy between cell types result in significant inhibition of cell death induced by Fas ligand in the BJAB cells but had no substantial effect on TRAIL-induced killing (Fig. 3d–f). Knockdown of Atg5 or Atg7 led to no differences in cell death with either Fas ligand or TRAIL in Jurkat cells (Fig. 3d–f). Furthermore, combined genetic and pharmacologic inhibition through Atg5 knockdown and chloroquine treatment had no additive effect on Fas-ligand-induced death in BJAB cells (Supplementary Fig. 4e). Autophagy inhibition through transient overexpression of autophagy dominant-negative Atg4b-C74A or Rab7-Q79L mutants also reduced BJAB cell death in response to Fas ligand (Supplementary Fig. 4d). Conversely, treatment of BJAB cells with autophagy inducers (EBSS and trehalose) led to more cell death in response to Fas ligand and TRAIL (Supplementary Fig. 4d). Together, these data show that autophagy is dispensable for Fas-ligand-induced apoptosis in Jurkat cells and is dispensable for TRAIL-induced apoptosis in both cell types.

**Figure 2** Differences in basal autophagic flux are transient but determine apoptotic response in a stimulus-specific manner. (a–c) BJAB lymphoma cells stably expressing mCherry-GFP-LC3 were serially cultured at log phase followed by fluorescence-activated cell sorting for cells with high and low autophagic flux using the ratio of mCherry/GFP. Cells were then re-plated in growth medium and autophagic flux was again measured by flow cytometry at the indicated time points (median ratio of mCherry/GFP fluorescence ± s.e.m., n = 3 wells). Lysates from cells collected at the indicated time points were immunoblotted with the indicated antibodies (b). Representative flow cytometry histograms for cells at 0 h and 24 h after sorting (c). (d) BJAB mCherry-GFP-LC3 cells were sorted for autophagic flux as in a (top and bottom 20%). Following treatment with Fas ligand (1.5 ng ml$^{-1}$) or TRAIL (4 ng ml$^{-1}$), cell viability was determined by MTS assay (a tetrazolium reduction-based metabolic measurement) at 24 h (percentage of untreated control, mean ± s.e.m., n = 3 wells, *P = 2.3 × 10$^{-4}$, **P = 0.0036). (e) Long-term growth of BJAB mCherry-GFP-LC3 cells sorted for autophagic flux followed by treatment with Fas ligand (4 ng ml$^{-1}$) or TRAIL (15 ng ml$^{-1}$) for 24 h. Cells were then re-plated and allowed to recover for 5 (Fas ligand) or 6 (TRAIL) days then assayed for viability (percentage of no ligand control, mean ± s.e.m., n = 3 wells, *P = 0.012). (f) BJAB mCherry–GFP-LC3 cells were sorted for autophagic flux as above and apoptosis was measured at 1 h by flow cytometry using AnnexinV and DAPI (mean ± s.e.m., n = 3 wells, *P = 0.0046). (g) Cells sorted as in a, were re-plated and, starting at the indicated times following sorting, treated with Fas ligand (4 ng ml$^{-1}$) for 24 h; cell viability was then determined by MTS (percentage of untreated control, mean ± s.e.m., n = 3 wells). Uncropped images of blots are shown in Supplementary Fig. 6.
Autophagy modulates Fas-induced death through the phosphatase Fap-1

The tyrosine phosphatase Fap-1 (Fas-associated phosphatase 1 or PTPN13, PTP-L1) specifically modulates Fas but not TRAIL signalling through dephosphorylation of Fas, which reduces its cell surface expression and activity. Fap-1 has been implicated in several cancers including liver cancer in which autophagy has been implicated as a tumour suppressor and Fas plays a major role in liver cell cancers including liver cancer in which autophagy has been implicated. Reduced Fap-1 expression in Type II cells is responsible (at least in part) for phenotypic differences between Fas Type I and Type II cells. Flow-sorting low-autophagic-flux BJAB cells (Type I) exhibited higher Fap-1 levels than high-flux cells that correlated with higher cell viability (Fig. 4a,b) and increased Fas receptor at the cell surface (Supplementary Fig. 5a). This was not seen in Jurkat cells (Type II), which express nearly undetectable levels of Fap-1 (ref. 43; Fig. 4a,b). Type I SKW6.4 cells and Type II CEM-CCRF (CEM) cells exhibited a similar pattern of cell viability with autophagy inhibition (Fig. 4c,d) and Fap-1 protein levels were substantially higher in the Type I cells than in the Type II cells (Fig. 4c). When autophagy was blocked by chloroquine or shRNA knockdown of Atg5, Atg7 or Vps34, Fap-1 levels increased in the Type I cells, correlating with the reduced level of Fas-ligand-induced apoptosis (Fig. 4e,f). No Fap-1 was detected with or without autophagy inhibition in the Type I Jurkat cells (Fig. 4c,f and Supplementary Fig. 5b). Conversely, autophagy acted in a cytoprotective manner in Jurkat cells with autophagy gene knockdown leading to decreased viability in response to Fas ligand. Together, these data led us to reason that autophagy was modulating Fas apoptosis by selectively degrading Fap-1.

