

# The endogenous caspase-8 inhibitor c-FLIP<sub>L</sub> regulates ER morphology and crosstalk with mitochondria

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Components of the death receptor-mediated pathways like caspase-8 have been identified in complexes at intracellular membranes to spatially restrict the processing of local targets. In this study, we report that the long isoform of the cellular FLICE-inhibitory protein (c-FLIP<sub>L</sub>), a well-known inhibitor of the extrinsic cell death initiator caspase-8, localizes at the endoplasmic reticulum (ER) and mitochondria-associated membranes (MAMs). ER morphology was disrupted and ER Ca<sup>2+</sup>-release as well as ER-mitochondria tethering was decreased in c-FLIP<sup>-/-</sup> mouse embryonic fibroblasts (MEFs). Mechanistically, c-FLIP ablation resulted in enhanced basal caspase-8 activation and in caspase-mediated processing of the ER-shaping protein reticulon-4 (RTN4) that was corrected by re-introduction of c-FLIP<sub>L</sub> and caspase inhibition, resulting in the recovery of a normal ER morphology and ER-mitochondria juxtaposition. Thus, the caspase-8 inhibitor c-FLIP<sub>L</sub> emerges as a component of the MAMs signaling platforms, where caspases appear to regulate ER morphology and ER-mitochondria crosstalk by impinging on ER-shaping proteins like the RTN4.

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Cellular FLICE inhibitory proteins (c-FLIP) inhibit death receptor (DR)-mediated apoptosis, by preventing caspase-8 activation.<sup>1</sup> Among the three identified c-FLIP splicing forms,<sup>2,3</sup> c-FLIP<sub>S,R</sub> were described as cytosolic, whereas c-FLIP<sub>L</sub> was also observed in the nucleus. A pool of membrane-bound c-FLIP<sub>L</sub> was also described<sup>4</sup> suggesting that caspase-8/c-FLIP<sub>L</sub> could re-distribute on stimulation, leading to a more subtle regulation of caspase-8 activity depending on substrates localization.<sup>5</sup> Furthermore, caspase-8 itself and Fas-Associated Death Domain adaptor protein (FADD) were found or were shown to re-localize in local complexes on ER<sup>6–8</sup> and mitochondria,<sup>9,10</sup> mediating the exchange of signals between the two organelles.<sup>11–13</sup> Several molecular platforms containing both membrane-bound proteins and cytosolic apoptosis modulators have been identified at the ER-mitochondria interface (the so-called mitochondria-associated membranes or MAMs),<sup>14</sup> controlling ER-mitochondria anchorage as well as lipid metabolism, Ca<sup>2+</sup> signaling and apoptosis.<sup>15</sup> MAMs have been recently described as lipid raft-like domains that orient proteins to promote the ER-mitochondria juxtaposition;<sup>16</sup> consequently, alterations in their composition may profoundly affect the physical and functional inter-organelle crosstalk. Furthermore, as mitochondrial and ER membranes are continuously and concertedly remodeled,<sup>17</sup> it is not surprising that membrane-

shaping proteins can also exert a function in regulating the ER-mitochondria coupling.<sup>12,18</sup> Different families of ER-shaping proteins control the organization of peripheral ER, which consists of sheet-like cisternae and tubules connected by three-way junctions.<sup>19</sup> Among these, Reticulons (RTN) and Deleted in Polyposis locus 1 (DP1) proteins cause the ER membrane to curve and tubulate,<sup>20,21</sup> whereas the GTPases Atlantins (ATL) promote the branching of ER tubules;<sup>22</sup> finally, ER sheet-enriched proteins such as the 63-kDa cytoskeleton-linking membrane protein (CLIMP63) control the width of ER cisternae, anchoring the organelle to microtubules and maintaining its spatial distribution.<sup>23,24</sup> Along with other components of the extrinsic apoptosis, here we described for the first time the enrichment of c-FLIP<sub>L</sub> at ER and ER-mitochondria interface. Furthermore, we observed that ER structure and tethering to mitochondria are impaired in cells lacking c-FLIP. Given the importance of membrane-shaping proteins and MAM complexes in regulating organelles structure and ER-mitochondria juxtaposition, we focused on the mechanism underlying this phenotype and we found that c-FLIP<sub>L</sub> deficiency induces the caspase-mediated processing of RTN4, thus affecting organelle shape and coupling to mitochondria. We therefore concluded that c-FLIP<sub>L</sub> is a novel regulator of ER morphology and ER-mitochondria crosstalk.

