The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death

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Summary

Autophagic cell death is morphologically characterized by an accumulation of autophagic vacuoles. Here, we show that inactivation of LAMP2 by RNA interference or by homologous recombination leads to autophagic vacuolization in nutrient-depleted cells. Cells that lack LAMP2 expression showed an enhanced accumulation of vacuoles carrying the marker LC3, yet a decreased colocalization of LC3 and lysosomes, suggesting that the fusion between autophagic vacuoles and lysosomes was inhibited. While a fraction of mitochondria from starved LAMP2-expressing cells colocalized with lysosomal markers, within autophagolysosomes, no such colocalization was found on removal of LAMP2 from the experimental system. Of note, LAMP1 depletion had no such effects and did not aggravate the phenotype induced by LAMP2-specific small interfering RNA. Serum and amino acid-starved LAMP2-negative cells exhibited an accumulation of autophagic vacuoles and then succumbed to cell death with hallmarks of apoptosis such as loss of the mitochondrial transmembrane potential, caspase activation and chromatin condensation. While caspase inhibition retarded cell death, it had no protective effect on mitochondria. Stabilization of mitochondria by overexpression of Bcl-2 or the mitochondrion-targeted cytomegalovirus protein vMIA, however, blocked all signs of apoptosis. Neither caspase inhibition nor mitochondrial stabilization antagonized autophagic vacuolization in LAMP2-deficient cells. Altogether, these data indicate that accumulation of autophagic vacuoles can precede apoptotic cell death. These findings argue against the clear-cut distinction between type 1 (apoptotic) and type 2 (autophagic) cell death.

Key words: Bcl-2, caspases, LAMP1, LAMP2, Lysosomes, Mitochondria

Introduction

According to conventional textbooks, there are two major types of programmed cell death (PCD), type 1 or apoptotic cell death and type 2 or autophagic cell death, both of which are defined by morphological criteria. Type 1 cell death is defined morphologically by nuclear condensation (pyknosis) and fragmentation (karyorhexis), without major ultrastructural changes of cytoplasmic organelles. Type 2 cell death is characterized by accumulation of autophagic vacuoles in the cytoplasm (Bursch, 2001; Edinger and Thompson, 2004; Leist and Jaattela, 2001; Lockshin and Zakeri, 2001). Type 1 cell death is thought to involve the activation of caspases as well as a stereotyped pattern of mitochondrial alterations leading to the release of caspase activators and caspase-independent death effectors that contribute to the acquisition of the apoptotic morphology (Adams, 2003; Green and Kroemer, 2004; Wang, 2002). It is less clear, however, to which extent macroautophagy actually contributes to type 2 cell death. Macroautophagy (which we will refer to as 'autophagy') involves the sequestration of cytosol or cytoplasmic organelles within double membranes, thus creating autophagosomes (also called autophagic vacuoles). Autophagosomes subsequently fuse with endosomes and eventually with lysosomes, thereby creating autophagolysosomes or autolysosomes. In the lumen of these latter structures, lysosomal enzymes operating at low pH then catabolize the autophagic material (Levine and Klionsky, 2004; Shintani and Klionsky, 2004). Several phylogenetically conserved genes (so called *Atg* genes) are strictly required for autophagy in yeast (Klionsky et al., 2003) and also in mammalian cells, as this has been shown for *Atg5* (Mizushima et al., 2001), *Atg6/Beclin* (Kihara et al., 2001; Qu et al., 2003; Yue et al., 2003), *Atg8/LC3* (Kabeya et al., 2000), *Atg10* (Nemoto et al., 2003) and *Atg12* (Boya et al., 2005).

Type 2 cell death has been described in clinically relevant circumstances – for example, in neurodegeneration, retinal degeneration (Shintani and Klionsky, 2004), bacterial infection (Nakagawa et al., 2004), and tumor cells succumbing to chemotherapy in vitro (Daido et al., 2004; Kanzawa et al., 2004; Opipari et al., 2004). However, functional reports indicating that silencing of *Atg* genes can reduce cell death are scarce. Thus, small interfering RNA (siRNA) specific for *Atg* genes can inhibit the autophagic cell death of L929 cells

induced by the pan-caspase inhibitor Z-VAD-fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) (Yu et al., 2004) as well as that of $Bax^{-/-}Bak^{-/-}$ mouse embryonic fibroblasts (MEF) treated with etoposide (Shimizu et al., 2004). Nonetheless, there is also evidence that autophagy can exert a cytoprotective function (Alva et al., 2004; Shintani and Klionsky, 2004) – for example, when the digestion of endogenous macromolecules compensates for the shortage of exogenous metabolites (Kuma et al., 2004) and/or when damaged organelles including mitochondria have to be removed (Rodriguez-Enriquez et al., 2004; Teckman et al., 2004). Thus, in conditions of nutrient depletion, inhibition of autophagy can induce cell death, and this cell death depends on mitochondrial outer membrane permeabilization and subsequent caspase activation (Boya et al., 2005). The pro-survival effect of autophagy was revealed by inhibiting the formation of autophagic vacuoles – for example, by siRNA specific for Atg5, Atg6/Beclin, Atg10 and Atg12 or by the addition of the pharmacological autophagy inhibitor 3-methyladenine (Boya et al., 2005). In these conditions (that is, starvation + siRNA specific for Atg5, Atg6/Beclin, Atg10 and Atg12), cells directly succumbed to apoptotic cell death, without any signs of autophagic vacuolization (Boya et al., 2005). However, lysosomotropic agents that reduce the lysosomal pH and/or destabilize lysosomal membranes (such as hydroxychloroquine, bafilomycin A1 or monensin) (Boya et al., 2003b) inhibited the fusion of lysosomes with autophagic vacuoles and hence produced a different morphotype. In the presence of these lysosomotropic agents, autophagic vacuoles progressively accumulated in the cytoplasm of starving cells, thus producing a morphology that initially resembled autophagic cell death. With prolonged starvation and lysosomal inhibition, such cells acquired features of apoptosis including nuclear pyknosis and karyorhexis (Boya et al., 2005). This result suggested a possible shift from type 1 cell death to type 2 cell death, yet was based on the utilization of pharmacological agents with limited specificity.

On the basis of these premises, we looked for a genetic manipulation that might block the fusion between autophagic vacuoles and lysosomes in a more specific fashion. As shown here, targeting of LAMP2 can inhibit the fusion of autophagosomes and lysosomes required for the late stage of the autophagic process. Lysosome-associated membrane proteins-1 and -2 (LAMP1, LAMP2) are homologous C-type transmembrane proteins (37% identity in humans) specific for lysosomes. LAMP1-deficient mice are viable and fertile (Andrejewski et al., 1999). However, the knockout of LAMP2 causes embryonic lethality and the massive accumulation of autophagic vacuoles in various tissues (Eskelinen et al., 2002; Tanaka et al., 2000). Loss of LAMP2 is also at the etiology of Danon disease, an X-linked lysosomal glycogen storage disease affecting infants or adolescents with a clinical triad of cardiomyopathy, myopathy and mental retardation (Nishino et al., 2000). LAMP2 loss does not lead to a major perturbation of lysosomal structure and function, except a mild accumulation of cholesterol (Eskelinen et al., 2002; Tanaka et al., 2000). The double knockout (DKO) of LAMP1 and LAMP2 causes a decrease in lysosomal density and a change in cholesterol traffic, which is detectable as cholesterol accumulation in late endosomal/lysosomal vesicles in MEF (Eskelinen et al., 2004).

Here, we show that targeting of LAMP2 expression by RNA interference inhibits the fusion of lysosomes and autophagic vacuoles, thereby increasing the number of autophagic vacuoles in conditions of prolonged nutrient starvation. In these conditions, cells progressively adopt characteristics of type 1 and type 2 cell death. This argues against the formal, sharp distinction between the two death modalities.