To determine the role of Fap-1 in autophagy modulation of autophagy, we sought to determine whether removal of Fap-1 could make BJAB cells respond to Fas ligand like Type II cells and whether exogenous expression of Fap-1 could make the Jurkat cells respond...
Autophagy facilitates Fas apoptosis in Type I cells and correlates with Fap-1 expression. (a,b) BJAB and Jurkat cells stably expressing mCherry–GFP–LC3 were flow sorted for high and low autophagic flux, treated with Fas ligand (1.5 ng ml⁻¹) for 24 h and viability was assessed by MTS (a; percentage of no ligand control, mean ± s.e.m., n = 3 wells, *P = 0.0046). (b) Immunoblots following sorting of the samples in a. (c,d) The indicated cell lines were treated with chloroquine (BJAB, CEM 20 μM; SKW6.4, 25 μM; Jurkat, 10 μM) for 16 h, followed by Fas ligand (BJAB, 12.5 ng ml⁻¹; SKW6.4, 50 ng ml⁻¹; Jurkat, 0.4 ng ml⁻¹; CEM, 40 ng ml⁻¹). Lysates from cells collected after chloroquine treatment were immunoblotted with the indicated antibodies (c). Cell viability was assessed by MTS 24 h following Fas ligand treatment (d; percentage of control (no ligand), mean ± s.e.m., n = 3 wells, *P = 2.7 × 10⁻⁴, **P = 1.6 × 10⁻⁴). (e,f) BJAB and Jurkat cells were transduced with control, Atg5, Atg7 or Vps34 shRNA lentiviruses, followed by three days of puromycin selection. Cells were then treated with Fas ligand (1.25 ng ml⁻¹) or TRAIL (1.25 ng ml⁻¹) for 24 h and viability was assessed by MTS (e; percentage of control (no ligand), mean ± s.e.m., n = 3 wells, *P = 1.4 × 10⁻⁶, **P = 2.3 × 10⁻⁹, ***P = 3.9 × 10⁻⁸, §§P = 9.2 × 10⁻⁴, §§§P = 3.4 × 10⁻⁴, §§§§P = 1.7 × 10⁻³). Immunoblots demonstrate Atg5, Atg7 and Vps34 knockdown, autophagy inhibition and altered Fap-1 levels. The blots are separated because different exposures were required to detect the proteins in one cell line without overexposing the lanes for the other; the Fap-1 blot is not separated to demonstrate the lack of Fap-1 protein in Type II Jurkat cells (f). Fap-1 blots (b,c,f) were run on separate gels owing to the quantity of protein required for detection (see Methods). Uncropped images of blots are shown in Supplementary Fig. 6.

