Published in final edited form as:
Cell Rep. 2014 April 10; 7(1): 45–52. doi:10.1016/j.celrep.2014.02.036.

Autophagy controls the kinetics and extent of mitochondrial apoptosis by regulating PUMA levels

Jacqueline Thorburn[1], **Zdenek Andrysik**[2], **Leah Staskiewicz**[1], **Jacob Gump**[1], **Paola Maycotte**[1], **Andrew Oberst**[3], **Douglas R. Green**[4], **Joaquín M. Espinosa**[2], and **Andrew Thorburn**[1,*]

[1]Dept. of Pharmacology, University of Colorado School of Medicine, Anschutz Medical Campus, Aurora, CO 80045

[2]Howard Hughes Medical Institute and Dept. of Molecular, Cellular and Developmental Biology, University of Colorado Boulder. Boulder, CO 80309

[3]Dept. of Immunology, University of Washington, Seattle, WA 98109-8059

[4]Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105-3678

Summary

Macroautophagy is thought to protect against apoptosis, however underlying mechanisms are poorly understood. We examined how autophagy affects canonical death receptor-induced mitochondrial outer membrane permeabilization (MOMP) and apoptosis. MOMP occurs at variable times in a population of cells and this is delayed by autophagy. Additionally, autophagy leads to inefficient MOMP after which some cells die through a slower process than typical apoptosis and, surprisingly, can recover and divide afterwards. These effects are associated with p62/SQSTM1-dependent selective autophagy causing PUMA levels to be kept low through an indirect mechanism whereby autophagy affects constitutive levels of PUMA mRNA. PUMA depletion is sufficient to prevent the sensitization to apoptosis that occurs when autophagy is blocked. Autophagy can therefore control apoptosis via a key regulator that makes MOMP faster and more efficient thus ensuring rapid completion of apoptosis. This identifies a molecular mechanism whereby cell fate decisions can be determined by autophagy.

Introduction

Macroautophagy and apoptosis are closely linked (Gump and Thorburn, 2011; Maiuri et al., 2007b; Rubinstein and Kimchi, 2012). Molecular mechanisms whereby autophagy can promote apoptosis in response to specific stimuli have been identified in Drosophila (Nezis et al., 2010) and, more recently in mammalian cells (Gump et al., 2014). However, because

*Correspondence: Andrew.Thorburn@ucdenver.edu Tel: 303-724-3290 Fax 303-724-3664.

autophagy is primarily a pro-survival mechanism, it is generally thought that the two processes regulate each other in opposite directions. Distinct molecular mechanisms have been identified whereby apoptosis can inhibit autophagy. For example, caspases cleave autophagy regulators such as Beclin 1 (BECN1) to suppress autophagy (Cho et al., 2009; Li et al., 2011; Luo and Rubinsztein, 2010). In contrast, mechanisms by which autophagy protects against apoptosis are poorly understood. The simplest way that autophagy can protect against apoptosis is by modulating the stresses that activate the apoptosis machinery. For example, autophagy protects against nutrient and growth factor deprivation-induced apoptosis (Boya et al., 2005; Lum et al., 2005). However, this needn't mean that autophagy inhibits the core apoptosis machinery; instead degradation of cellular components by autophagy can supply macromolecular precursors and energy until nutrients are restored and this might avoid activation of apoptosis signaling at all. Although many proteins regulate both autophagy and apoptosis (Gump and Thorburn, 2011; Rubinstein and Kimchi, 2012), general mechanisms by which the process of autophagy controls the core apoptosis machinery have not been identified. Nevertheless, because diverse apoptotic stimuli (e.g. different classes of chemotherapy drugs (Levy and Thorburn, 2011)) can all be sensitized when autophagy is inhibited, it is widely suggested that regulation of the core apoptosis machinery by autophagy must exist.

Even when cells in a clonal population die in response to a defined stimulus, there is considerable variation in the timing of apoptosis (Albeck et al., 2008; Goldstein et al., 2000; Spencer et al., 2009). Single cell imaging of well-understood apoptosis stimuli such as the Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) have shown how this cell-to-cell variation comes about. TRAIL induces apoptosis in most cells via "Type II apoptosis" whereby TRAIL receptor signaling leads to activation of caspase-8, which cleaves the BH3 domain-containing protein BID to create a truncated protein called tBID. tBID translocates to mitochondria causing BAX- and BAK-dependent permeabilization of the outer mitochondrial membrane releasing cytochrome c, Smac/Diablo and other mitochondrial proteins. Mitochondrial Outer Membrane Permeabilization (MOMP), is completed within a few minutes (Goldstein et al., 2000; Rehm et al., 2003) and is widely considered a point of no return that marks commitment to death because it is followed within five to ten minutes by a burst of effector caspase activity that causes rapid contraction and fragmentation of the cell. The variation in timing of apoptosis is due to variation in the time when MOMP occurs and has been extensively studied in TRAIL-treated HeLa cells (Albeck et al., 2008; Spencer et al., 2009).

Here, we examined the effects of autophagy on this well understood canonical apoptosis pathway. We found that autophagy controls the core apoptosis machinery by regulating the timing of MOMP and the cell response after MOMP. These effects can be explained by autophagy's ability to regulate low constitutive levels of the BH3 domain-containing protein PUMA. Surprisingly, autophagy's effects on PUMA can also lead to slow and incomplete cellular degradation after MOMP from which some cells can recover and go on to divide and the pro-apoptotic effect of autophagy inhibition on viability of cells after apoptosis stimulation is blocked by removal of PUMA. This defines a mechanism by which autophagy can control the apoptotic threshold to govern cell fate.