Materials and Methods

Cell lines and culture conditions

HeLa cells were stably transfected with pcDNA3.1 control vector (Neo), with human Bcl-2 (Bcl-2) or the cytomegalovirus UL37 exon 1 gene coding for vMIA (viral mitochondrial inhibitor of apoptosis, kindly provided by V. Goldmacher, Immunogen Inc., Cambridge, MA) (Belzacq et al., 2001; Goldmacher et al., 1999; Vieira et al., 2001). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% fetal calf serum (FCS), 1 mM pyruvate and 10 mM Hepes at 37°C under 5% CO_2. Mouse embryonic fibroblasts (MEF) were immortalized by transfection with a plasmid containing the Simian Virus 40 large T antigen. The MEF genotype was either wild type (WT) or double knockout (DKO) for LAMP1 and LAMP2 (Eskelinen et al., 2004). These cells were grown in DMEM supplemented with 10% FCS and antibiotics at 37°C under 5% CO_2. Serum and amino acid starvation of cells was performed using serum-free Earle's Balanced Salt Solution medium (Sigma) (Boya et al., 2005). For caspase inhibition, a sublethal dose of Z-VAD-fmk (25 μM, Bachem) was added at the same time as amino acid starvation of cells.

Transfection and RNA interference

siRNAs for human *lamp1* and *lamp2* (National Center for Biotechnology Information, accession numbers NM_005561 and NM_002294) were synthesized by Proligo France, or Eurogentec Belgium. For *lamp1*, the siRNA sequence of the + strand started at position 311 (AGAAAUGCAACACGUUA, LAMP1A) or at position 904 (GGAAUCCAGUUGAAUACAA, LAMP1B). For *lamp2* the siRNA sequences were chosen to start at position 64 (GCUGUGCGGUCUUAUGCAU, LAMP2-64) and at position 69 (GCGGUCUUAUGCAUUGGAA, LAMP2-69). As a control, we targeted the nuclear envelope protein Emerin, whose knockdown does not induce any known phenotype (Harborth et al., 2001). The concentrations of LAMP siRNA was 100 nM in single siRNA experiments, as well as in double siRNA experiments, whereas that of Emerin siRNA was 200 nM. Cells were cultured and transfected with siRNAs at 80% confluence with Oligofectamine reagent (InVitrogen). Transient transfections with cDNAs was performed with Lipofectamine 2000™ (Invitrogen) to label mitochondria with mt-dsRed plasmid (Clontech), lysosomes with SytVII-GFP (synaptotagmin VII-green fluorescent protein) plasmid (kindly provided by N. W. Andrews, Yale University, New Haven, CT) (Martinez et al., 2000) and autophagic vacuoles with LC3-GFP plasmid (Kabeya et al., 2000).

Flow cytometry

To determine apoptosis-associated changes by cytofluorometry we used 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 40 nM) for the mitochondrial transmembrane potential ($\Delta\Psi_m$) quantification, and propidium iodide (PI, 1 μg/ml) for determination of cell viability (all of them from Molecular Probes) (Boya et al., 2003c; Castedo et al., 2002a). After different experimental conditions, cells were trypsinized and incubated with the fluorochromes for 15 minutes at 37°C, followed by cytofluorometric analysis with a FACS Scan (Becton Dickinson).

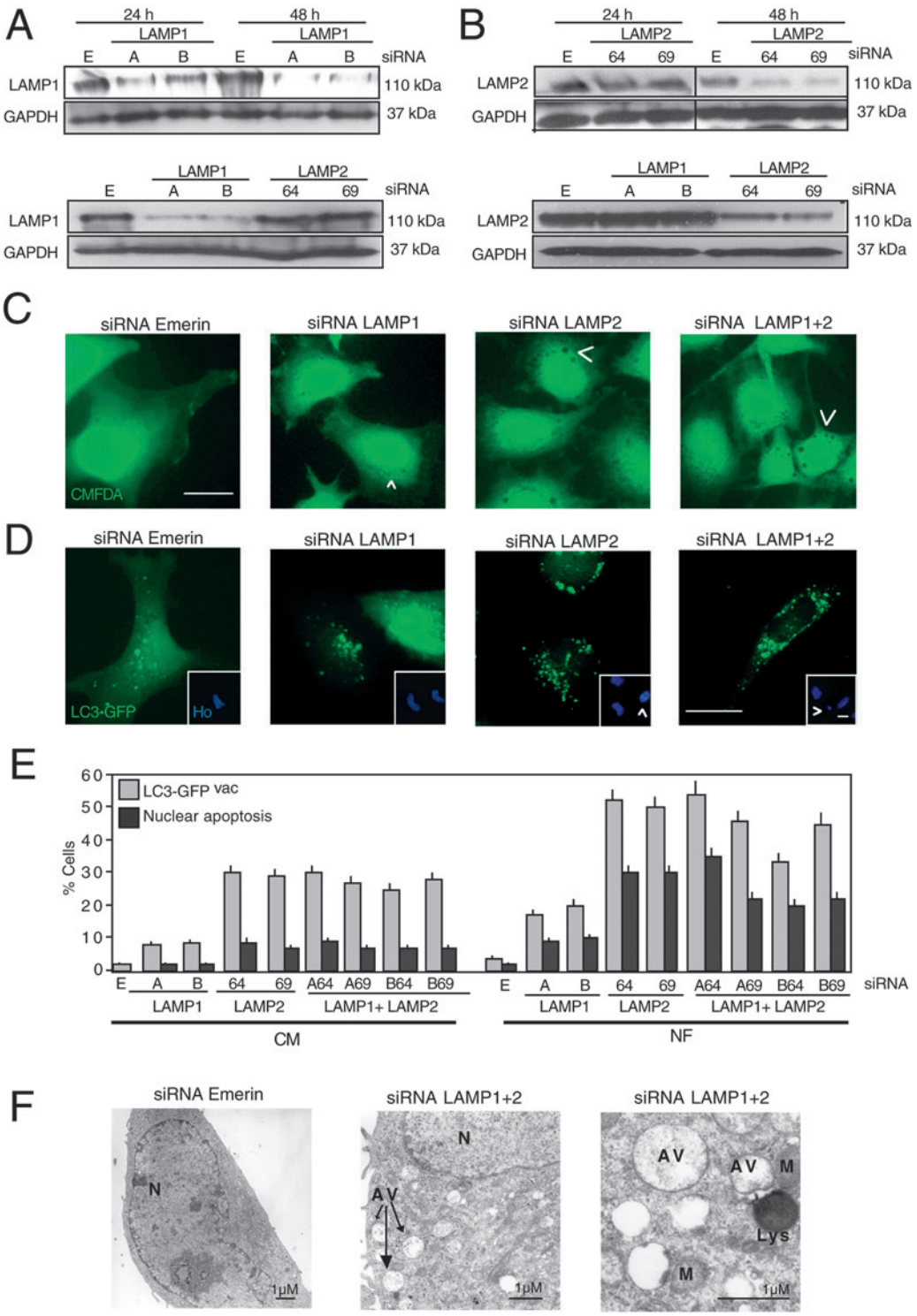

Fig. 1. LAMP2 knockdown sensitizes to vacuolization and cell death by nutrient depletion. (A,B) Immunoblot confirmation of the efficacy of LAMP1- (A) or LAMP2-specific (B) small interfering RNA (siRNA). HeLa cells were transfected with the indicated constructs specific for emerin (E), LAMP1 (A,B), and LAMP2 (64, 69), and the expression level of LAMP1 or LAMP2 was determined by immunoblot, after 24 or 48 hours. GAPDH expression was determined as a loading control. (C) Vacuolization induced by LAMP2 depletion. Twenty-four hours after transfection, the cells were cultured for 16 hours in nutrient-free (NF) medium – that is, in the absence of serum and amino acids, and then stained with CMFDA. Cytoplasmic vacuoles are visualized as holes. (D,E) Vesicular LC3 accumulation induced by LAMP2 depletion. Cells were transfected with siRNAs, then 24 hours later with an LC3-GFP fusion construct, and were nutrient deprived for 16 hours. Representative fluorescence microphotographs are shown in D, and the frequency of cells exhibiting the accumulation of LC3-GFP in vacuoles and apoptotic chromatin condensation (as determined by counterstaining with Hoechst 33342) was quantified (X±s.d., n=3) in E. Data in C and D are shown for LAMP1-A and LAMP2-64 siRNAs, and similar results were obtained for the LAMP1-B and LAMP2-69 siRNAs. (F) Electron microscopy of representative cells 6 hours after nutrient and serum depletion after siRNA specific for emerin (right) or siRNA specific for LAMP2 (64)+LAMP1 (A). Bars in C and D, 10 μm.