Figure 4 Autophagy facilitates Fas apoptosis in Type I cells and correlates with Fap-1 expression. (a,b) BJAB and Jurkat cells stably expressing mCherry–GFP–LC3 were flow sorted for high and low autophagic flux, treated with Fas ligand (1.5 ng ml⁻¹) for 24 h and viability was assessed by MTS (a; percentage of no ligand control, mean ± s.e.m., n = 3 wells, *P = 0.0046). (b) Immunoblots following sorting of the samples in a. (c,d) The indicated cell lines were treated with chloroquine (BJAB, CEM 20 μM; SKW6.4, 25 μM; Jurkat, 10 μM) for 16 h, followed by Fas ligand (BJAB, 12.5 ng ml⁻¹; SKW6.4, 50 ng ml⁻¹; Jurkat, 0.4 ng ml⁻¹; CEM, 40 ng ml⁻¹). Lysates from cells collected after chloroquine treatment were immunoblotted with the indicated antibodies (c). Cell viability was assessed by MTS 24 h following Fas ligand treatment (d; percentage of control (no ligand), mean ± s.e.m., n = 3 wells, *P = 2.7 × 10⁻⁴, **P = 1.6 × 10⁻⁴). (e,f) BJAB and Jurkat cells were transduced with control, Atg5, Atg7 or Vps34 shRNA lentiviruses, followed by three days of puromycin selection. Cells were then treated with Fas ligand (1.25 ng ml⁻¹) or TRAIL (1.25 ng ml⁻¹) for 24 h and viability was assessed by MTS (e; percentage of control (no ligand), mean ± s.e.m., n = 3 wells, *P = 1.4 × 10⁻⁶, **P = 2.3 × 10⁻⁹, ***P = 3.9 × 10⁻⁸, §§P = 9.2 × 10⁻⁴, §§§P = 3.4 × 10⁻⁴, §§§§P = 1.7 × 10⁻³). Immunoblots demonstrate Atg5, Atg7 and Vps34 knockdown, autophagy inhibition and altered Fap-1 levels. The blots are separated because different exposures were required to detect the proteins in one cell line without overexposing the lanes for the other; the Fap-1 blot is not separated to demonstrate the lack of Fap-1 protein in Type II Jurkat cells (f). Fap-1 blots (b,c,f) were run on separate gels owing to the quantity of protein required for detection (see Methods). Uncropped images of blots are shown in Supplementary Fig. 6.

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To Fas ligand like Type 1 cells. As predicted, overexpression of wild-type Fap-1 caused Jurkat cells to behave more like Type I cells in response to autophagy inhibition and Fas ligand, whereas expression of catalytically inactive (ΔCD) Fap-1 had no effect; wild-type or ΔCD Fap-1 had no effect on the BJAB cells (Fig. 5a,b). Conversely, Fap-1 shRNA knockdown abrogated the increase in cell death caused by chloroquine in response to Fas ligand in Type I BJAB cells (Fig. 5c–e and Supplementary Fig. 5c,d) but did not have a substantial effect on TRAIL-induced viability (Fig. 5c). These results indicate that Fap-1 is necessary and sufficient for autophagy to modulate Fas-induced apoptosis.

**Fap-1 is targeted for autophagic degradation through the selective autophagy adaptor p62**

The adaptor protein p62 (also known as SQSTM1) and its family members regulate selective autophagy of specific cargoes. Furthermore, p62 associates with the death-inducing signalling complex (DISC) and modulates death receptor signalling. To determine whether Fap-1 autophagic degradation occurs through p62, we depleted p62 protein, which resulted in increased Fap-1 and concomitant inhibition of Fas-ligand-induced apoptosis (Fig. 6a,b). We were also able to co-immunoprecipitate endogenous Fap-1 and p62 in a chloroquine and Fas-ligand-dependent manner (Fig. 6c, Supplementary Fig. 5e). Therefore, p62 associates directly with Fap-1 enabling it to recruit Fap-1 for autophagic degradation in response to Fas receptor activation. Together, these data show that p62-mediated recruitment of Fap-1 to autophagosomes regulates the apoptotic response to Fas activation.

**DISCUSSION**

Our results demonstrate that transient and significant differences in autophagic flux are present at baseline in a popula-
Autophagy facilitates apoptosis through selective degradation of Fap-1. (a,b) BJAB and Jurkat cells expressing the indicated Fap-1 wild-type (WT) and catalytically inactive (ΔCD) constructs were treated with chloroquine (BJAB, 20 μM; Jurkat, 10 μM) for 16 h followed by Fas ligand (BJAB, 1.5 ng ml⁻¹; Jurkat 15 ng ml⁻¹) for 24 h. Cell viability was determined by MTS (a; percentage of control (no ligand), mean ± s.e.m., n = 3 wells, *P = 2.7 × 10⁻⁴, **P = 2.4 × 10⁻⁵, ***P = 0.0024). Cell lysates were blotted and probed with the indicated antibodies (b). (c) BJAB cells expressing control or Fap-1 shRNAs were treated with 20 μM chloroquine for 16 h followed by Fas ligand (12.5 ng ml⁻¹) or TRAIL (25 ng ml⁻¹) for 24 h; cell viability was then determined by MTS (c; percentage of control (no ligand), mean ± s.e.m., n = 3 wells, *P = 4.8 × 10⁻⁶, **P = 8.1 × 10⁻⁵, ***P = 8.0 × 10⁻⁴, †P = 0.013, NS P > 0.05). (d) Immunoblots of lysates collected following chloroquine treatment. Long-term growth was assessed in cells from c by re-plating them at low density and allowing them to recover for 5 days, followed by viability assay (percentage of untreated control, mean ± s.e.m., n = 3 wells, *P = 0.043; e). Fap-1 blots (b,d) were run on separate gels owing to the quantity of protein required for detection (see Methods). Uncropped images of blots are shown in Supplementary Fig. 6.