Results and Discussion

Timing of MOMP is regulated by autophagy

We treated HeLa cells with TRAIL and monitored MOMP using time-lapse microscopy to determine when a mitochondrial-targeted Red Fluorescent Protein (Albeck et al., 2008) was released into the cytoplasm. Autophagy was increased by treatment with trehalose, which activates autophagy via a mTOR-independent mechanism (Sarkar et al., 2007) and, unlike other autophagy stimulators like mTOR inhibitors or starvation, has no known other effects on cell viability. Autophagy was inhibited by RNAi knockdown of essential autophagy regulators such as ATG12 as shown by reduced accumulation of LC3-II in cells where autophagic flux was blocked (Figure 1A). As expected (Albeck et al., 2008), MOMP was completed within 5 minutes (Figure 1B) starting at variable times (Figure 1C) from approximately 50 minutes to over two hours following treatment with TRAIL at doses sufficient to induce MOMP in all the cells in the population. Trehalose treated cells still underwent MOMP, however, its timing was markedly delayed when autophagy was stimulated. This delay was blocked when autophagy was inhibited by knockdown of ATG12 (Figure 1C) indicating that although trehalose has other properties that stabilize proteins and organelles (Jain and Roy, 2009), the effects we see here were due to increased autophagy. Delayed MOMP was also prevented when other autophagy regulators (ATG7, ATG5 and BECN1) were knocked down (Figure S1A-C) indicating that this is an autophagy-dependent effect and not other functions of the targeted genes.

Increased autophagy might delay MOMP by inhibiting signaling at the TRAIL receptor. Indeed, it has been reported that autophagy can degrade activated caspase-8 (Hou et al., 2010). Trehalose did not noticeably reduce or slow TRAIL-induced caspase-8 or BID cleavage (Figure 1D). This suggests that autophagy does not delay MOMP by altering the initial steps in TRAIL receptor signaling. To test this, we monitored the effects of autophagy induction on MOMP induced by Fas Ligand or an artificially dimerized caspase-8 molecule (Oberst et al., 2010), which both also showed autophagy-dependent delay (Figure S1 D,E). The lack of delay in BID cleavage and similar delay in MOMP after activation of caspase-8 by other stimuli or even artificial dimerization suggests that autophagy's ability to delay MOMP is due to an effect on mitochondrial permeabilization by tBID rather than an effect on signaling at the TRAIL receptor itself.

Autophagy regulates PUMA levels to control timing of MOMP

To test mechanisms at the mitochondria, we asked if BH3-domain proteins were altered when autophagy was inhibited. Inhibition of autophagy by knockdown of ATG5, ATG7, ATG12, Vps34/PIK3C3 or the selective autophagy mediator p62/SQSTM1 (Johansen and Lamark, 2011; Noda et al., 2010) all caused an increase in PUMA levels (Figure 1E). PUMA increase following ATG knockdown was seen in a variety of tumor cell lines of different tissue origin in addition to HeLa cells (Figure S2A) including those with wildtype or mutant p53. This effect was selective and other BH3 proteins (e.g. BAD, BIM, NOXA) showed no increase when autophagy was blocked (Figure S2C). PUMA protein levels might increase after ATG gene knockdown because autophagy directly degrades PUMA or because autophagy degrades something else that inhibits PUMA expression. We examined

PUMA mRNA and found that knockdown of essential autophagy regulators and p62/SQSTM1 could increase PUMA mRNA levels suggesting that an indirect mechanism applies. These effects were seen in HeLa cells, which have low levels of p53 and in MDA MB 231 cells (Figure S2B), which have mutant p53. These data suggest that the mechanism is due to modulation of constitutive PUMA expression not through induced PUMA transcription as occurs in response to p53 activators. Because efficient MOMP requires that proteins like tBID cooperate with PUMA (Chipuk et al., 2008; Chipuk and Green, 2009), we next determined the effect of PUMA knockdown on the timing of MOMP after TRAIL treatment. PUMA shRNA was sufficient to delay MOMP similarly to that seen with trehalose treatment and this delay was not extended if the PUMA knockdown cells were also treated with trehalose (Figure 1F).

Higher autophagy and PUMA degradation allows cellular recovery after MOMP

Cell contraction and breakup usually occurs in less than 5 minutes after MOMP has taken place (Albeck et al., 2008). Consistent with this, control cells where no autophagy manipulation was applied usually contracted quickly after MOMP so that within 20 minutes the vast majority of cells had a surface area less than 60% of their area immediately before MOMP. However some cells contract more slowly and, when autophagy was stimulated with trehalose, it was more common for dying cells to take longer to contract after MOMP as measured by this metric (Figure 2A). This effect was reversed by knockdown of ATG5 or ATG7 (Figure 2B) indicating that these effects were also autophagy-dependent. Surprisingly, continued imaging showed that although, as expected, most cells underwent MOMP, fast contraction and then death (Figure 2C), cells undergoing slow contraction after MOMP could sometimes recover and display a normal morphology (Figure 2D). To test if these effects were due to autophagy's ability to degrade PUMA, we assessed "slow" death in PUMA knockdown cells. Figure 3A shows that PUMA knockdown alone, like autophagy induction, increases the number of cells displaying slow contraction after incomplete MOMP. Moreover, continued monitoring demonstrated that these cells (in the absence of any artificial caspase inhibition) could recover normal morphology and even divide afterwards (Figure 3B and Movies S1 and S2).