Light microscopy and immunofluorescence

Cells cultured on coverslips were stained with CMFDA (5-chloromethylfluorescein diacetate, 1 μM) (Molecular Probes) and Hoechst 33342 (Ho) (Sigma, 2 μM). To label lysosomes, Lysotracker Red (500 nM) (Molecular Probes) was added to cultures for 30 minutes. Alternatively, cells were fixed with paraformaldehyde (4% w:v) for LC3-GFP, SytVII-GFP, mt-dsRed and immunofluorescence assays (Castedo et al., 2001; Castedo et al., 2002b). Cells were stained for the detection of activated caspase-3 with a polyclonal antibody from Cell Signaling Technology developed with an anti-rabbit immunoglobulin Alexa® fluor conjugate (Molecular Probes). Fluorescence microscopy was analyzed with a Leica IRE2 equipped with a DC300F camera. Confocal microscopy was performed with a LSM 510 Zeiss microscope equipped with a 63× objective. To determine the percentage of colocalization images were loaded into Image J software (http://rsb.nih.gov/ij).

Western blot analysis

Cells were washed by cold PBS at 4°C and lysed in a buffer containing 50 mM Tris HCl pH 6.8, glycerol 10%, 2% SDS, 10 mM DTT and 0.005% bromophenol blue. Forty micrograms of protein were loaded on a 10% SDS-PAGE and transferred to nitrocellulose. The membrane was incubated for 1 hour in PBS-Tween 20 (0.05%) containing 5% nonfat milk. Primary antibodies (anti-LAMP2 mAb H4B4 and LAMP1 mouse IgG2b) (from ABR Affinity BioReagents and BD Transduction Laboratories, respectively) were incubated for 15 hours at 4°C and detected with the appropriated horseradish peroxidase-labeled secondary antibodies (Southern Biotechnologies Associates) and revealed by SuperSignal West Pico chemoluminiscent substrate (Pierce). Anti-GAPDH (Chemicon) was used to control equal loading.

Electron microscopy

Cells were fixed for 1 hour at 4°C in 1.6% glutaraldehyde in 0.1 M Sörensen phosphate buffer (pH 7.3), washed, fixed again in aqueous 2% osmium tetroxide and finally embedded in Epon. Electron microscopy was performed with a Zeiss EM 902 transmission electron microscope, at 90 kV, on ultrathin sections (80 nm) stained with lead citrate and uranyl acetate.

Statistical analysis

All the data are representative of at least three independent experiments (with comparable results). Statistical significance of the data was evaluated after the calculation of one way analysis of variance (ANOVA). Values of $P<0.05$ were considered statistically significant.

Results

LAMP2 depletion sensitizes to starvation-induced cell death with autophagic and apoptotic characteristics

HeLa cells were subjected to RNA interference specific for the two lysosomal proteins LAMP1 and LAMP2 (or Emerin as a control) (Harborth et al., 2001) using a panel of small interfering RNAs (siRNA), which acted in a specific fashion (Fig. 1A,B). While such LAMP1- or LAMP2-specific siRNAs had no major effects on cellular proliferation, morphology or viability within the first 48 hours after transfection, they did induce the formation of cytoplasmic vacuoles in conditions of starvation (Fig. 1C). In nutrient-free (NF) medium – that is, in the absence of serum and amino acids – control cells did not develop any discernible vacuoles. In these conditions, LAMP1 knockdown cells manifested rather small cytoplasmic vacuoles, while LAMP2 knockdown cells exhibited a higher degree of vacuolization. No additive effect was observed for