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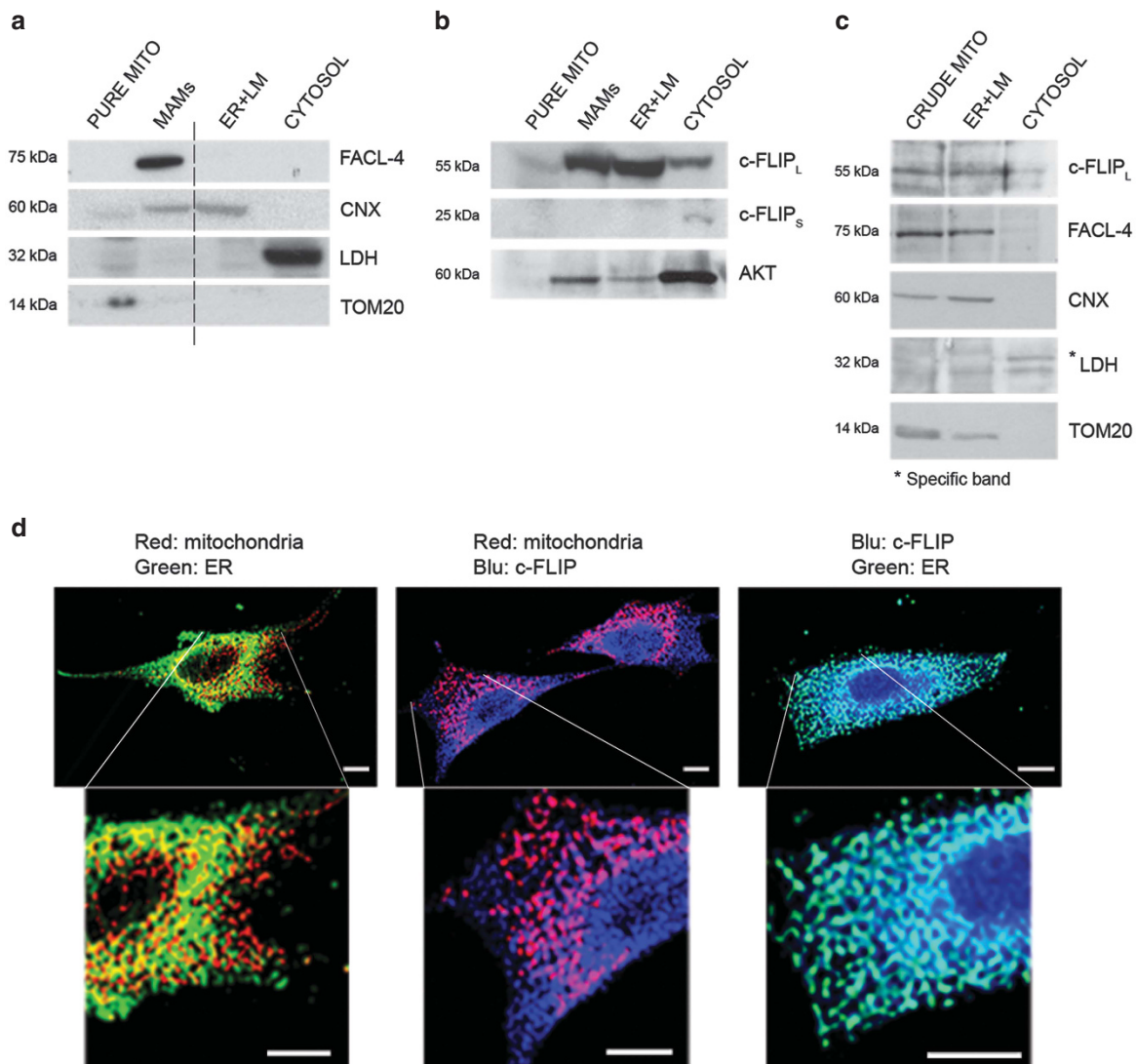
**Abbreviations:** ATL, atlastin; BAP31, B-cell associated protein 31; BAPTA, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester); Bid, BH3-interacting domain death agonist; c-FLIP, cellular FLICE inhibitory protein; CLIMP63, cytoskeleton-associated protein 4; DP1, deleted in polyposis locus 1; ERYFP, ER-targeted yellow fluorescent protein; FADD, Fas-associated death domain adaptor protein; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; MAMs, mitochondria-associated membranes; mtRFP, mitochondria-targeted red fluorescent protein; PARP, poly-(ADP-ribose) polymerase; RTN4, reticulon-4; STS, staurosporine; TG, thapsigargin; WT, wild type

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## Results

**c-FLIP<sub>L</sub> localizes at ER and MAMs.** We investigated c-FLIP subcellular localization by performing cell fractionation and indirect immunofluorescence. Percoll-purified fractions from the mouse liver, devoid of cross-contaminations as indicated by western blotting (WB) analysis of specific markers (Figure 1a), were assayed. In agreement with previous evidence,<sup>25,26</sup> c-FLIP<sub>S</sub> was found in the cytosol (Figure 1b). Conversely, c-FLIP<sub>L</sub> localized also at the ER+LM (Light Membranes) and MAMs fractions (Figure 1b). The pattern of distribution of calnexin (CNX)<sup>27</sup> and AKT,<sup>28</sup> both previously identified at MAMs, further substantiated the specificity of c-FLIP<sub>L</sub> subcellular localization (Figures 1a and b). In homogenates from mouse embryonic fibroblasts (MEFs),

c-FLIP<sub>L</sub> was similarly retrieved in both ER+LM and crude mitochondria still containing ER and MAMs as contaminants (Figure 1c). Confocal analysis corroborated c-FLIP distribution in MEFs. In the left panel of Figure 1d, yellow discrete areas represented the ER-mitochondria contact points. A similar pattern of co-localization was observed for c-FLIP and mitochondria (Figure 1d, central panel), whereas a wider overlapping was reported between the ER-protein calreticulin (CRT) and c-FLIP (Figure 1d, right panel), confirming c-FLIP distribution at the ER and ER-mitochondria contacts points. According to biochemical and morphological evidence, we concluded that ER and MAMs contain c-FLIP<sub>L</sub>, which is conversely absent from mitochondria devoid of the interacting ER.