The previous experiments were performed with doses of TRAIL high enough to ensure that MOMP eventually occurred in all or almost all the cells. However, if these mechanisms affect the long term viability of cells, they would be expected to manifest as modulation of the response to different doses of TRAIL. Therefore, we continuously monitored long term survival in mixed populations of isogenic HeLa cells with specific knockdowns that were marked with GFP-NLS or Cherry-NLS tags and treated with different doses of TRAIL. As expected knockdown of BID completely prevented TRAIL-induced apoptosis (Figure 4A). PUMA knockdown did not prevent apoptosis but did reduce its efficiency as shown by consistent increased viability at different doses of TRAIL (Figure 4B). This effect was selective for PUMA because knockdown of another BH3 protein (NOXA) did not affect cell viability (Figure S3). Inhibition of autophagy by knockdown of ATG7 (Figure 4C) or Vps34/PIK3C3 (Figure 4D) had the opposite effect as knockdown of PUMA and sensitized to apoptosis indicating a protective effect of autophagy. Importantly, autophagy's protective effect as shown by sensitization to apoptosis upon knockdown of ATG7 (Figure 4E) or

ATG12 (Figure 4F) was abolished by PUMA knockdown. Consistent with a modulatory role, protection against potentiation by autophagy inhibition by PUMA depletion was less pronounced when higher doses of TRAIL were used. Thus TRAIL induced apoptosis is, as expected, completely dependent on BID but the efficiency of BID-induced MOMP and subsequent apoptosis is modulated by PUMA. Additionally, the ability of autophagy to selectively regulate PUMA levels explains how autophagy protects against apoptosis and modulates the apoptotic threshold. This effect also occurred in TRAIL-treated MDA MB 231 cells (Figure S4C). PUMA knockdown also partially rescued from the potentiation of death induced by autophagy inhibition in response to the general apoptosis inducer staurosporine in both HeLa (Figure S4 A, B) and MDA MB 231 cells (Figure S4C). These data suggest that this mechanism applies generally during canonical mitochondrial apoptosis but that when stimuli, such as staurosporine, that are not pure apoptosis inducers like TRAIL are used, protection by PUMA knockdown is reduced.

BCL-family proteins stimulate or inhibit autophagy and apoptosis and these activities are at the center of the mutual regulation of these two processes by each other. For example, anti-apoptotic BCL family proteins like BCL-2 and BCL-xL inhibit autophagy (Pattingre et al., 2005). BH3-only proteins such as BAD (Maiuri et al., 2007a), NOXA (Elgendy et al., 2011) or PUMA (Zhang et al., 2012) can activate autophagy while others such as BIM inhibit autophagy (Luo et al., 2012) showing that autophagy is both positively and negatively regulated by proapoptotic BH3 proteins. PUMA has been reported to regulate mitophagy through a Bax-dependent mechanism (Yee et al., 2009); this activity is different from that observed here because it appears to promote rather than inhibit apoptosis. Our results expand this picture by showing that autophagy regulates BH3 protein function in canonical apoptosis through a p62-dependent mechanism that selectively and likely indirectly controls PUMA levels. It is known that PUMA can synergize with activator BH3 proteins such as BID to promote rapid and efficient MOMP followed quickly by complete apoptosis (Chipuk et al., 2008; Chipuk and Green, 2009). We propose that this mechanism is exploited to allow autophagy to inhibit apoptosis. Constitutive PUMA expression is kept low by selective autophagy through an indirect mechanism, which ultimately affects PUMA mRNA and this allows changes in PUMA levels to regulate the kinetics and efficiency of MOMP. This mechanism provides a way for autophagy to modulate the rate-limiting step in apoptosis. Moreover, when MOMP does happen in cells with low PUMA, the process is inefficient and cells are more likely to undergo a slow contraction following caspase activation that is different from that seen in the majority of cells where MOMP is rapidly followed by complete apoptosis and breakup of the cell. Surprisingly, some of these slow cells can recover and even go on to divide. This identifies a mechanism by which autophagy can directly control the core apoptosis machinery and suggests that autophagy’s ability to regulate PUMA levels controls synergy with MOMP activators like BID to regulate the apoptotic threshold that determines cell fate.

EXPERIMENTAL PROCEDURES

Cell Culture and reagents

Tumor cell lines cells were cultured in media recommended by ATCC and maintained at 37°C, 5% CO_2. hTRAIL/TNFSF10 was obtained from R & D Systems (Minneapolis, MN, USA) and used at a final concentration of 25 μg/ml or as indicated. Leucine zipper-tagged FasL construct was expressed in mammalian cells for the production of soluble active oligomerized FasL. Trehalose was obtained from Sigma (St. Louis, MO, USA) and used at a final concentration of 75mM.

Western Blotting and Antibodies

Proteins were separated by SDS-PAGE and transferred onto either nitrocellulose or PVDF membranes. Blots were probed with antibodies that recognize ATG5, ATG7, ATG12, BECN-1, BAD, BID, BIM, CASP 3, CASP 8, PI3K Class III (Vps34), PUMA (Cell Signaling, Beverley, MO, USA), LC3, NOXA (Novus, Littleton,CO, USA), p62/SQSTM1 (Abnova, Taipei City, Taiwan), β-actin (Sigma-Aldrich, St. Louis, USA).