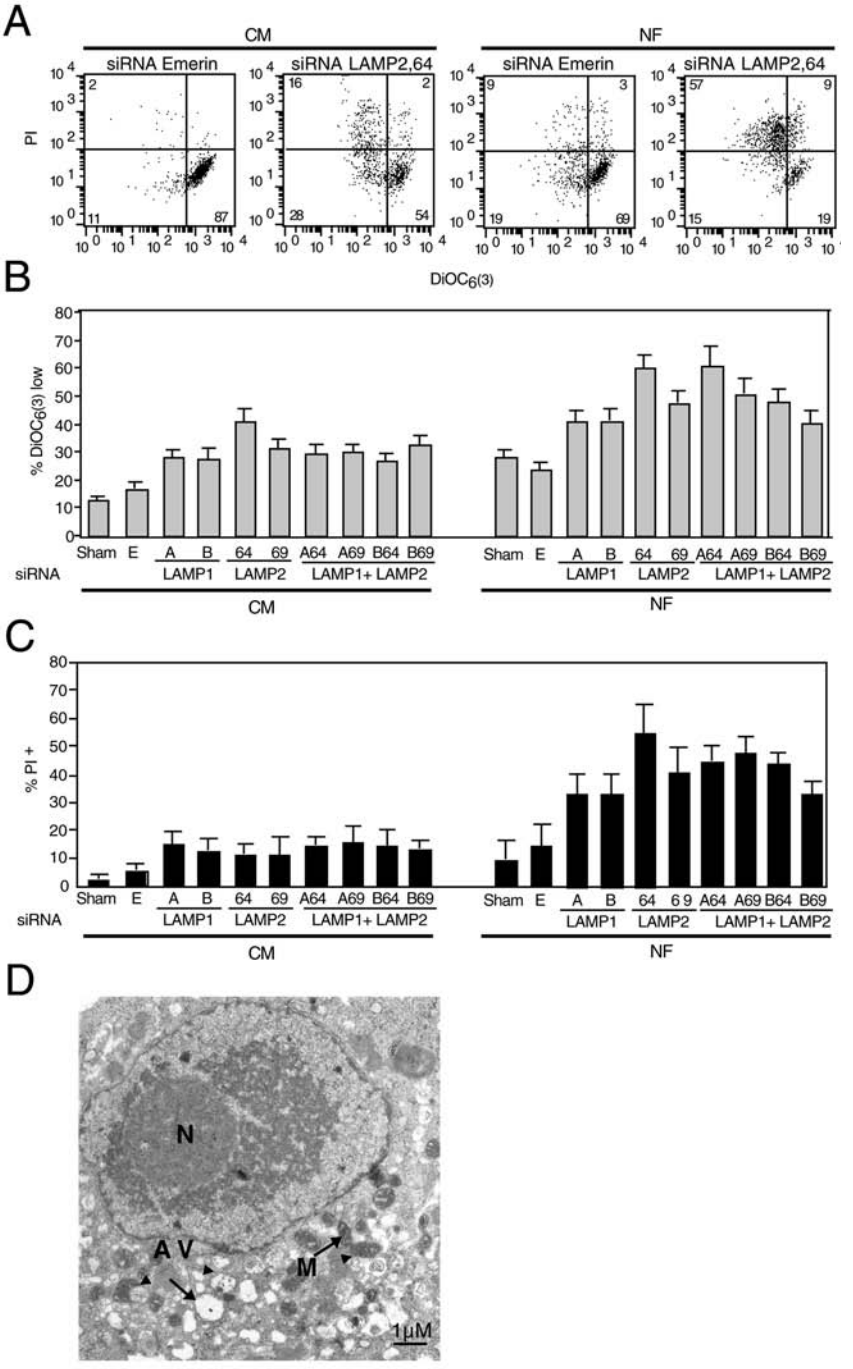

Fig. 2. Effect of siRNA specific for LAMP1 and/or LAMP2 on cell death induced by serum and amino acid depletion. Cells transfected with the indicated siRNAs (24 hours) were cultured in complete medium (CM) or nutrient-free (NF) medium for 24 hours, followed by staining with propidium iodine (PI) and $DiOC_6(3)$ for determination of viability and the mitochondrial transmembrane potential, respectively. Representative FACS pictograms are shown in A. Numbers refer to the percentage of cells in each quadrant. Note that PI^+ cells tend to be $DiOC_6(3)^{low}$, meaning that the former can be represented as a subpopulation of the latter. Results are means (±s.d.) of three independent experiments. The percentage of $DiOC_6(3)^{low}$ cells and PI^+ cells obtained in response to a variety of different siRNAs are shown in B and C, respectively. An electron microscopic picture of a representative cell subjected to LAMP2 siRNA (64) and nutrient depletion (20 hours) is shown in D. N, nucleus; AV, antophagic vacuole; M, mitochondrion.

the double knockdown of LAMP1 and LAMP2 (Fig. 1C). Similarly, after 16 hours of serum and amino acid starvation, LAMP1- and LAMP2-specific siRNA both induced the cytoplasmic accumulation of the autophagic vacuole marker LC3-GFP in discrete foci (Kabeya et al., 2000; Mizushima et al., 2004). These LC3-GFP foci were more pronounced for LAMP2 siRNA than for LAMP1 siRNA, and no synergic effects were observed when both LAMP1 and LAMP2 were knocked down (Fig. 1D,E). The knockdown of LAMP2 (alone or together with LAMP1) clearly induced the accumulation of autophagic vacuoles with their characteristic double membranes, as visible by transmission electron microscopy (Fig. 1F).

Upon close inspection, we found that a subpopulation of LAMP2-depleted cells exhibited chromatin condensation, as detectable with the nuclear stain Hoechst 33342 (Fig. 1D,E). Cytofluorometric quantification revealed that a significant fraction of starved LAMP2 knockdown cells lost the capacity to retain the dye $DiOC_6(3)$ and hence dissipated the mitochondrial transmembrane potential ($\Delta\Psi_m$) (Fig. 2A,B). Among this $\Delta\Psi_m^{low}$ population, a fraction of cells incorporated the vital dye propidium iodide (PI) and thus lost the barrier function of their plasma membrane (Fig. 2A). The frequency of dead (PI^+) cells increased with concurrent depletion of LAMP2 and nutrients (Fig. 2C). Again, these effects (enhanced nuclear condensation, $\Delta\Psi_m$ loss and cell death in response to starvation) were less pronounced for LAMP1 depletion than for LAMP2 knockdown (Fig. 2). Electron microscopic examination of cells dying after LAMP2 depletion and nutrient depletion revealed typical signs of nuclear apoptosis (pyknosis and karyorhexis), as well as a pronounced vacuolization of the cytoplasm (Fig. 2D).

To exclude cell type- or species-specific effects and siRNA-associated artifacts, we repeated these experiments with mouse embryonic fibroblasts (MEF) with a double knockout (DKO) genotype for both LAMP1 and LAMP2. When compared with wild-type controls, starved DKO cells exhibited an increase in cytoplasmic vacuolization (Fig. 3A) and in the accumulation of LC3-GFP in autophagic vacuoles (Fig. 3B). Moreover, starved DKO cells showed an increase in Hoechst 33342-detectable chromatin condensation (Fig. 3C), an accelerated $\Delta\Psi_m$ loss and an elevated PI permeability, as compared with

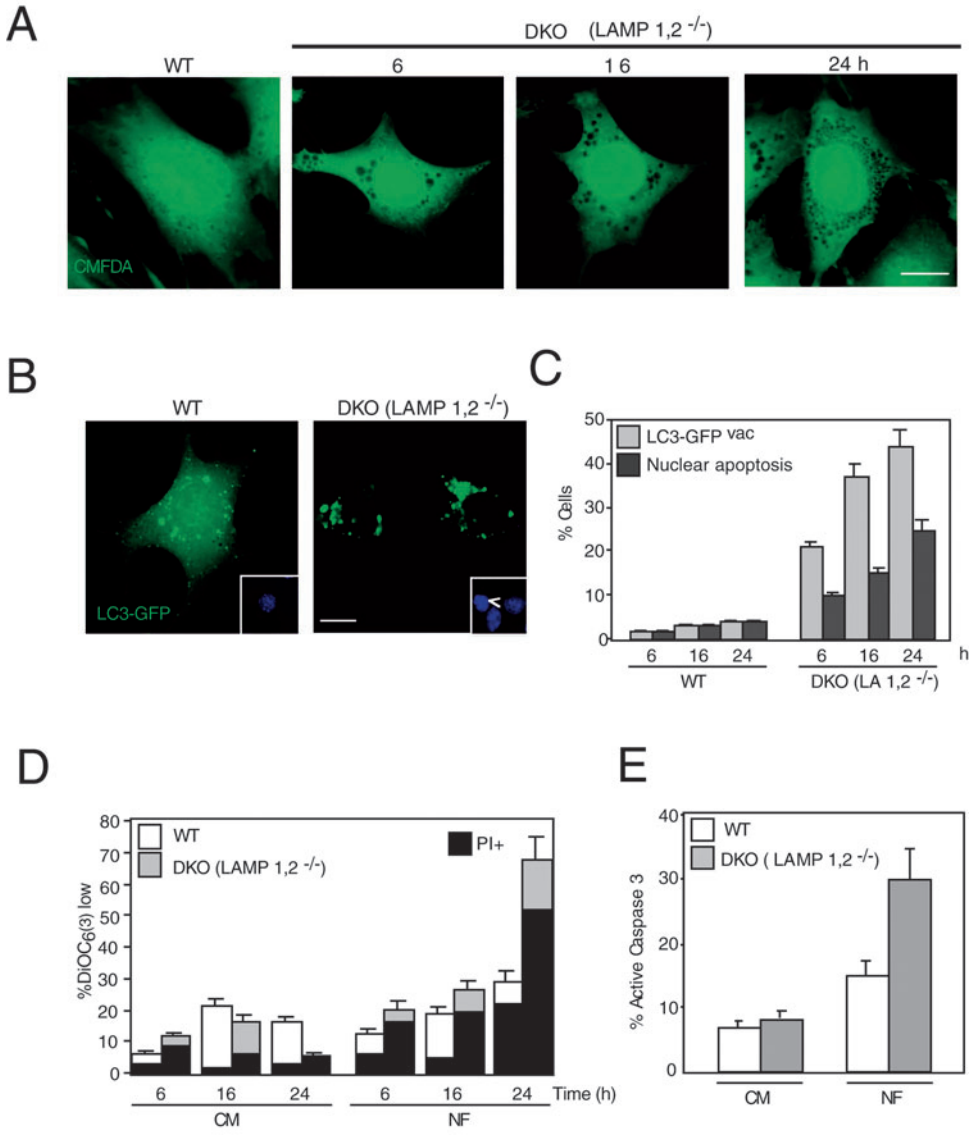

Fig. 3. Knockout of LAMP1 and LAMP2 sensitizes to nutrient depletion-induced cell death. (A) Cytoplasmic vacuolization induced by nutrient depletion in mouse embryonic fibroblasts (MEF) with a normal genotype (WT) or lacking both LAMP1 and LAMP2 (double knockout, DKO). Cytoplasmic vacuoles are visualized as CMFDA-negative holes. (B, C) LC3 accumulation observable in DKO cells. WT or DKO cells were first transfected with LC3-GFP (24 hours) and then nutrient-depleted for 15 hours (B) or for the indicated period (C), followed by quantitation of the frequency of cells with LC3 accumulation in cytoplasmic vacuoles and that of cells with manifest chromatin condensation, as detectable by counterstaining with Hoechst 33342. (D) WT or DKO cells were cultured in complete medium (CM) or nutrient-free (NF) medium for the indicated time, stained with PI and $DiOC_6(3)$ and subjected to cytofluorometric analysis (X±s.d., *n*=3). (E) Activation of caspase-3 WT and DKO cells, 24 hours after culture in the presence or absence of nutrients. Cells were stained with an antibody recognizing mature, cleaved caspase-3 and the percentage of cells containing active caspase-3 was quantified by immunofluorescence. Bar in A and B, 10 μm.