Autophagy can thus determine which cells live and which cells die in a population but these effects are both stimulus- and cell-type-specific. Our work addresses an area that has been confusing—whether autophagy protects or kills cells⁴⁹. Our data show that even in the same population of cells, it can do both and it is the context (that is, specific stimulus and cell type) that determines whether autophagy is pro- or anti-apoptotic. We do not know specifically or generally how autophagy is able to generally protect against apoptosis in our system, although it has been reported that autophagy can degrade activated caspase-8 to protect against TRAIL-induced death in other contexts⁵⁰. However, our work does reveal a defined mechanism by which autophagy promotes apoptosis in mammalian cells. For Fas ligand, autophagy-dependent differences in apoptosis in response depend on p62-mediated selective autophagic degradation of Fap-1 (Fig. 6d). This model explains both the cell-type-specific differences whereby only Type I cells require autophagy for efficient apoptosis and the stimulus-specific effect whereby autophagy promotes Fas-induced death but not TRAIL-induced death. Our work here establishes a molecular mechanism for an autophagy-dependent switch from apoptosis inhibition to promotion and provides an example whereby stochastic variability in autophagy can determine cell fate. These findings also underscore the importance of autophagy in cancer therapy, specifically in the case of cancers where Fap-1 is implicated⁵¹ and more generally by demonstrating that autophagy can switch from a death promoter to a death inhibitor in different contexts. Present attempts to modulate autophagy in a clinical setting⁵² are focused on...
p62 is required for Fap-1 degradation by autophagy and p62 and Fap-1 interact directly. (a, b) Cell viability was determined by MTS assay following Fas ligand (4 ng ml\(^{-1}\)) treatment in BJAB cells expressing control or p62 lentiviral shRNAs (a); percentage of control (no ligand), mean ± s.e.m., \(n = 3\) wells, \(*P = 3.2 \times 10^{-5}, \quad **P = 1.8 \times 10^{-5}\). (c) Co-immunoprecipitation of endogenous p62 and Fap-1 in BJAB cells treated with 20 \(\mu\)M chloroquine for 16 h, followed by treatment with Fas ligand (50 ng ml\(^{-1}\)) for 2 h at 4 \(^\circ\)C. (Model) The mechanism for autophagy promotion of Fas apoptosis in Type I cells. MOMP, mitochondrial outer membrane permeabilization. Fap-1/bc were run on separate gels owing to the quantity of protein required for detection (see Methods). Uncropped images of blots are shown in Supplementary Fig. 6.

Figure 6 p62 is required for Fap-1 degradation by autophagy and p62 and Fap-1 interact directly. (a, b) Cell viability was determined by MTS assay following Fas ligand (4 ng ml\(^{-1}\)) treatment in BJAB cells expressing control or p62 lentiviral shRNAs (a); percentage of control (no ligand), mean ± s.e.m., \(n = 3\) wells, \(*P = 3.2 \times 10^{-5}, \quad **P = 1.8 \times 10^{-5}\). (c) Co-immunoprecipitation of endogenous p62 and Fap-1 in BJAB cells treated with 20 \(\mu\)M chloroquine for 16 h, followed by treatment with Fas ligand (50 ng ml\(^{-1}\)) for 2 h at 4 \(^\circ\)C. (Model) The mechanism for autophagy promotion of Fas apoptosis in Type I cells. MOMP, mitochondrial outer membrane permeabilization. Fap-1/bc were run on separate gels owing to the quantity of protein required for detection (see Methods). Uncropped images of blots are shown in Supplementary Fig. 6.