Expression constructs, siRNA, shRNA, Transfection and Transduction

pBabe-Puro-IMS-RP was provided by Dr. John Albeck. Retrovirus particles were produced by co-transfecting GP2-293 cells (Clontech, Mountain View, CA, USA) pBbabe-Puro-IMS-RP with VSVG using T*rans*IT-LT1 (Mirus, Madison, WI, USA). Media containing viral particles was collected at 48 hours and at 72 hours post-transfection and stored at −80°C. Cells were plated at 10,000–30,000 cells/well in a 6-well plate, 24 hours prior to transduction. For IncuCyte experiments, HeLa cells were plated at 820 cells/well in a 48-well plate and transduction was performed to scale for single and double knockdown experiments. HeLa cells stably expressing IMS-RP (HeLa-IMS-RP) were generated by puromycin selection to produce single clones. HeLa-IMS-RP cells stably expressing pEGFP-LC3 or pmCherry-LC3 were generated by transfection with T*rans*-IT-LT1 and hygromycin selection.

siRNA and shRNA knockdowns were performed using SMARTpool:ON TARGET PLUS and pLKO.1 shRNA lentiviruses (sequences are in Supplementary Experimental Procedures). After infection, cells transduced with shRNA lentivirus were allowed to grow and passaged only three times before being discarded to avoid selection for loss of *ATG* gene knockdown-mediated inhibition of autophagy (Staskiewicz et al., 2013).

Time-Lapse Video Microscopy and Image Analysis

Time-lapse movies were generated using a Zeiss Axiovert 100 fluorescence microscope enclosed in an environmental chamber (Carl Zeiss,Thornwood, NY, USA) at 10x, 32x or 40x magnification. Images were captured with a Hamamatsu Orca CCD camera every 4–6 minutes using Volocity imaging software (Perkin Elmer, Waltham, MA, USA). Cells were grown in either 6-well plates or 24-well plates (Costar Incorporated, Tewksbury, MA, USA and MatTek, Corporation, Ashland, MA, USA). IMS-RP release was analyzed visually and by using Volocity software to measure the timing of the drop in standard deviation of the mean red fluorescence intensity when the fluorescent signal changed from more punctate

mitochondrial IMS-RP fluorescence to diffuse cytoplasmic fluorescence after mitochondrial outer membrane permeabilization. The area of each cell was determined using Volocity software. Time-lapse movies in Figure 2A were generated using a Nikon A1R-Si spectral imaging confocal microscope equipped with an environmental chamber (Nikon, Inc. Melville, KY, USA) at 40x magnification. Images were taken every 4 minutes using ImageJ software. In Figure 4 and in Figure S3A, quantitative live cell imaging to assess long term viability was performed using an IncuCyte Zoom imaging system (Essen Biosciences, Ann Arbor, MI). Images were obtained every 4 hours using a 10x objective. Live cell content was determined using the custom parameters for measuring cell confluence and/or fluorescence. For fluorescence imaging of mixed cultures, HeLa cells expressing nuclear GFP or mCherry were used expressing control or targeted shRNA lentiviruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by NIH grants 2RO1 CA111421 and 1RO1 CA150925 (A.T.) 2RO1 CA117907 (J.M.E.) and the University of Colorado Cancer Center Functional Genomics, Flow Cytometry and Tissue Culture Shared Resources that are supported by NIH grant P30 CA046934.

REFERENCES

Albeck JG, Burke JM, Aldridge BB, Zhang M, Lauffenburger DA, Sorger PK. Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. Mol Cell. 2008; 30:11–25. [PubMed: 18406323]

Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Metivier D, Meley D, Souquere S, Yoshimori T, et al. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 2005; 25:1025–1040. [PubMed: 15657430]

Chipuk JE, Fisher JC, Dillon CP, Kriwacki RW, Kuwana T, Green DR. Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. Proc Natl Acad Sci USA. 2008; 105:20327–20332. [PubMed: 19074266]

Chipuk JE, Green DR. PUMA cooperates with direct activator proteins to promote mitochondrial outer membrane permeabilization and apoptosis. Cell cycle. 2009; 8:2692–2696. [PubMed: 19652530]

Cho DH, Jo YK, Hwang JJ, Lee YM, Roh SA, Kim JC. Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells. Cancer Lett. 2009; 274:95–100. [PubMed: 18842334]

Elgendy M, Sheridan C, Brumatti G, Martin SJ. Oncogenic rasinduced expression of noxa and beclin-1 promotes autophagic cell death and limits clonogenic survival. Molecular Cell. 2011; 42:23–35. [PubMed: 21353614]

Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. Nat Cell Biol. 2000; 2:156–162. [PubMed: 10707086]

Gump JM, Staskiewicz L, Morgan MJ, Bamberg A, Riches DWH, Thorburn A. Autophagy variation within a cell population determines cell fate through selective degradation of Fap-1. Nat Cell Biol. 2014; 16:47–54. [PubMed: 24316673]

Gump JM, Thorburn A. Autophagy and apoptosis: what is the connection? Trends Cell Biol. 2011; 21:387–392. [PubMed: 21561772]

Hou W, Han J, Lu C, Goldstein LA, Rabinowich H. Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. Autophagy. 2010; 6:891–900. [PubMed: 20724831]

Jain NK, Roy I. Effect of trehalose on protein structure. Protein Sci. 2009; 18:24–36. [PubMed: 19177348]

Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy. 2011; 7:279–296. [PubMed: 21189453]

Levy JM, Thorburn A. Targeting autophagy during cancer therapy to improve clinical outcomes. Pharmacol Ther. 2011; 131:130–141. [PubMed: 21440002]

Li H, Wang P, Yu J, Zhang L. Cleaving Beclin 1 to suppress autophagy in chemotherapy-induced apoptosis. Autophagy. 2011; 7

Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, Thompson CB. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell. 2005; 120:237–248. [PubMed: 15680329]

Luo S, Garcia-Arencibia M, Zhao R, Puri C, Toh PPC, Sadiq O, Rubinsztein DC. Bim inhibits autophagy by recruiting Beclin 1 to microtubules. Molecular Cell. 2012; 47:359–370. [PubMed: 22742832]

Luo S, Rubinsztein DC. Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ. 2010; 17:268–277. [PubMed: 19713971]

Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinaki K, Tavernarakis N, et al. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. Embo J. 2007a; 26:2527–2539. [PubMed: 17446862]

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007b; 8:741–752. [PubMed: 17717517]

Nezis IP, Shravage BV, Sagona AP, Lamark T, Bjørkøy G, Johansen T, Rusten TE, Brech A, Baehrecke EH, Stenmark H. Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late Drosophila melanogaster oogenesis. J Cell Biol. 2010; 190:523–531. [PubMed: 20713604]

Noda NN, Ohsumi Y, Inagaki F. Atg8-family interacting motif crucial for selective autophagy. FEBS Lett. 2010; 584:1379–1385. [PubMed: 20083108]

Oberst A, Pop C, Tremblay AG, Blais V, Denault J-B, Salvesen GS, Green DR. Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. J Biol Chem. 2010; 285:16632–16642. [PubMed: 20308068]

Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit beclin 1-dependent autophagy. Cell. 2005; 122:927–939. [PubMed: 16179260]

Rehm M, Dussmann H, Prehn JH. Real-time single cell analysis of Smac/DIABLO release during apoptosis. J Cell Biol. 2003; 162:1031–1043. [PubMed: 12975347]

Rubinstein AD, Kimchi A. Life in the balance - a mechanistic view of the crosstalk between autophagy and apoptosis. J Cell Sci. 2012; 125:5259–5268. [PubMed: 23377657]

Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem. 2007; 282:5641–5652. [PubMed: 17182613]

Spencer SL, Gaudet S, Albeck JG, Burke JM, Sorger PK. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. Nature. 2009; 459:428–432. [PubMed: 19363473]

Staskiewicz L, Thorburn J, Morgan MJ, Thorburn A. Inhibiting autophagy by shRNA knockdown: Cautions and recommendations. Autophagy. 2013; 9

Yee K, Wilkinson S, James J, Ryan K, Vousden K. PUMA- and Bax-induced autophagy contributes to apoptosis. Cell Death Differ. 2009

Zhang F, Li Y, Tang Z, Kumar A, Lee C, Zhang L, Zhu C, Klotzsche-von Ameln A, Wang B, Gao Z, et al. Proliferative and survival effects of PUMA promote angiogenesis. Cell Rep. 2012; 2:1272–1285. [PubMed: 23122957]

Highlights

- Autophagy controls the timing of mitochondrial permeabilization (MOMP) in apoptosis.
- Autophagy promotes inefficient MOMP and can allow cellular recovery.
- Autophagy selectively regulates PUMA levels.
- PUMA depletion prevents sensitization to apoptosis by autophagy inhibition.