wild-type controls (Fig. 3D). The combination of starvation and LAMP2 removal induced activation of caspase-3, both in MEF (Fig. 3E) and in HeLa cells (see below). Altogether, the microscopic observations, as well as the biochemical analyses, indicate that starvation plus LAMP2 knockdown induces autophagic vacuolization followed by chromatin condensation and characteristics of apoptotic cell death. This process thus combines features of type 1 and type 2 cell death.

LAMP2 depletion blocks the colocalization of autophagosomal and lysosomal markers

LAMP2 may be involved in lysosomal biogenesis and/or the fusion between autophagosomes and lysosomes required for the final catabolism of autophagic material (Eskelinen et al., 2002; Eskelinen et al., 2004; Levine and Klionsky, 2004; Shintani and Klionsky, 2004; Tanaka et al., 2000). To discriminate between these possibilities, we labeled cells with an acidophilic, lysosomotropic agent, Lysotracker® Red (LTR), and simultaneously tracked autophagic vacuoles with LC3-GFP. LAMP2 (or LAMP1) knockdown had no negative effect on the intensity of LTR staining, neither in normal nor in starving conditions (Fig. 4A and data not shown). However, LAMP2-specific siRNA, alone or in combination with LAMP1-specific siRNA (but not LAMP1-specific siRNA alone), reduced the colocalization of LC3-GFP and LTR induced by starvation (Fig. 4A,B). This suggests that LAMP2 (but not LAMP1) is required for the formation of autophagolysosomes. Mitochondria are known to undergo progressive autophagic depletion in conditions of nutrient or growth factor depletion (Chang et al., 2003; Rodriguez-Enriquez et al., 2004; Xue et al., 2001). We therefore measured the colocalization of a mitochondrial marker (mt-DsRed) and that of a lysosomal marker (synaptotagmin VII fused to GFP, SytVII-GFP) (Martinez et al., 2000) in starving cells in which either of the two LAMP proteins were knocked down. After nutrient depletion, a fraction of mitochondria colocalized with SytVII-GFP in control cells treated with the control siRNA

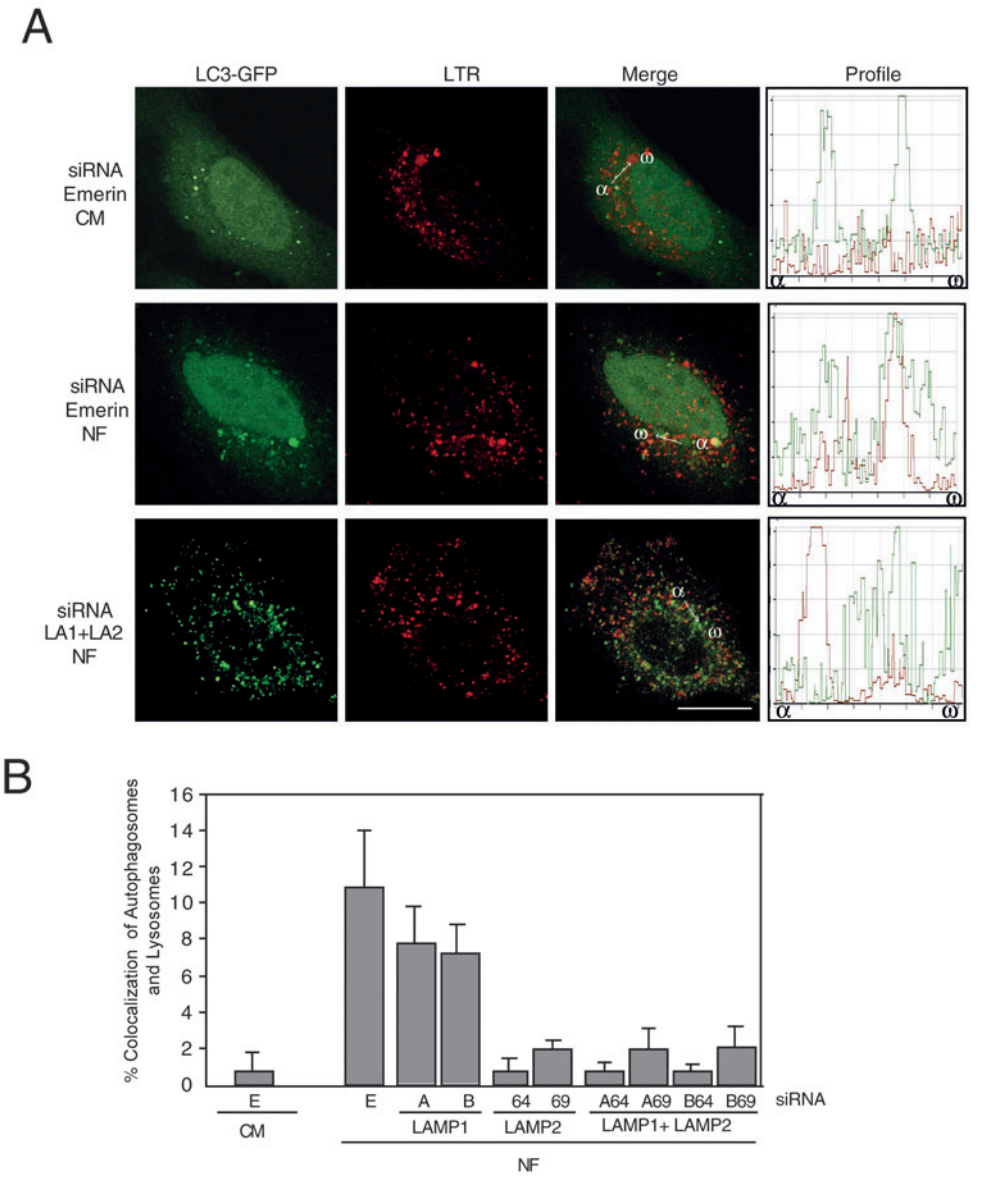

Fig. 4. Colocalization of the autophagic marker LC3-GFP and LTR on nutrient depletion in controls and LAMP1 or LAMP2 knockdown cells. HeLa cells were transfected with siRNA specific for the indicated gene products (24 hours), transfected with LC3-GFP (24 hours), and cultured in complete medium (CM) of nutrient-free medium (NF) for 4 hours, followed by staining with lysotracker (LTR, 30 minutes) red and confocal scanning fluorescence microscopy. Representative cells are shown in A. Bar, 10 μm. The graphs (right panels) represent the fluorescence distribution determined for section of the cells, as indicated by the orientation of the arrow. The degree of colocalization (X±s.d.) was determined by image analysis for 50 cells each, as indicated in Materials and Methods.