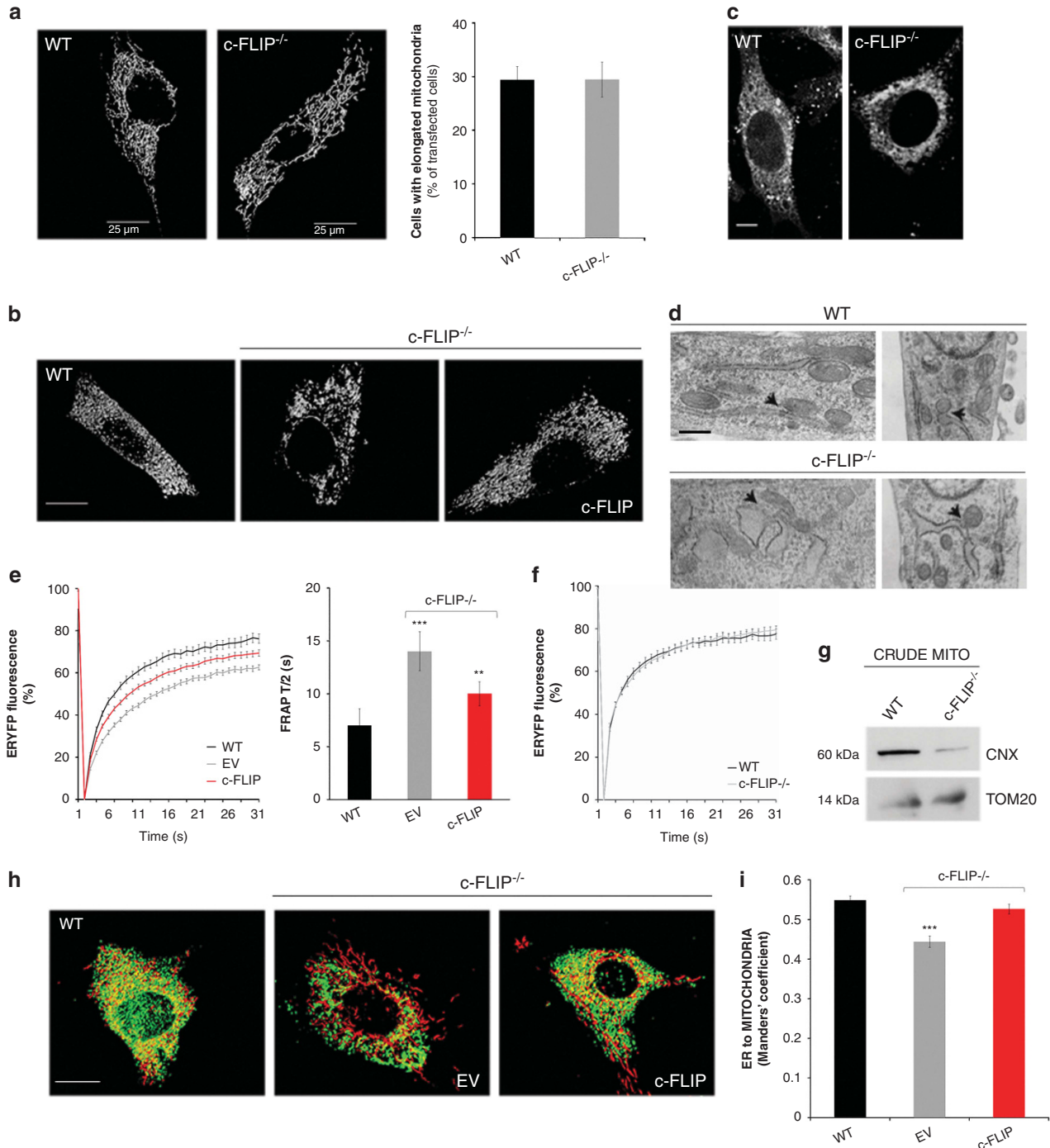


**Figure 1** c-FLIP<sub>L</sub> is retrieved at ER and MAMs. (a, b) Proteins (20 μg) from Percoll-purified subcellular fractions of the mouse liver were separated by SDS-PAGE and immunoblotted with the indicated antibodies, specific for each fraction. The translocase of the mitochondrial outer membrane 20 (TOM20) was used as marker for mitochondria, the lactate dehydrogenase (LDH) as marker for cytosolic fraction. Calnexin (CNX) is considered as a marker of both ER and MAMs, whereas the long-chain fatty-acid CoA synthases (FACL4) is MAM-enriched. Distant parts from the same gel were joined at dashed lines. (c) Subcellular fractions and immunoblot analysis from WT MEFs. (d) Immunofluorescence and confocal analysis of endogenous c-FLIP subcellular localization. WT MEFs were transfected with pDsRed2-mito encoding for the mitochondrial-targeted fluorescent protein (mtRFP) to label mitochondria and immunostained for the endogenous ER marker calreticulin (CRT) and for c-FLIP. Left and central panels indicate co-localization of ER with mitochondria, and of mitochondria with c-FLIP. Right panel indicates co-localization of ER and c-FLIP. Boxes denote magnified areas. Scale bar, 20 μm