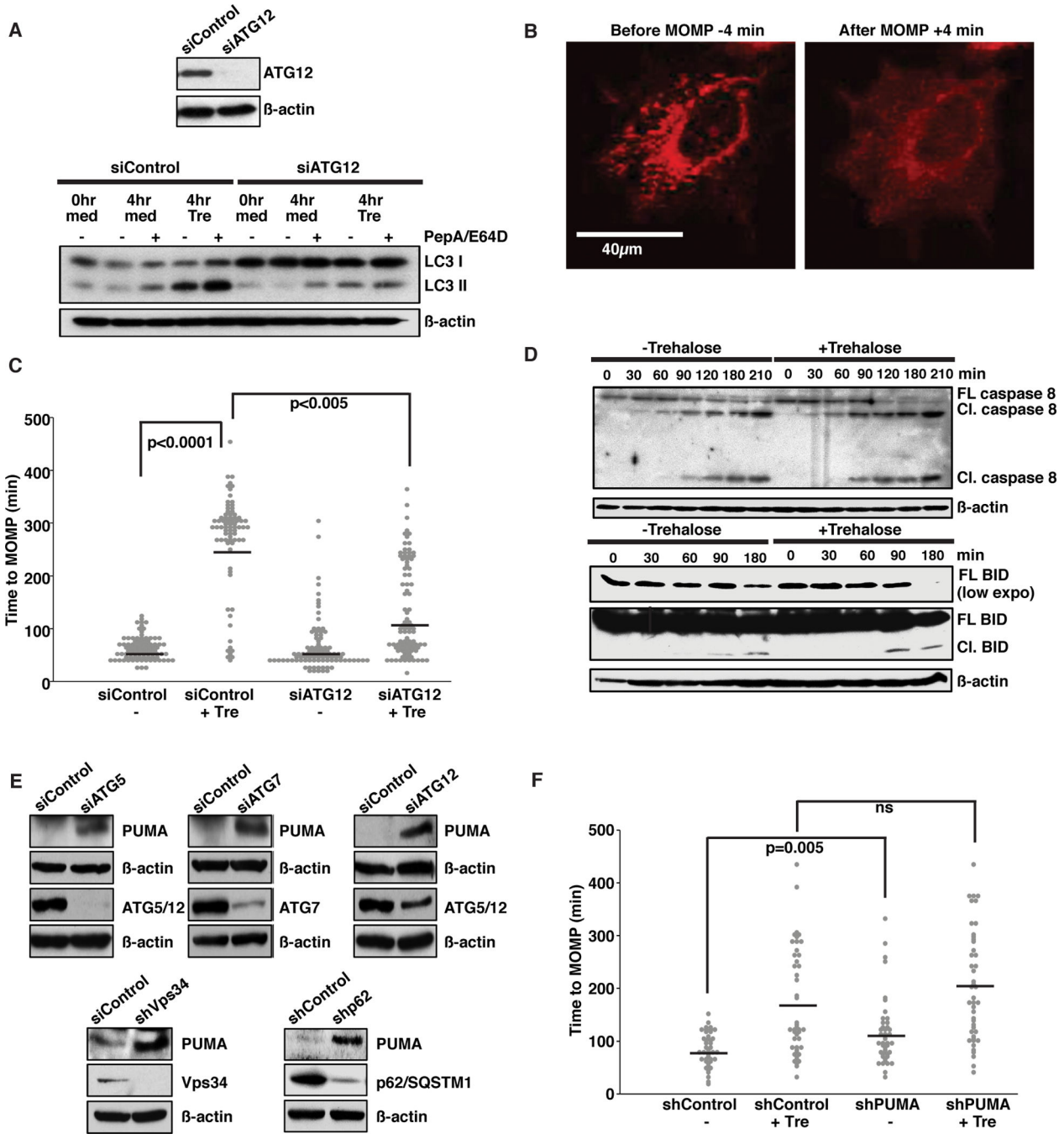

Figure 1. Autophagy regulates the timing of MOMP and PUMA levels

(A) siRNA targeting ATG12 in HeLa cells inhibits trehalose-induced (Tre) autophagy shown by reduced accumulation of LC3-II after treatment with lysosomal protease inhibitors Pepstatin and E64D.

(B) An example of complete and rapid MOMP visualized in HeLa cells stably expressing IMS-RP. Loss of punctate IMS-RP staining was used to determine when MOMP took place after TRAIL treatment.

(C) Trehalose treatment delays MOMP after TRAIL treatment (25ng/ml) and this delay is blocked by autophagy inhibition using ATG12 siRNA. Cells were imaged individually after treatment with or without trehalose and siRNA as indicated. Bars indicate the mean time to MOMP, p values by Student's t- test.

(D) Western blot analysis of time course of TRAIL-treated HeLa cells −/+ Trehalose to analyze cleavage of initiator caspase-8 or BID. Autophagy induction by trehalose did not delay caspase-8 or Bid cleavage.

(E) Autophagy inhibition by shRNA knockdown of ATG5, ATG7, ATG12, Vps34 or p62/SQSTM1 all cause an increase in PUMA levels.

(F) PUMA knockdown is sufficient to delay MOMP in response to TRAIL treatment. Cells were infected with shRNA targeting PUMA in the presence or absence of trehalose as indicated and time to MOMP determined by imaging individual cells. PUMA knockdown is sufficient delay MOMP similarly to trehalose. Bars indicate mean time to MOMP, p values were calculated by t-test, ns indicates $p>0.05$.

See also Supplementary Figures 1 and 2.

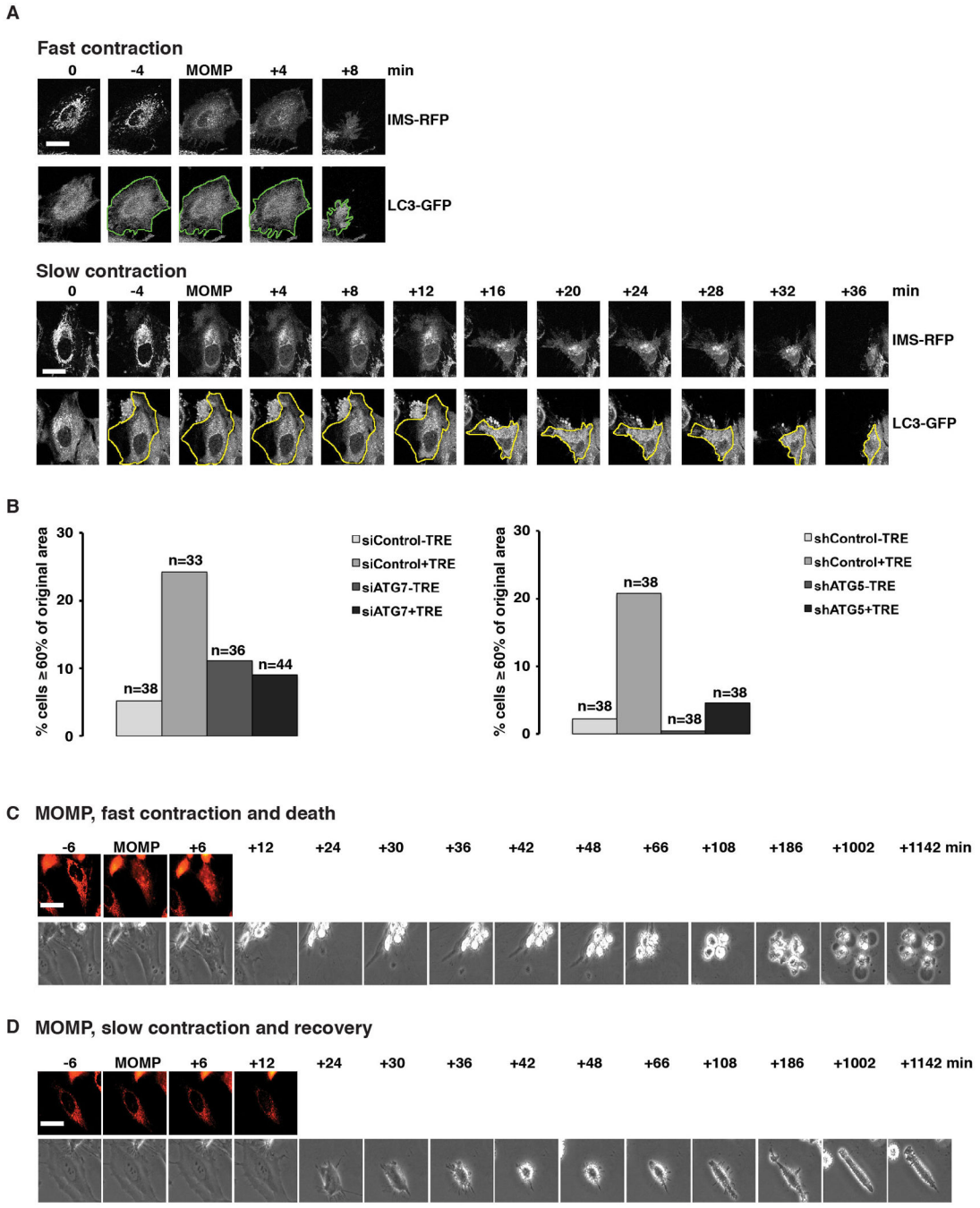

Figure 2. Autophagy is associated with slow cellular contraction after MOMP and cellular recovery