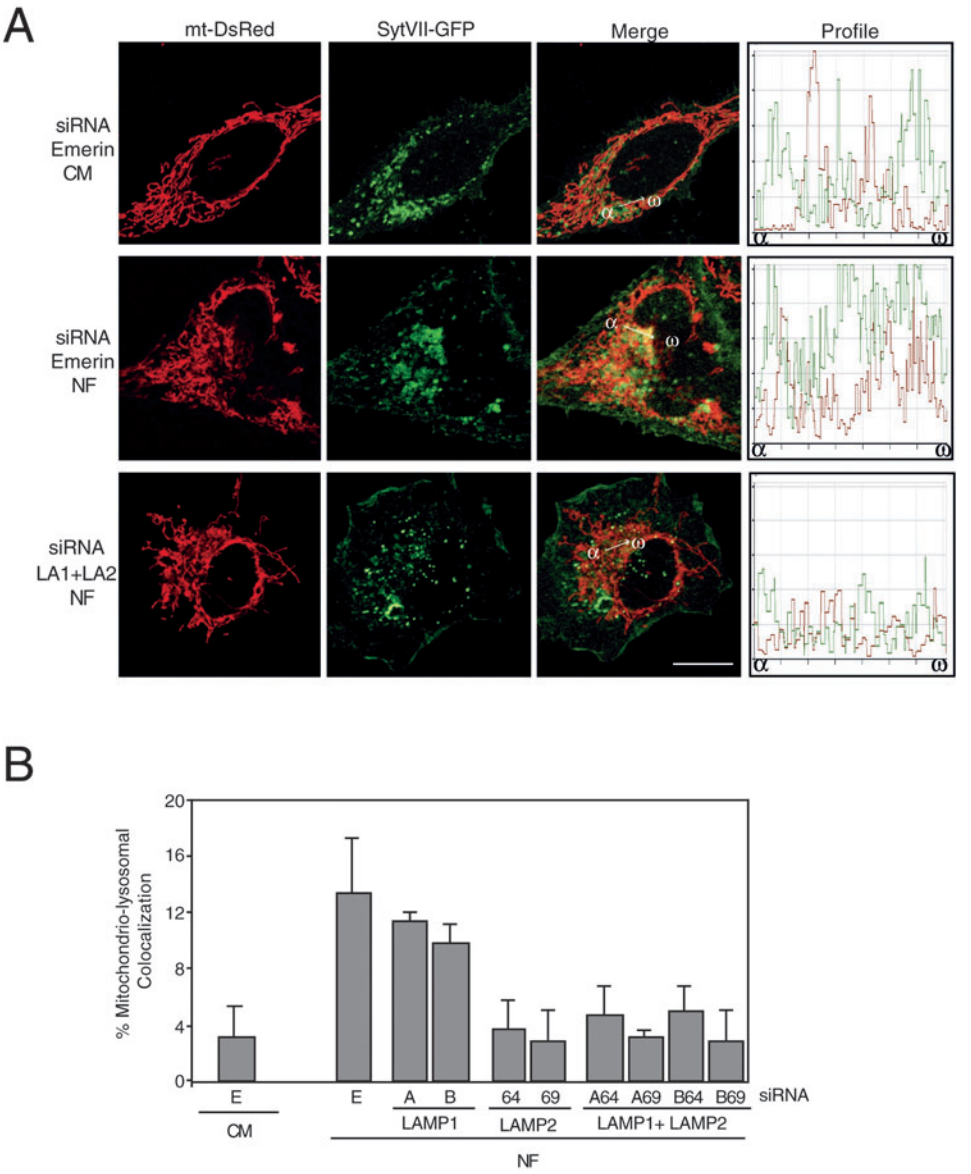

Fig. 5. Colocalization of mitochondrial and lyosomal markers on nutrient depletion in controls and LAMP1 (LA1) or LAMP2 (LA2) knockdown cells. HeLa cells were treated with siRNA specific for emerin, LAMP1 and/or LAMP2 (24 hours), then transfected with mt-DsRed (which localizes to mitochondria) and SytVII-GFP (which localizes to lysosomes), cultured for 24 hours and then incubated in CM or NF medium for 4 hours, followed by confocal microscopy as in Fig. 3. Representative cells are shown in A and the degree of colocalization (X±s.d., *n*=50) between mt-DsRed and SytVII-GFP is shown in B. The graphs (right panels) represent the fluorescence distribution determined for section of the cells, as indicated by the orientation of the arrow. Bar, 10 μm.

specific for emerin. LAMP2-specific siRNA reduced the colocalization of both organellar markers (Fig. 5A,B), again suggesting that LAMP2 is involved in the fusion of lysosomes with autophagic vacuoles. Very similar results were obtained in LAMP1/2 DKO fibroblasts from murine origin (Fig. 6A,B), underscoring that LAMP proteins have similar roles in different species and cell types. Data indicating a failure in the colocalization of autophagic vacuoles and lysosomes or mitochondrial and lysosomes induced by LAMP2 deficiency were obtained at different time points after starvation, namely 4 hours after nutrient depletion (shown in Figs 4-6), as well as after 16 hours (not shown), when a significant fraction of cells were dead or dying (Fig. 2).

Cell death-induced accumulation of autophagic vacuoles involves hallmarks of apoptosis

The accumulation of autophagic vacuoles induced by starvation and LAMP2 depletion was followed by caspase-3 activation, as shown for HeLa cells (Fig. 7A,B) and MEF (Fig. 3E, see above). We therefore wondered whether inhibition of caspases with Z-VAD-fmk would prevent cell death in this model. Z-VAD-fmk partially reduced the mortality (as assessed with the vital dye PI) of cells depleted from LAMPs and nutrients (Fig. 7D). However, Z-VAD-fmk did not stabilize the $\Delta\Psi_m$, which was lost in an increasing fraction of such cells (Fig. 7C), presumably as a sign of metabolic insufficiency (Zong et al., 2004) or of incipient apoptosis (Zamzami et al., 1995). Next, we addressed the question as to whether the preservation of mitochondrial function by overexpressed Bcl-2 and the mitochondrion-targeted Cytomegalovirus protein vMIA would prevent apoptosis induced by the accumulation of autophagic vacuoles. Transfection-enforced overexpression of either of these two proteins stabilized the $\Delta\Psi_m$ of cells depleted from nutrients and from LAMP proteins (Fig. 8A). Moreover, Bcl-2 and vMIA prevented cell death in these circumstances (Fig. 8B). Thus, two different strategies of apoptosis inhibition, caspase