using autophagy inhibition combined with other anti-cancer drugs and are the focus of over three dozen clinical trials. Such trials should consider the implications of context-specific promotion and inhibition of apoptosis by autophagy.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.M.G. and A.T. designed the study; J.M.G. and L.S. performed experiments; A.B. and D.W.H.R. provided Fap-1 reagents and expertise; J.M.G. designed experiments and analysed data; J.M.G. and A.T. wrote the manuscript, which was commented on by all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Materials. The following antibodies were used: LC3 no. NB100-2220, 1:2,000 (Novus Biologicals); Fap-1 no. AF3577 (TPPN13), 1:1,000 (R&D Systems); p62 no. H0000887-M01, 1:5,000 (Abnova); Atg5 no. 2630, 1:1,000; Atg7 no. 8538, 1:800; Vps34 no. 3811, 1:800 (Cell Signalling); β-actin no. A5441, 1:10,000 (Sigma-Aldrich); CD95-APC no. 558814 (anti-Fas; BD Biosciences), CellEvent Green Caspase-3/7 reagent was from Invitrogen. zVAD-fmk was from R&D Systems. Doxorubicin, etoposide, chloroquine and DCMI (norlociprimapirene) and all other chemicals used were from Sigma-Aldrich.

Cell lines. Parental BJAB cells, an EBV-negative B-cell lymphoma line, were provided previously by M. Peter (University of Chicago, USA). CEM-CCRF cell line was a gift from the Laboratory of D. Graham (University of Colorado, USA). Other cell lines were obtained from ATCC. The cells were most recently fingerprinted using the ABI Identifier kit in January 2010, and were distinct from other cells in the merged DMSZ, ATCC, JCRB and Riken databases and are therefore not contaminated with any other cell line. All cell lines were tested regularly for the presence of mycoplasma. Cell lines were cultured in either RPMI 1640 or MEM (Invitrogen) with 10% FBS (Sigma) in a 5% CO₂, humidified atmosphere at 37°C.

Immunoblots. Western blotting was performed using standard methods with antibodies in 3% milk in TBS/1%Tween-20 at the above dilutions. Owing to difficulty detecting Fap-1 with the best available antibody, western blots were performed with 50 μg of total protein on an 8% 1.5 mm mini gel; electrophoresis started at 10 milliamperes with stepwise 10 milliamp increases in amperage every 10 minutes to 80 milliamps and was run for approximately 2 1/2 hours total, followed by transfer on a semi-dry apparatus for 1.5 h at 15 V. Fap-1 western blots were always run on separate gels from the other proteins in each experiment due to the amount of protein required to detect Fap-1 and the low percentage of gel used to detect this large protein. Fap-1 antibody specificity was determined on the basis of the disappearance of a band with a relative molecular mass of 230,000 following Fap-1 shRNA knockdown in BJAB cells and the appearance of the same band in Jurkat cells following ectopic expression of the PTPN13 (Fap-1) gene. Quantification by integrated optical density analysis on scanned images was performed with ImageJ using the Measure function on isolated bands in inverted images.

Immunoprecipitation. Dishes (15 cm) of 80% confluent BJAB cells were treated with 20 μM chloroquine for 16 h at 37°C, then incubated at 4°C for 30 min before adding 50 ng ml⁻¹ Fas ligand and incubating at 4°C for a further 2 h. Cell lysis and immunoprecipitation of endogenous p62 and Fap-1 was performed as described previously. The data herein are representative of three separate experiments.

RNA interference. Protein depletion through RNA-mediated interference (RNAi) was mediated using the pLKO shRNA system (Sigma-Aldrich). Plasmids were obtained from the Functional Genomics Core at the University of Colorado Boulder. Lentiviruses were generated by co-transfection of pLKO-shRNA plasmids using the Measure function on isolated bands in inverted images.

Immunoprecipitation of endogenous p62 and Fap-1 was performed as described previously. The data herein are representative of three separate experiments.

Fluorescence microscopy. Cells were sorted for autophagic flux as described above, cytoplasm onto coated slides, fixed with 4% formaldehyde at room temperature for 10 min, washed and mounted using gelvatol. Epifluorescent images were acquired with Volocity 5 (Perkin Elmer) on a Zeiss Axiovert 200M microscope (Carl Zeiss) outfitted with a x63 objective. Epifluorescent image analysis and quantification were automated using Volocity 5. Briefly, cell area was quantified by regions of interest as defined by fluorescent intensity threshold 1/2 standard deviation above background and puncta were defined as areas with intensity three standard deviations above background within the defined regions of interest. Field choice and acquisition were not blinded; fields were chosen on the basis of the presence of 1 or more entire cells as detected by Volocity. Automated calculations were performed using a macro for each image, exported from Volocity and analysed in Excel. Confocal images were acquired on an Olympus FV1000 laser scanning confocal microscope with a x63 objective. Images were acquired by an individual blinded to the experimental conditions and with no knowledge of the expected outcome. Image j v1.46 (NIH) was used to adjust intensity using identical settings for each image and to analyse the raw images. Images used in the figures are representative of trends observed in all of the images obtained. Co-localization images were created using the ImageJ co-localization plugin (http://rsweb.nih.gov/ij/plugins/colocalization.html) and quantified by integrated optical density analysis using the ImageJ Measure function.