(A) Images from time-lapse series showing cellular contraction after MOMP displaying two distinct phenotypes. An example of rapid contraction and cellular breakup is shown in the upper series of images. An example of slower contraction after MOMP.

(B) Quantification of the percent of cells displaying slow contraction after MOMP defined as cell area ≥60% of the original area 20 minutes after MOMP/incomplete MOMP. Cells were treated with trehalose in the presence or absence of ATG5 or ATG7 shRNAs as indicated. Trehalose increases the number of slow contracting cells and this is abolished by autophagy inhibition.

(C) Continued monitoring of fast contracting cells after MOMP shows irreversible cell breakup and death from which they cannot recover.

(D) Continued monitoring of slow contracting cells shows that they can recover normal morphology hours after MOMP.

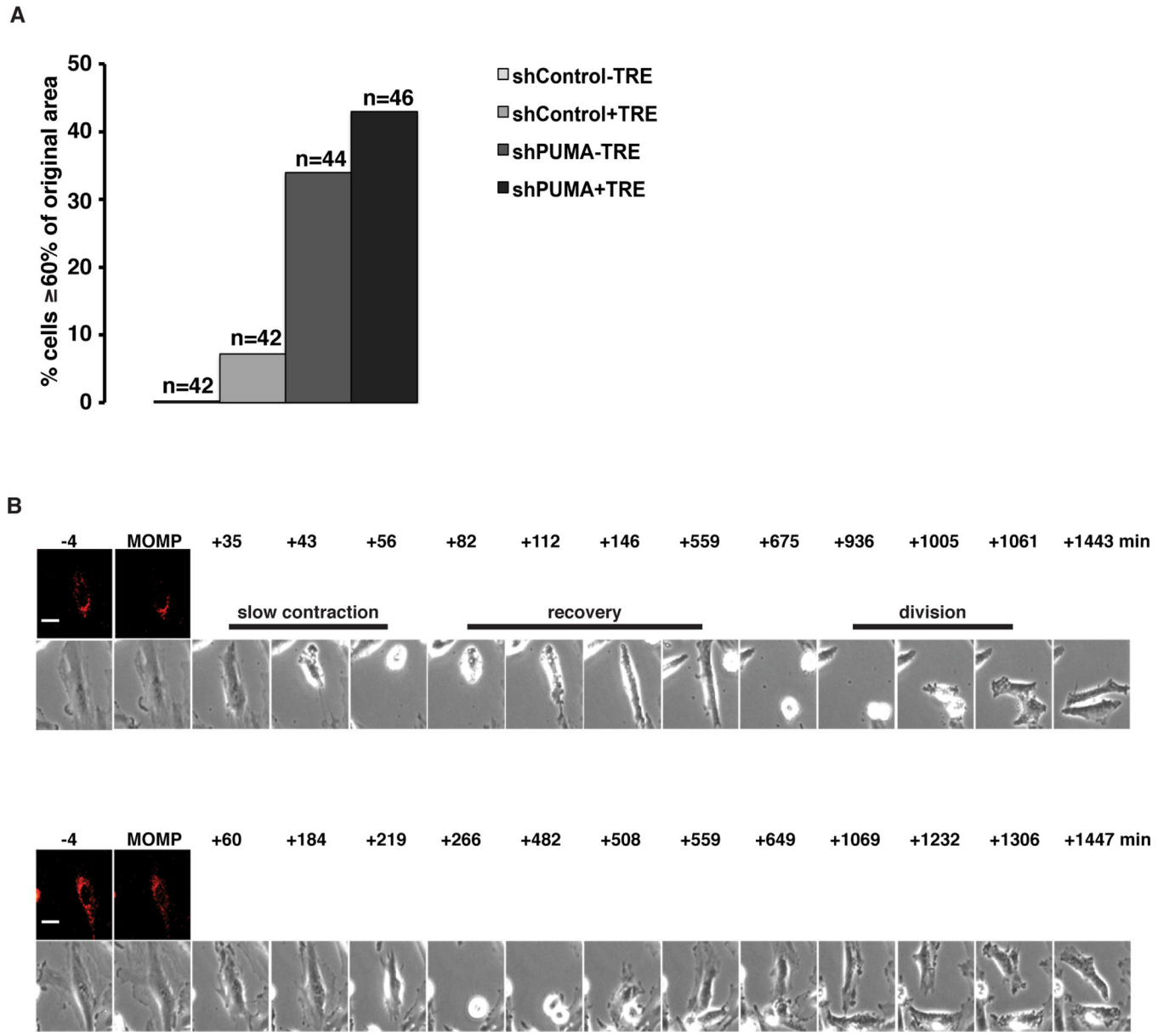

Figure 3. PUMA depletion is sufficient to increase the likelihood of slow cellular contraction, cell recovery and can allow division after MOMP

(A) Quantitation of cell area 20 minutes after MOMP in cells treated with trehalose in the presence or absence of PUMA knockdown. The percentage of cells displaying slow contraction is increased by PUMA knockdown alone.