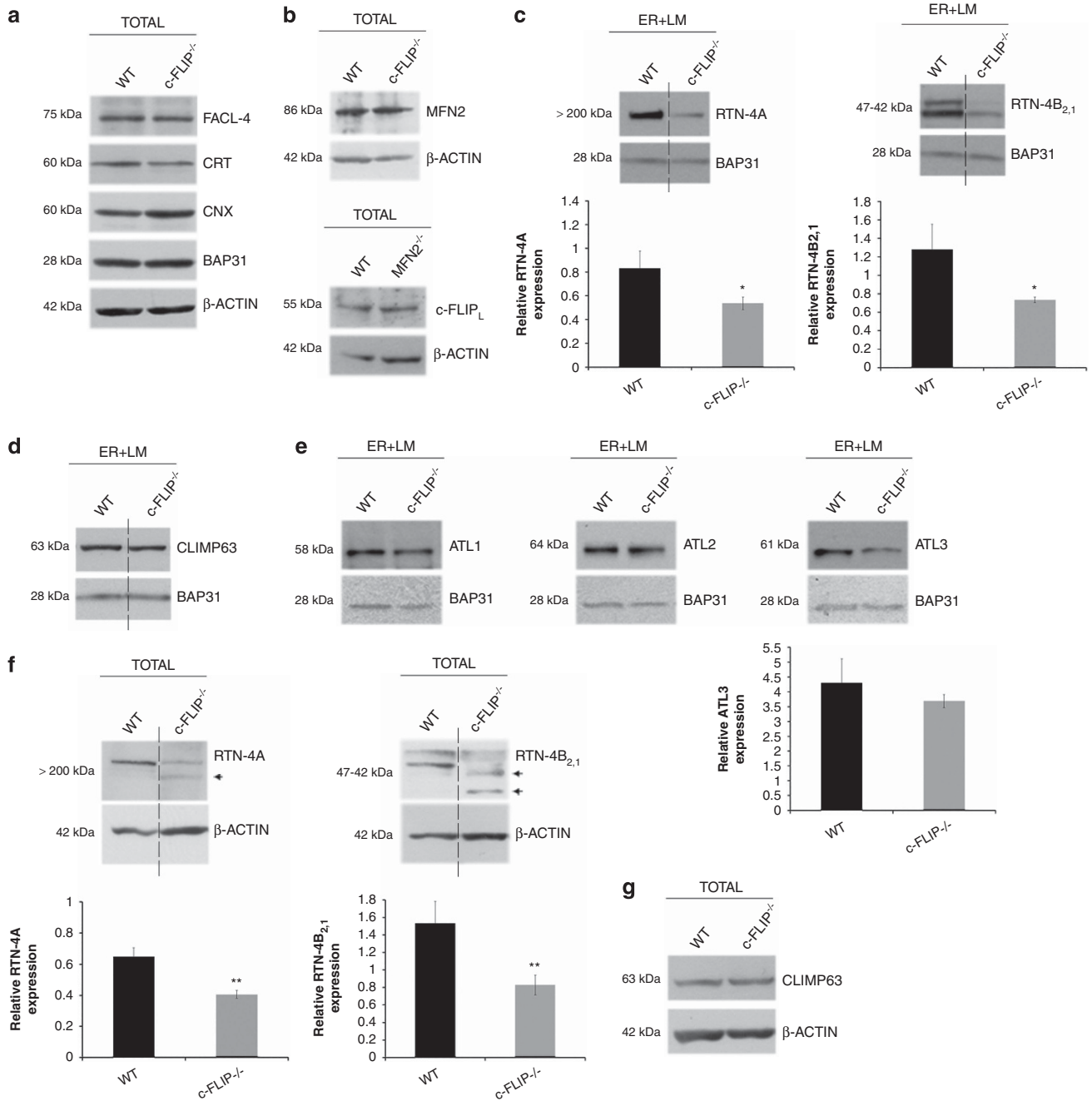
**Lack of c-FLIP<sub>L</sub> controls ER integrity and ER-mitochondria tethering.** The subcellular distribution of c-FLIP<sub>L</sub> prompted us to investigate its involvement in the regulation of ER morphology and tethering to mitochondria. To this aim, WT and c-FLIP<sup>-/-</sup> MEFs<sup>29</sup> transfected with mitochondria- and ER-targeted fluorescent proteins (respectively mtRFP and ERYFP) were used, and ER and mitochondrial morphology was investigated by comparing three-dimensional (3D) reconstructions of volume-rendered confocal stacks of mtRFP and ERYFP. c-FLIP depletion did not significantly alter mitochondrial morphology or distribution (Figure 2a). Conversely, whereas WT cells showed a well-organized network of interconnected tubules spanning the whole cytosol (Figure 2b, left panel), c-FLIP<sup>-/-</sup> cells displayed a deranged ER, characterized by fragmented and enlarged cisternae, seemingly to the detriment of reticular ER at the cell periphery (Figure 2b, central panel). Such defects were partially restored on c-FLIP<sub>L</sub> re-introduction in ablated MEFs (Figure 2b, right panel). To ensure that ER defects were not a result of transfections toxicity, immunofluorescence analysis of the endogenous ER-protein CRT (Figure 2c) and electron microscopy (EM) analysis were performed (Figure 2d), confirming the enlargement of ER cisternae in c-FLIP<sup>-/-</sup> cells with respect to the tubular ER in WT MEFs. Fluorescence recovery after photobleaching (FRAP) of ERYFP was finally used to quantitatively assess the ER integrity, as the speed with which fluorescence returns to the photobleached region is dependent on the degree of ER luminal contiguity.<sup>30</sup> In accordance with morphological evidence, FRAP analysis further substantiated ER impairment and lack of integrity at the cell periphery of c-FLIP<sup>-/-</sup> cells (Figure 2e), which were partially restored by c-FLIP<sub>L</sub> re-introduction, hence corroborating c-FLIP role in this phenotype. Confirming the ER fragmentation mainly at the cell periphery, no major differences in ER luminal contiguity were observed in the perinuclear area (Figure 2f). In view of c-FLIP<sub>L</sub> localization at MAMs and of its involvement in ER shape, we investigated whether c-FLIP could have a role also in ER-mitochondria tethering. To address this point, we firstly measured the ability of crude mitochondria from WT and c-FLIP<sup>-/-</sup> MEFs to interact with microsomal components as CNX. As shown in Figure 2g, crude mitochondria from ablated MEFs were less contaminated by microsomes than those isolated from WT, indicating diminished ER-mitochondria interactions. Successively, we measured the ER-mitochondria distance by semi-quantitative confocal microscopy analysis of volume-rendered 3D reconstructions of z-axis stacks of confocal images of ERYFP and mtRFP.<sup>17,18</sup> Whereas WT MEFs showed several yellow areas of co-localization, representing the ER-mitochondria contact points, c-FLIP<sup>-/-</sup> cells revealed lower ER-mitochondria juxtaposition (Figure 2h), which was partially restored on c-FLIP<sub>L</sub> re-introduction. Quantitatively, Manders' colocalization coefficient<sup>31</sup> confirmed these evidence, revealing ~15% reduction in the ER-to-mitochondria tethering in c-FLIP<sup>-/-</sup> MEFs, that was counterbalanced by c-FLIP<sub>L</sub> re-expression (Figure 2i). The occurrence of inter-organelle contacts in WT and c-FLIP<sup>-/-</sup> MEFs was also verified by EM analysis (Figure 2d).

**ER-shaping protein Reticulon-4 is reduced in c-FLIP ablated cells.** Defective ER morphology indicated that c-FLIP<sub>L</sub> could have a role in maintaining the ER structure. To characterize the molecular mechanism involved, we first compared the expression levels of different ER proteins to discern whether ER expansion could be related to excessive ER membrane proliferation. However, we did not detect significant differences between WT and c-FLIP<sup>-/-</sup> cells in any of the tested ER-resident proteins (Figure 3a). We further compared MFN2 levels, as its involvement in ER-mitochondria tethering and morphology was previously reported.<sup>18</sup> However, we did not notice major differences either in MFN2 levels between WT and c-FLIP<sup>-/-</sup> MEFs, or in c-FLIP<sub>L</sub> between WT and MFN2<sup>-/-</sup> cells, suggesting a MFN2-independent mechanism (Figure 3b). As aberrant ER enlargement observed in c-FLIP<sup>-/-</sup> cells was similar to ER defects associated with RTN4 depletion,<sup>20</sup> or to overexpression of ATLS<sup>32</sup> or CLIMP63,<sup>23</sup> we compared the expression levels of these ER-shaping proteins in microsomal fractions from WT and c-FLIP<sup>-/-</sup> MEFs (Figures 3c and e). Interestingly, c-FLIP-depleted cells displayed diminished expression of RTN-4A,B<sub>2,1</sub> isoforms (Figure 3c), whereas no significant variations were observed in CLIMP63 or in ATLS<sub>1,2</sub> (Figures 3d and e). Only a slight, but not statistically significant, reduction in ATLS<sub>3</sub> was noticed (Figure 3e), indicating the involvement of RTN4 in ER alterations affecting c-FLIP<sup>-/-</sup> MEFs. Total lysates from the two cell lines, besides evidencing the differences in RTN-4A,B<sub>2,1</sub>, also revealed additional lower-molecular weight bands, compatible with cleaved fragments of this protein and visible predominantly in ablated MEFs (Figure 3f). Conversely, no extra bands of CLIMP63 were observed (Figure 3g); therefore, proposing that cleavage events could affect the RTN4 integrity in c-FLIP<sup>-/-</sup> MEFs, in turn the disrupted ER morphology.