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Transmission electron microscopy. Flux-sorted HeLa mCherry–GFP–LC3 cells were washed with PBS, fixed in 10% glutaraldehyde for 15 min at room temperature, washed with PBS and suspended in 1% Sea-prep agarose before being frozen at −80°C for cryosectioning. Cryosections were fixed and stained for electron microscopy using standard protocols before being visualized on a Tecnai G1 12 BioTwin microscope (Fei) equipped with a Gatan CCD (charge-coupled device; Gatan) imaging system. Images were acquired by an individual blinded to the experimental conditions and expected outcome. Image intensity normalization and annotation were performed with ImageJ with identical settings for each image. Autophagosomes and autolysosomes were counted manually using previously defined criteria.

Statistics. Statistical $P$ values were calculated using a two-tailed Student’s $t$-test. All test assumptions regarding distribution and variance were met for each data set. All statistics are based on biological replicates from the same independent experiment, not technical replicates or from combined experiments, with the exception of the western blotting quantification in Fig. 1c, which is based on three separate western blots from two independent experiments.

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Supplementary Figure 1 Method to sort cells with high and low autophagic flux by flow cytometry accurately measures basal and induced autophagic flux. 

a, Cells constitutively expressing mCherry-GFP-LC3 are sorted based on the relative ratio of mCherry/GFP fluorescence which changes in response to the pH gradient as autophagosomes fuse with lysosomes to form autolysosomes (autophagic flux). 

b, BJAB mCherry-GFP-LC3 cells were treated with EBSS (100%) or trehalose (75 mM) for 4 hours followed by flow cytometry to quantitate autophagosome/autolysosome number (normalized fold over unsorted control, mean ± s.e.m., n=3 wells).

c, HeLa mCherry-GFP-LC3 cells expressing control or Atg5 shRNA were treated with EBSS for 4 hours followed by flow cytometry for autophagic flux. Data are representative of at least 3 independent experiments. 

d, BJAB mCherry-GFP-LC3 cells were sorted by flow cytometry for high or low autophagic flux, then treated with Lysotracker Blue for 30 min. at 37 °C, followed by flow cytometry (median GFP, mCherry or Lysotracker Blue fluorescence normalized to fold over unsorted control, mean ± s.e.m., n=3 wells). 

e, BJAB mCherry-GFP-LC3 cells were sorted as in (d) followed by cytoplasmic extraction with 0.1 % saponin to eliminate non-lipidated LC3 and flow cytometry to quantitate autophagosome/autolysosome number (normalized folded over unsorted control median GFP or mCherry fluorescence, mean ± s.e.m., n=3 wells).

f, Flow cytometry histograms of representative data from (e).
**Supplementary Figure 2** Confocal microscopy reveals differences in the number of autophagosomes and autolysosomes in flux sorted cells; High and low flux sorted cells exhibit similar size, cell cycle and apoptotic profile. **a**, HeLa mCherry-GFP-LC3 cells were sorted for autophagic flux, treated with Lysotracker Blue for 30 min. at 37 °C, adhered to slides by cytopin centrifugation, fixed with formaldehyde and visualized by spinning disc confocal microscopy; these are the same fields depicted in Fig. 1d. Colocalization images were produced using ImageJ (see methods). **b**, Quantification of the colocalization images in (a). Data are representative of 20 separate fields and have been repeated 5 times. **c**, BJAB mCherry-GFP-LC3 cells were sorted for autophagic flux by flow cytometry. **d**, **e**, Forward and side scatter of sorted cells in (c). **f**, Cell cycle profiles of sorted cells in (c) stained with Hoechst 33342.
Supplementary Figure 3  Autophagy modulation of Fas ligand-induced apoptosis and cell killing. a, b, BJAB mCherry-GFP-LC3 cells were sorted for autophagic flux by flow cytometry followed by treatment with Fas ligand (a) or TRAIL (b) for 24 hours and MTS assay for viability (fold over no ligand control, mean ± s.e.m., n=3 wells). These are the full data sets from the same experiment depicted in Figure 2d. c-e, BJAB mCherry-GFP-LC3 cells were sorted as in (a) followed by treatment with Fas ligand for the indicated times in the presence of fluorogenic Caspase-3/7 substrate CellEvent Green (5 μM) and analyzed by flow cytometry. c, Dot plot of Caspase-3/7 activity vs. DAPI fluorescence in unsorted cells. d, Quantitation of Caspase-3/7 activity in sorted cells at the indicated time points (median CellEvent Green fluorescence, mean ± s.e.m., n=3 wells, *p=0.0091). e, Representative flow cytometry histograms of Caspase-3/7 activity in (c, d). f, BJAB mCherry-GFP-LC3 cells were sorted for autophagic flux as above, followed by treatment with Fas ligand at the indicated concentrations in the presence of fluorogenic CellEvent Caspase-3/7 substrate (5 μM) and analyzed on an IncuCyte ZOOM for the indicated times (mean CellEvent Green fluorescence per field, mean, n=12: 4 fields for each of 3 wells).
Supplementary Figure 4 Effect of autophagy inhibition or autophagy induction on Fas ligand-induced death. 