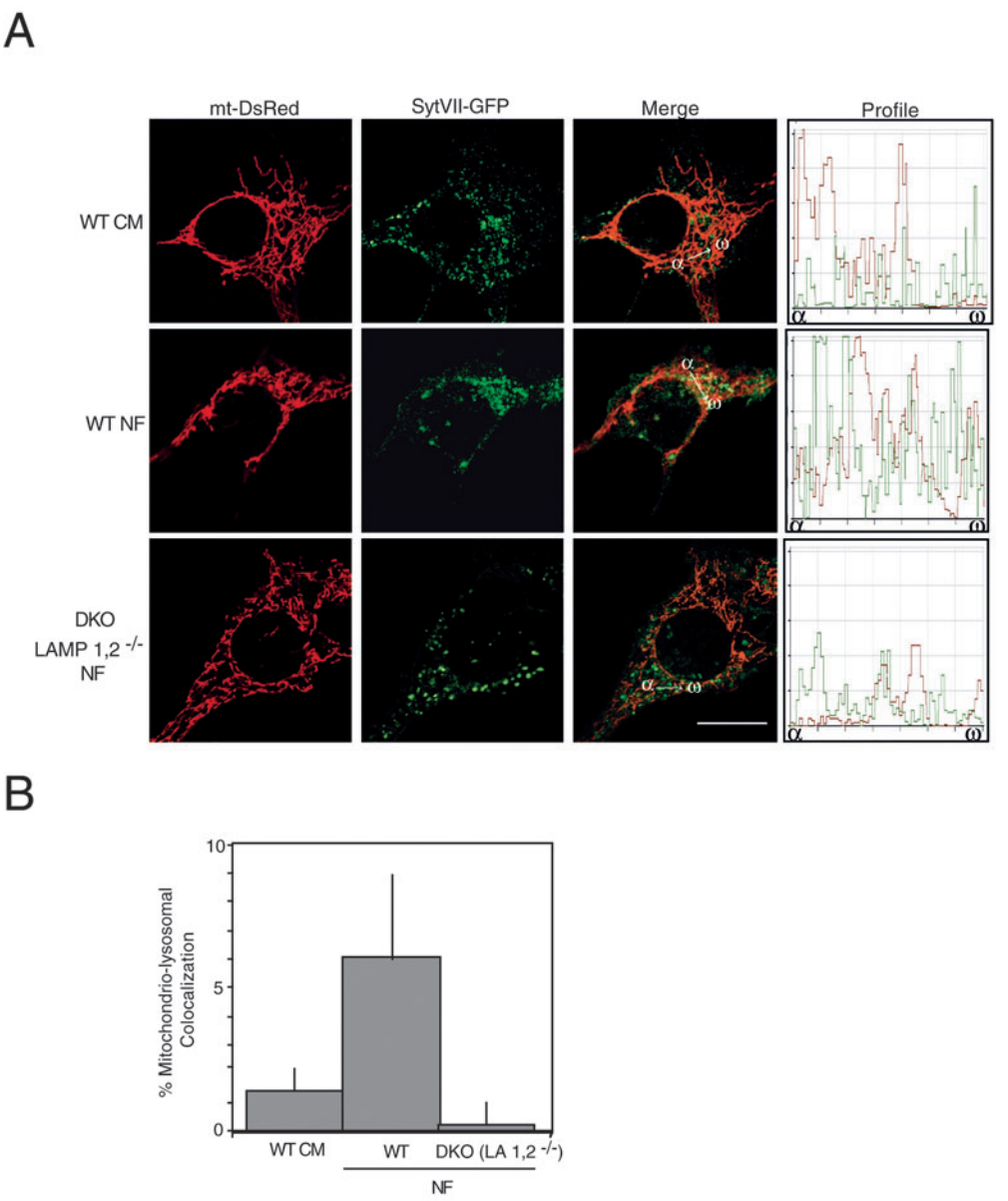

Fig. 6. Colocalization of mt-DsRed and SytVII-GFP in wild-type (WT) and LAMP1$^{-/-}$ LAMP2$^{-/-}$ (DKO) MEF. The degree of colocalization was measured similarly as in Fig. 4, before and after overnight starvation of amino acids and serum. Examples of the normal separation of mitochondria and lysosomes are shown in the upper part of A, whereas examples of how these organelles colocalize on starvation in WT but not DKO cells are shown in the middle and lower parts of A, respectively. Bar, 10 μm. The graphs (right panels) represent the fluorescence distribution determined for section of the cells, as indicated by the orientation of the arrow. Quantitative data are shown in B.

inhibition and mitochondrial stabilization, both preserved the cellular viability yet differed in their capacity to maintain the $\Delta\Psi_m$. Of note, apoptosis inhibition did not affect the accumulation of autophagic vacuoles (as measured with LC3-GFP) (Fig. 8C), supporting the contention that autophagic vacuolization occurs upstream and independently of the apoptotic process.

Discussion

Type 2 cell death is defined by the accumulation of autophagic vacuoles (Bursch, 2001; Edinger and Thompson, 2004; Leist and Jaattela, 2001; Lockshin and Zakeri, 2001). Here, we develop an experimental model in which the accumulation of autophagic vacuoles is induced by two simultaneous manipulations, namely the depletion of nutrients, which stimulates the sequestration of cytoplasmic material in autophagic vacuoles, and the knockdown or knockout of LAMP2, a manipulation that reduces the removal of autophagic vacuoles by fusion with lysosomes (Figs 4-6). Nutrient depletion alone or depletion of LAMP2 alone, however, do not provoke the accumulation of autophagic vacuoles. Although the concurrent depletion of nutrients and LAMP2 initially induced the morphological appearance of type 2 cell death (Fig. 1C-F; Fig. 3A,B), it finally resulted in morphological and biochemical changes that are typical for type 1 cell death, namely a $\Delta\Psi_m$ dissipation (Fig. 2 and Fig. 3D), cytochrome *c* release from mitochondria (not shown), caspase activation (Fig. 3E and Fig. 7A,B), and chromatin condensation (Fig. 1D,E, Fig. 2D and Fig. 3B,C). More importantly, inhibition of caspases retarded cell death (Fig. 7D) yet had no influence on autophagic vacuolization and mitochondrial change (Fig. 7C). Stabilization of mitochondria with Bcl-2 or vMIA preserved the $\Delta\Psi_m$ (Fig. 8A) while maintaining cellular viability (Fig. 8B), yet had no influence on autophagic vacuolization (Fig. 8C). These functional