**TNF $\alpha$  induces RTN4 processing.** Previous evidence indicated that caspases and their inhibitors could impinge on levels of endogenous ER morphogenic proteins. In particular, caspase-7 was reported to mediate the cleavage of RTN-4B.<sup>33</sup> Owing to the ability of c-FLIP to control the apical caspase-8, which activates the caspase-7, we investigated whether increased caspase activation has occurred in cells lacking c-FLIP, in turn accounting for RTN4 processing. In agreement with this hypothesis, the basal caspase-8 activity was enhanced in c-FLIP<sup>-/-</sup> MEFs (Figure 4a). Successively, to better characterize the involvement of caspase-8/c-FLIP axis and extrinsic apoptotic response in RTN4 cleavage and ER alterations, we exposed c-FLIP<sup>-/-</sup> cells to increasing doses of TNF $\alpha$ , known to strongly induce the extrinsic pathway of apoptosis through caspase activation.<sup>29</sup> As shown, we observed a rapid, but defective cleavage of RTN4 (Figure 4b, upper panel: RTN-4A; lower panel: RTN-4B<sub>2,1</sub>), decreasing at higher doses of TNF $\alpha$  despite the dose-dependent increase in caspase-8 activity (Figure 4c) and cleavage of caspase-3 and poly-(ADP-ribose) polymerase (PARP) (Figure 4d), which suggested an efficient activation of caspase-mediated pathways. As non-neural cells as MEFs abundantly express RTN-4B<sub>2,1</sub> as compared with the little amount of RTN-4A, evaluation of RTN-4B<sub>2,1</sub> was chosen for the following experiments. As reduction of RTN-4B<sub>2,1</sub>



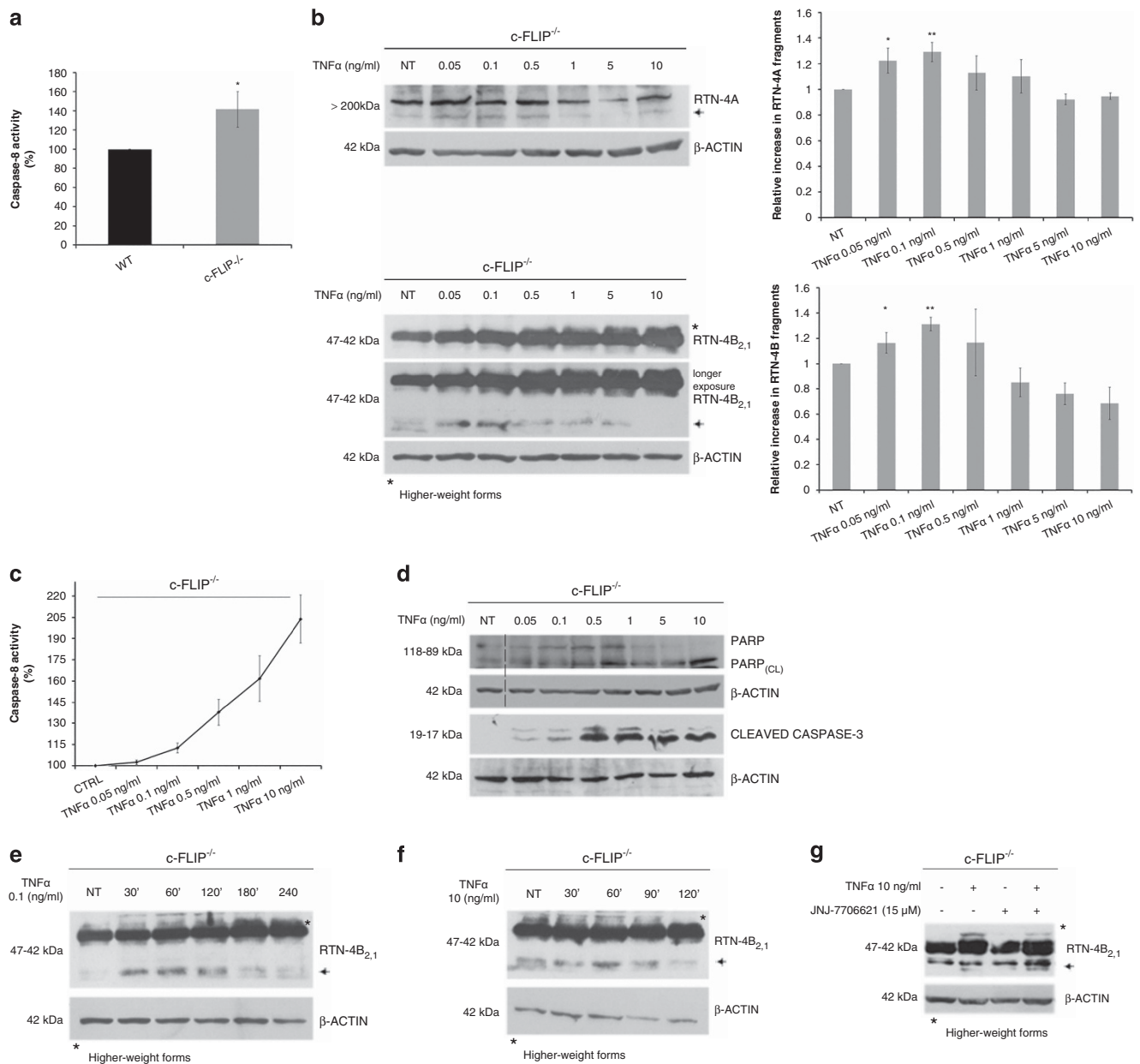
**Figure 2** c-FLIP<sub>L</sub> depletion affects ER morphology and tethering to mitochondria. **(a) Left:** Representative 3D-reconstructions of mitochondria in MEFs of the indicated genotype transfected with mtRFP. **Right:** mean  $\pm$  S.E.M. ( $n = 4$ , 25 cells per experiment) of data from **a**. **(b)** Representative 3D-reconstructions of ER in MEFs of the indicated genotype co-transfected with ERYFP and with plasmids encoding for c-FLIP<sub>L</sub> or for the empty vector. 'c-FLIP' indicates c-FLIP<sub>L</sub> re-introduction in depleted MEF. Scale bar, 25  $\mu$ m. **(c)** Immunofluorescence analysis of the resident ER protein CRT in WT and c-FLIP<sup>-/-</sup> MEFs. **(d)** Representative EM images of ER and ER-mitochondria contact points in WT and c-FLIP<sup>-/-</sup> cells. Scale bar, 0.54  $\mu$ m in the upper, right panel; 0.35  $\mu$ m in other panels. Arrows indicate ER-mitochondria proximity. **(e, f)** Fluorescence recordings from real-time sequence of ERYFP FRAP in c-FLIP<sup>-/-</sup> and WT MEFs at the cell periphery **(e)** and in the perinuclear region **(f)**. The histogram reported mean  $\pm$  S.E.M. ( $n = 5$ , 10 cells per experiment) of data from **e** (\*\* $P \leq 0.001$ ; \*\*\* $P \leq 0.0001$ ). In **e, f**, 16- $\mu$ m<sup>2</sup> regions were photobleached for a total of 3 s, using 100% laser power of the 488 nm line. The post-bleaching images were taken for a total of 120 s. 'c-FLIP' indicates c-FLIP<sub>L</sub> re-introduction in depleted MEF, 'EV' the empty vector. **(g)** WB of the indicated proteins in crude mitochondrial fractions (20  $\mu$ g) from WT and c-FLIP<sup>-/-</sup> MEFs. **(h)** Representative 3D-reconstructions of ER and mitochondria in MEFs of the indicated genotype co-transfected with mtRFP, ERYFP and the reported plasmids. Yellow areas indicate that organelles are closer than 270 nm. Scale bar, 25  $\mu$ m. **(i)** Mean  $\pm$  S.E.M. ( $n = 5$ , 10 cells per experiment) of interaction data from **h** (\*\* $P \leq 0.001$ ).