- **a**, BJAB cells expressing control or Atg12 shRNA were treated with Fas ligand (15 ng/mL) for 24 hours in the presence or absence of zVAD-fmk (100 μM). Cell viability was then quantitated by MTS absorbance (fold over untreated control, mean ± s.e.m., n=3 wells).

- **b**, BJAB cells expressing control or Bid shRNA were treated with autophagy inhibitor chloroquine (25 μM) for 12 hours followed by Fas ligand (4 ng/mL) for 24 hours. Cell viability was then quantitated by MTS (fold over untreated control, mean ± s.e.m., n=3 wells).

- **c**, BJAB cells were treated with autophagy inhibitors chloroquine (20 μM) or desmethyldesipramine (DCMI, 10 μM) for 12 hours followed by treatment with the indicated cytotoxic agents for 24 hours. Cells were then assayed for viability by MTS (% of control (no cytotoxic drug), mean ± s.e.m., n=3 wells, *p=0.0024, **p=0.017).

- **d**, BJAB cells expressing vector control or the indicated autophagy dominant-negative constructs were treated with Fas ligand (4 ng/mL) for 24 hours; cell viability was then quantitated by MTS absorbance (fold over untreated control, mean ± s.e.m., n=3 wells). Data are representative of 2 independent experiments.

- **e**, BJAB cells expressing control or Atg5 shRNA were treated with chloroquine (20 μM) for 12 hours followed by Fas ligand (4 ng/mL) for 24 hours. Cell viability was then quantitated by MTS (fold over no ligand control, mean ± s.e.m., n=3 wells). Data are representative of 3 independent experiments.

- **f, g**, BJAB cells were treated with the indicated autophagy inducers overnight (EBSS, 100%; trehalose, 75 mM) followed by treatment with Fas ligand at the indicated concentrations for 24 hours. Cell viability was then quantitated by MTS absorbance. **f**, MTS data normalized (fold over untreated (no Fas ligand) control, mean ± s.e.m., n=3 wells). **g**, Raw MTS values (absorbance at 490 nm, mean ± s.e.m., n=3 wells).
**Supplementary Figure 5** Autophagy controls cell surface expression of Fas receptor via Fap-1. a, BJAB mCherry-GFP-LC3 cells were sorted for autophagic flux by flow cytometry, and then stained with APC-conjugated anti-Fas antibody at 4 °C for 30 minutes. Cells were then washed and analyzed by flow cytometry. Data are representative of 3 replicates from 3 independent experiments. b, Additional exposures of immunoblots from experiment in Fig. 4f. c-d, BJAB cells expressing control or Fap-1 shRNAs were treated with 20 µM chloroquine for 12 hours followed by treatment with Fas ligand (c) or TRAIL (d) at the indicated concentrations for 24 hours. Cell viability was quantitated by MTS (absorbance at 490 nm, mean ± s.e.m., n=3 wells). These are the full dose response curves (without normalization) from the data in Figure 5c. e, Additional exposures from immunoprecipitation experiment depicted in Fig. 6c.
Supplementary Figure 6 Full immunoblot images. Red boxes indicate the cropped portion of each western blot presented in the corresponding main figures.