(B) PUMA knockdown cells displaying slow contraction and recovery can go on to divide. Images from a time lapse experiment showing two cells that underwent MOMP followed by slow contraction, recovery of normal morphology and then cell division. See also Supplemental Movies 1 and 2.

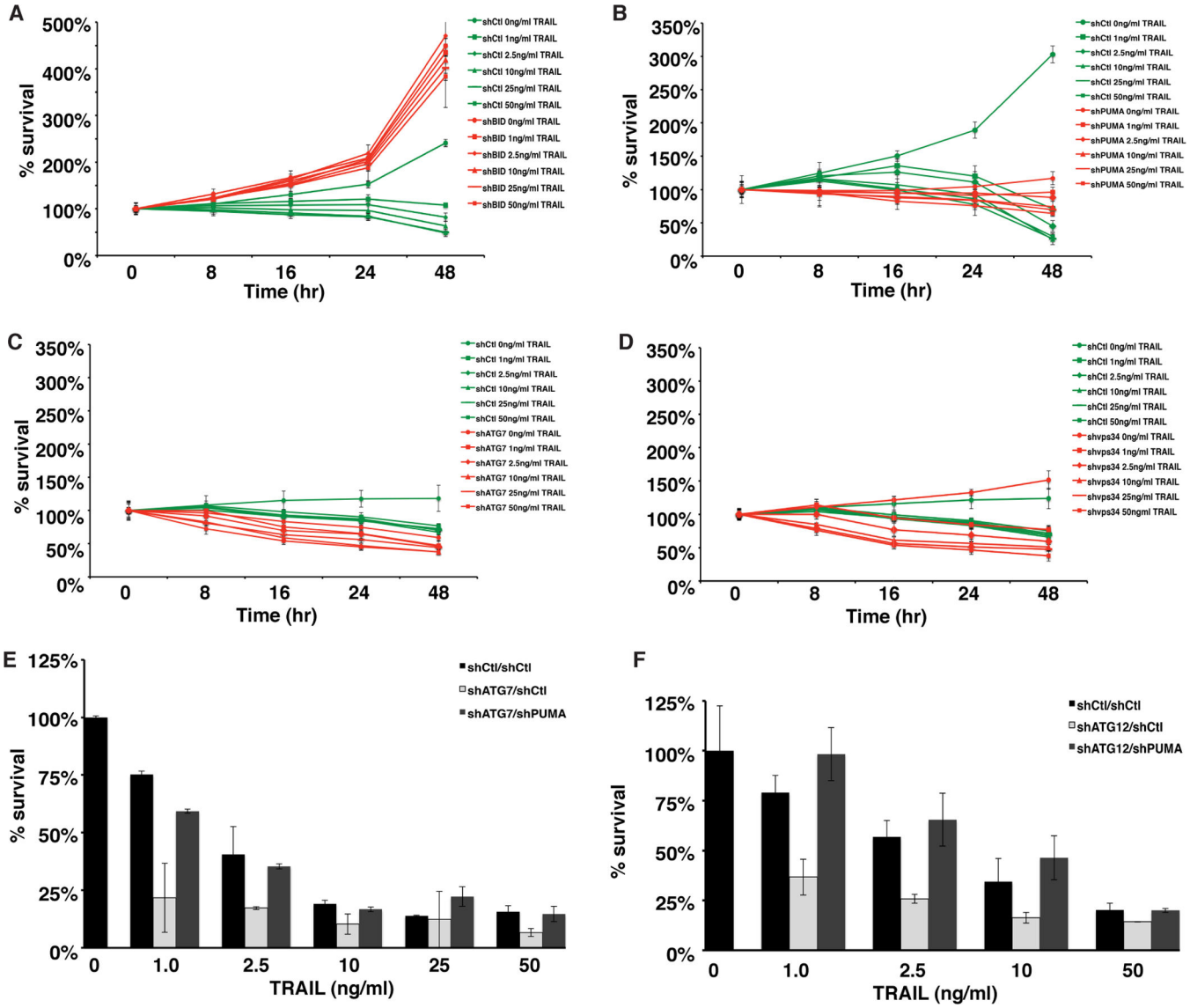

Figure 4. Autophagy regulation of long term cell viability after TRAIL-induced apoptosis is explained by regulation of PUMA

(A) Quantitation of cell viability by continuous monitoring of mixed populations of control (Green) or shRNA knockdown (Red) cells. HeLa cells were marked with nuclear GFP or mCherry and cell viability after treatment with different doses of TRAIL was determined by continual monitoring using the IncuCyte system. shRNA knockdown of BID completely protects against TRAIL induced death.

(B) shRNA knockdown of PUMA partially inhibits TRAIL induced apoptosis.

(C) shRNA knockdown of ATG7 to inhibit autophagy potentiates TRAIL-induced apoptosis.

(D) shRNA knockdown of Vps34 to inhibit autophagy potentiates TRAIL-induced apoptosis.

(E) Cell viability quantified by the IncuCyte at different doses of TRAIL was determined in matched control, ATG7 or ATG7/PUMA knockdown cells. Autophagy inhibition by ATG7 knockdown increases TRAIL induced apoptosis and this is rescued by simultaneous knockdown of PUMA. At higher doses of TRAIL, the protective effect of autophagy inhibition is reduced.

(F) Cell viability quantified by the IncuCyte in matched control, ATG12 or ATG12/PUMA knockdown cells. Autophagy inhibition by ATG12 knockdown increases TRAIL induced apoptosis that is rescued by simultaneous knockdown of PUMA.

See also Supplemental Figures 3 and 4.