**Figure 3** Evaluation of different ER markers in WT and c-FLIP-ablated cells. (a) Total lysates (50 μg) from WT and c-FLIP<sup>-/-</sup> MEFs were separated by SDS-PAGE and immunoblotted for different ER markers. (b) **Upper panel:** representative WB analysis of MFN2 level in total lysates from WT and c-FLIP<sup>-/-</sup> MEFs (n=4). **Lower panel:** representative WB analysis of c-FLIP<sub>L</sub> level in total lysates from WT and MFN2<sup>-/-</sup> MEFs (n=3). (c, d) Representative WB analysis of expression levels of RTN-4A and -B<sub>2,1</sub> (c) and CLIMP63 (d) in ER fractions isolated from WT and c-FLIP<sup>-/-</sup> MEFs. The same amount of proteins was used and protein loading was controlled by evaluating the expression of the ER-resident protein BAP31. Histograms indicate the mean ± S.E.M. of full length, ER+LM-associated RTNs densitometric analysis (n=3 for RTN-4A; n=3 for RTN-4B<sub>2,1</sub>). (e) WB analysis of expression levels of ATL1,2,3 in ER fractions. BAP31 was used as loading control. The densitometric analysis reported in the histogram, represented the mean ± S.E.M. (n=3) of ATL3 level. (f, g) WB analysis of RTN-4A, B<sub>2,1</sub> (f) and CLIMP63 (g) levels in total lysates from c-FLIP<sup>-/-</sup> and WT MEFs. Histograms indicate the mean ± S.E.M. of full-length RTN densitometric analysis (n=3 for RTN-4A; n=6 for RTN-4B<sub>2,1</sub>). In c, d, f broken lines indicate where a lane was deleted

cleavage at increasing TNFα doses (Figure 4b) or at longer time points (Figures 4e and f) occurred concomitantly to the formation of a higher-weight form of RTN-4B<sub>2,1</sub> (indicated by the asterisk in the less-exposed lane) compatible with the phosphorylated form of the protein, we hypothesized that in our model RTN-4B<sub>2,1</sub> processing could be prevented by an