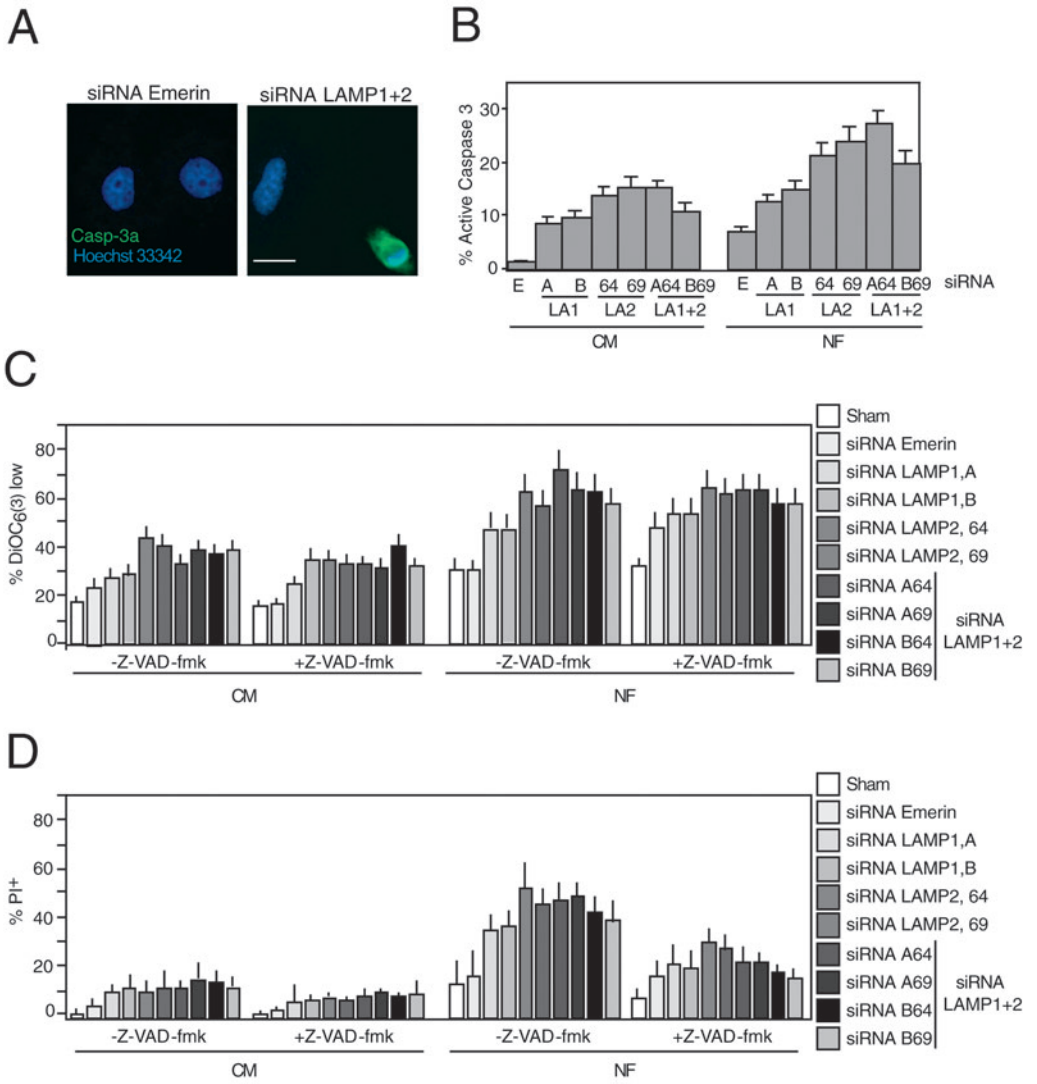

Fig. 7. Contribution of caspases to vacuolization-associated death. (A,B) Activation of caspase-3 induced by a combination of LAMP-specific siRNA and starvation. Cells were treated as indicated and stained with an antibody recognizing activated caspase-3. Representative fluorescence microphotographs are shown in A and quantitative data (X±s.d., *n*=3) are shown in B. Bar, 10 μm. (C,D) Effect of the pan-caspase inhibitor Z-VAD-fmk. Cells were subjected to the indicated LAMP1 and/or LAMP2 knockdown and then cultured in the absence or presence of nutrients and/or Z-VAD-fmk for 24 hours. Then, the loss of the $\Delta\Psi_m$ (C) and viability (D) was measured with the indicated dyes, as in Fig. 2.

experiments, together with the kinetic studies, suggest a scenario in which autophagic vacuolization precedes mitochondrion-dependent caspase activation with nuclear apoptosis. Thus, a lethal process marked by an initial change characteristic of type 2 cell death shifts to a process marked by the final steps of type 1 cell death, shedding doubts on the general validity of the strict type 1/2 dichotomy postulated by the literature.

It should be noted that a shift from an initial type 2 to a final type 1 morphology has been observed in another model system. In *Drosophila*, developmental cell death in the salivary gland occurs in a stepwise fashion, with a caspase-independent, reversible type 2 morphology that drifts to a caspase-dependent, irreversible type 1 pattern (Lee and Baehrecke, 2001; Martin and Baehrecke, 2004). Thus, at least in some paradigms of type 2 cell death, cells may ultimately succumb to biochemical processes such as caspase activation, which are typically associated with apoptosis. However, it remains to be determined whether type 2 cell death shifts to type 1 cell death in other examples of cell death, for instance in human pathologies.

Another important aspect emerging from this study is that lesions affecting distinct organellar systems can trigger cell death through a final mitochondrial pathway (Ferri and Kroemer, 2001; Green and Kroemer, 2004). vMIA is a predominantly mitochondrial protein that suppresses outer membrane permeabilization through its capacity to interact with Bax and to convert Bax into an apoptosis inhibitor (Arnoult et al., 2004; Poncet et al., 2004). Similar to Bcl-2, vMIA can inhibit cell death induced by damage that primarily affects nuclei (Andreau et al., 2004; Castedo et al., 2004), the endoplasmic reticulum (Boya et al., 2002), lysosomes (Boya et al., 2003a; Boya et al., 2003c) and, as shown here, the

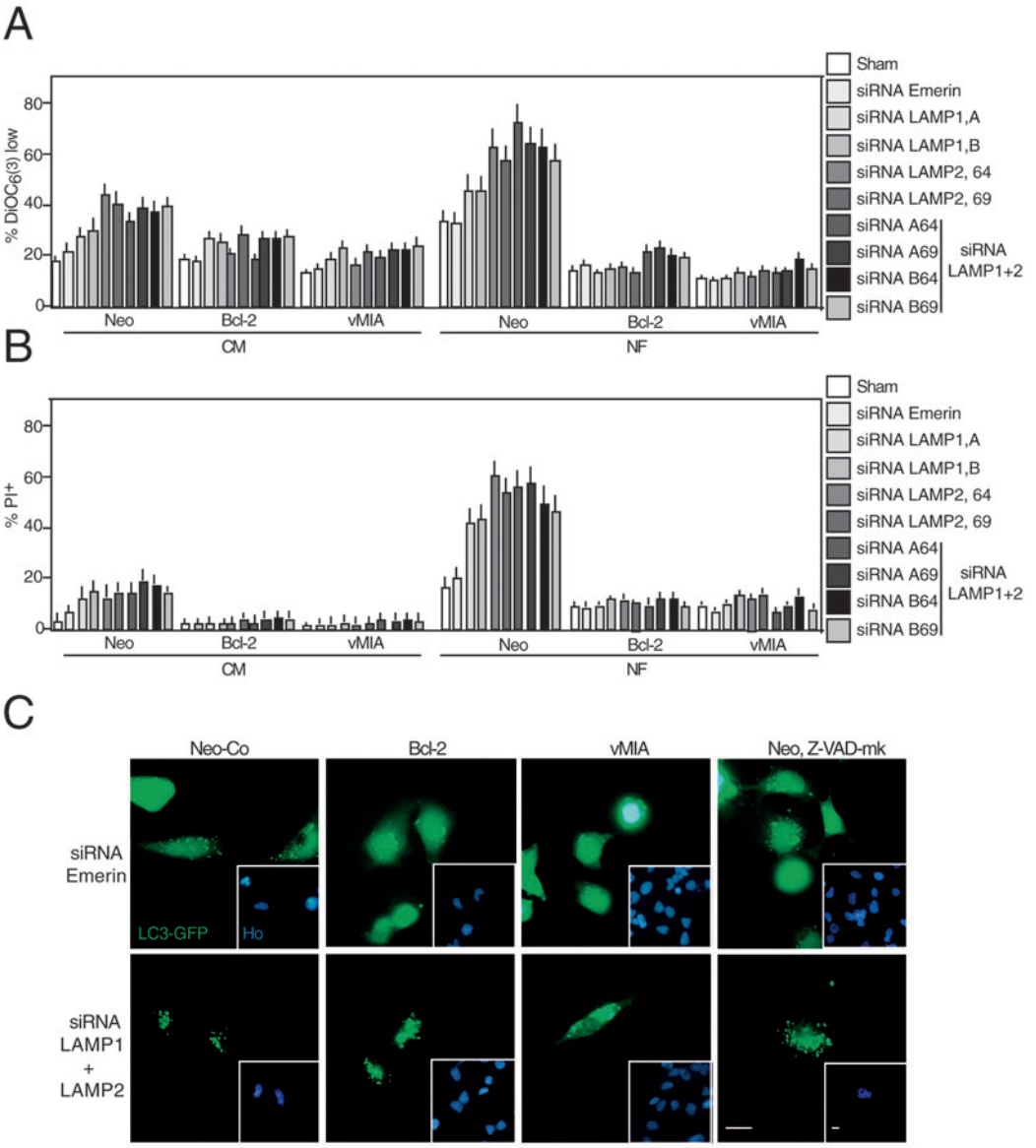

Fig. 8. Effect of mitochondrial apoptosis inhibitors on vacuolization-associated death. (A,B) Effect of Bcl-2 and vMIA on cell death. HeLa cells stably transfected with Bcl-2 or vMIA were subjected to the indicated LAMP-specific siRNAs, and the frequence of $\Delta\Psi_m^{low}$ (A) or dead (B) cells was measured by staining with $DiOC_6(3)$ or PI, respectively. (C) Accumulation of the autophagic vacuole marker LC3-GFP in vesicles from Neo controls, Bcl-2-transfected and vMIA-transfected HeLa cells or Neo control vector-only transfected cells treated with Z-VAD-fmk. Note that none of the apoptosis inhibitors influenced the vacuolar accumulation of LC3-GFP. Bar, 10 μm.

autophagic compartment. This underscores the importance of mitochondrial events in sealing irreversible cell fate, even when the primary lethal lesion affects other organelles.

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