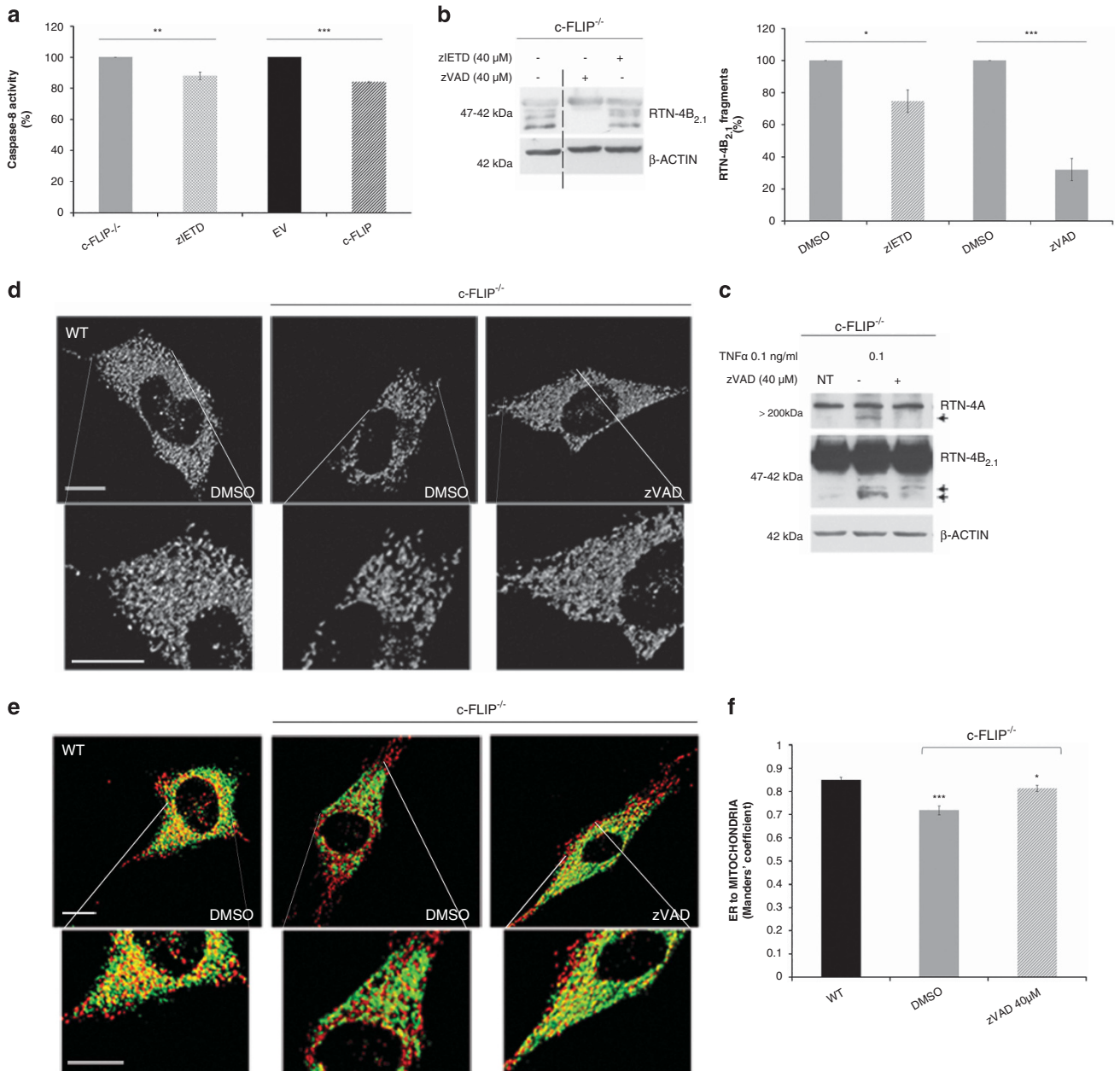
unidentified TNFα-induced phosphorylation event. Interestingly, cyclin-dependent kinases 1/2 (CDK1/2)-mediated phosphorylation of RTN-4B was reported to negatively control RTN4 processing.<sup>33</sup> In accordance with our hypothesis, co-treatment of c-FLIP<sup>-/-</sup> MEFs with TNFα and the CDK1/2 pharmacological inhibitor JNJ-7706621 lowered the



**Figure 4** Caspase activation impacts on RTN4 levels in c-FLIP<sup>-/-</sup> MEFs. **(a)** Basal caspase-8 activity was measured in c-FLIP<sup>-/-</sup> and WT MEFs. Mean  $\pm$  S.E.M. ( $n = 7$ ) ( $*P \leq 0.05$ ). **(b)** Evaluation of RTN-4A and -B<sub>2,1</sub> cleavage by WB analysis in c-FLIP<sup>-/-</sup> MEFs treated for 2 h with the indicated doses of TNF $\alpha$ . Histograms indicate the mean  $\pm$  S.E.M. of RTN4 fragments densitometric analysis ( $n = 3$  for RTN-4A in the upper panel;  $n = 3$  for RTN-4B<sub>2,1</sub> in the lower panel). **(c)** Colorimetric evaluation of caspase-8 activity on treatment of c-FLIP<sup>-/-</sup> cells with the indicated doses of TNF $\alpha$ . **(d)** WB analysis of caspase-3 and PARP cleavage in c-FLIP<sup>-/-</sup> MEFs treated with the indicated doses of TNF $\alpha$ . **(e, f)** Representative WB analysis of RTN-4B<sub>2,1</sub> cleavage in c-FLIP<sup>-/-</sup> MEFs treated, respectively, with TNF $\alpha$  0,1 ng/ml **(e)** and 10 ng/ml **(f)** at the indicated time points. In **b, d-f**,  $\beta$ -actin was used to normalize. **(g)** Evaluation of RTN-4B<sub>2,1</sub> cleavage by WB analysis in c-FLIP<sup>-/-</sup> MEFs treated with TNF $\alpha$  10 ng/ml alone or in combination with JNJ-7706621 15  $\mu$ M

accumulation of the higher-weight form of RTN-4B<sub>2,1</sub> and increased the formation of cleavage fragments with respect to c-FLIP<sup>-/-</sup> cells treated with TNF $\alpha$  alone (Figure 4g). We also observed mild RTN-4B<sub>2,1</sub> cleavage in WT cells on TNF $\alpha$  treatment. Such cleavage increased when TNF $\alpha$  was combined with the *de novo* protein synthesis inhibitor cycloheximide (data not shown). Together our data highlight a role for TNF $\alpha$ -induced caspase activation in RTN4 cleavage, in c-FLIP-deficient cells as well as in their WT counterpart.

**A caspase-mediated pathway is involved in RTN4 alterations and influences ER morphology and tethering to mitochondria.** As already reported in other experimental models,<sup>34,35</sup> we verified that c-FLIP ablation in MEFs enhanced the basal caspase-8 activity (Figure 4a), which was restored by both the specific caspase-8 inhibitor zIETD and c-FLIP<sub>L</sub> re-introduction (Figure 5a). To confirm the involvement of caspases in TNF $\alpha$ -mediated RTN4 processing in c-FLIP<sup>-/-</sup> cells, zIETD and the pan-caspases inhibitors zVAD were used. Although both inhibitors were efficient in



**Figure 5** Caspase-inhibition rescues RTN4 processing and ameliorates ER morphology and tethering to mitochondria. **(a)** Caspase-8 activity was measured in c-FLIP<sup>-/-</sup> MEFs on caspase-8 inhibition with zIETD and c-FLIP<sub>L</sub> re-introduction. Mean ± S.E.M. ( $n = 3$  experiments) (\*\* $P \leq 0.001$ ; \*\*\* $P \leq 0.0001$ ). **(b)** WB evaluation of basal RTN4 cleavage, on pharmacological caspase inhibition with zIETD or zVAD. Broken lines indicate that we joined together distant parts from the same gel. The histogram represents the mean ± S.E.M. of RTN-4B<sub>2.1</sub> fragments densitometric analysis ( $n = 3$ ). **(c)** WB analysis of RTN4 fragments formation in c-FLIP<sup>-/-</sup> cells on treatment with the indicated dose of TNF $\alpha$ , alone or in combination with the inhibitor zVAD. **(d)** Representative 3D-reconstructions of ER in MEFs of the indicated genotype transfected with ERYFP, and treated with DMSO or zVAD (40  $\mu$ M). Scale bar, 20  $\mu$ m. **(e)** Representative 3D-reconstructions of ER and mitochondria in MEFs of the indicated genotype co-transfected with mtRFP, ERYFP and treated with DMSO or zVAD (40  $\mu$ M). Scale bar, 20  $\mu$ m. **(f)** Mean ± S.E.M. ( $n = 5$ , 10 cells per experiment) of interaction data from **e** (\*\*\* $P \leq 0.0001$ ). In **b**, **c**  $\beta$ -actin was used to normalize

reducing the basal RTN4 proteolysis, even though to a different extent (Figure 5b), zVAD efficaciously diminished also the TNF $\alpha$ -induced RTN-4B<sub>2.1</sub> processing (Figure 5c). This evidence further corroborated the role of caspases and components of DR-mediated pathway in controlling the RTN4 cleavage. In view of the essential role of reticulons in ER tubulogenesis, as well as of the involvement of caspases in RTN4 regulation, we wondered whether the altered ER

morphology of c-FLIP<sup>-/-</sup> cells could be associated with the caspase-dependent RTN4 cleavage. To test this hypothesis, ER morphology and ER-mitochondria tethering of c-FLIP<sup>-/-</sup> MEFs pre-treated with zVAD were compared with those of WT and c-FLIP<sup>-/-</sup> cells exposed to the vehicle DMSO. Strengthening the proposed model, semi-quantitative confocal microscopy analysis of volume-rendered 3D reconstructions of z-axis stacks of confocal images of WT and c-FLIP<sup>-/-</sup>

cells transfected with ERYFP revealed that caspase-inhibition was sufficient to rescue the ER morphology of c-FLIP<sup>-/-</sup> MEFs (Figure 5d). Moreover, as estimated by measuring the quantitative Manders' co-localization coefficient, zVAD was also efficient in restoring the ER-mitochondria distance of c-FLIP<sup>-/-</sup> MEFs to levels comparable to those of WT (Figure 5e and f), hence underlying the potential role of caspases in regulating membrane-localized proteins and ER-mitochondria interactions.

**c-FLIP ablation results in disturbed Ca<sup>2+</sup> signaling.** Integrity of ER and ER-mitochondria contact points have been demonstrated to be crucial for Ca<sup>2+</sup> signaling.<sup>36</sup> In view of the role of c-FLIP<sub>L</sub> at the ER and MAMs, we investigated its involvement in Ca<sup>2+</sup> exchange between the two organelles. We firstly compared the increase in cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>c</sub>) in Fura-2AM-loaded WT and c-FLIP<sup>-/-</sup> MEFs on treatment with the sarcoplasmic-endoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA) inhibitor thapsigargin (TG), causing the passive ER discharge, and with ATP which induces ER Ca<sup>2+</sup> release from inositol 1,4,5-trisphosphate (IP<sub>3</sub>)-sensitive stores. EGTA was added to all solutions to assess only intracellular calcium fluxes. c-FLIP<sup>-/-</sup> MEFs displayed severely blunted ER Ca<sup>2+</sup> release in response to both TG and ATP (Figures 6a and b). In addition, as [Ca<sup>2+</sup>]<sub>c</sub> is buffered by mitochondria, along with the blunted ER Ca<sup>2+</sup> release, in Rhod-2AM loaded c-FLIP<sup>-/-</sup> cells also the mitochondrial Ca<sup>2+</sup> peak evoked by ATP was significantly lower than that in WT (Figure 6c). Nevertheless, such defects were not restored by c-FLIP<sub>L</sub> re-introduction, as we did not observe any amelioration in either ER Ca<sup>2+</sup> release (Figure 6d) or mitochondrial Ca<sup>2+</sup> entry (Figure 6e) in c-FLIP<sup>-/-</sup> cells re-expressing c-FLIP<sub>L</sub> with respect to c-FLIP<sup>-/-</sup> MEFs transfected with the EV. According to our data, c-FLIP<sup>-/-</sup> MEFs displayed reduced ER-mitochondria juxtaposition and diminished Ca<sup>2+</sup> exchange. We therefore wondered whether the reduced ability of ER and mitochondria to exchange signals and calcium could impact on Ca<sup>2+</sup>-mediated apoptosis. In agreement with this hypothesis, cells lacking c-FLIP were more resistant than WT to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which is known to induce a Ca<sup>2+</sup>-dependent apoptotic response (Figure 6f).<sup>37</sup> Conversely, c-FLIP<sup>-/-</sup> MEFs were as susceptible as WT to staurosporine (STS), which activates the mitochondrial pathway, but much more sensitive to DR-stimulation induced by TNF $\alpha$ , as previously reported (Figure 6g). Altogether our data demonstrate that c-FLIP ablation loosens ER-mitochondria juxtaposition, lowering Ca<sup>2+</sup> transfer to mitochondria and impacting on sensitivity to Ca<sup>2+</sup>-dependent apoptosis.

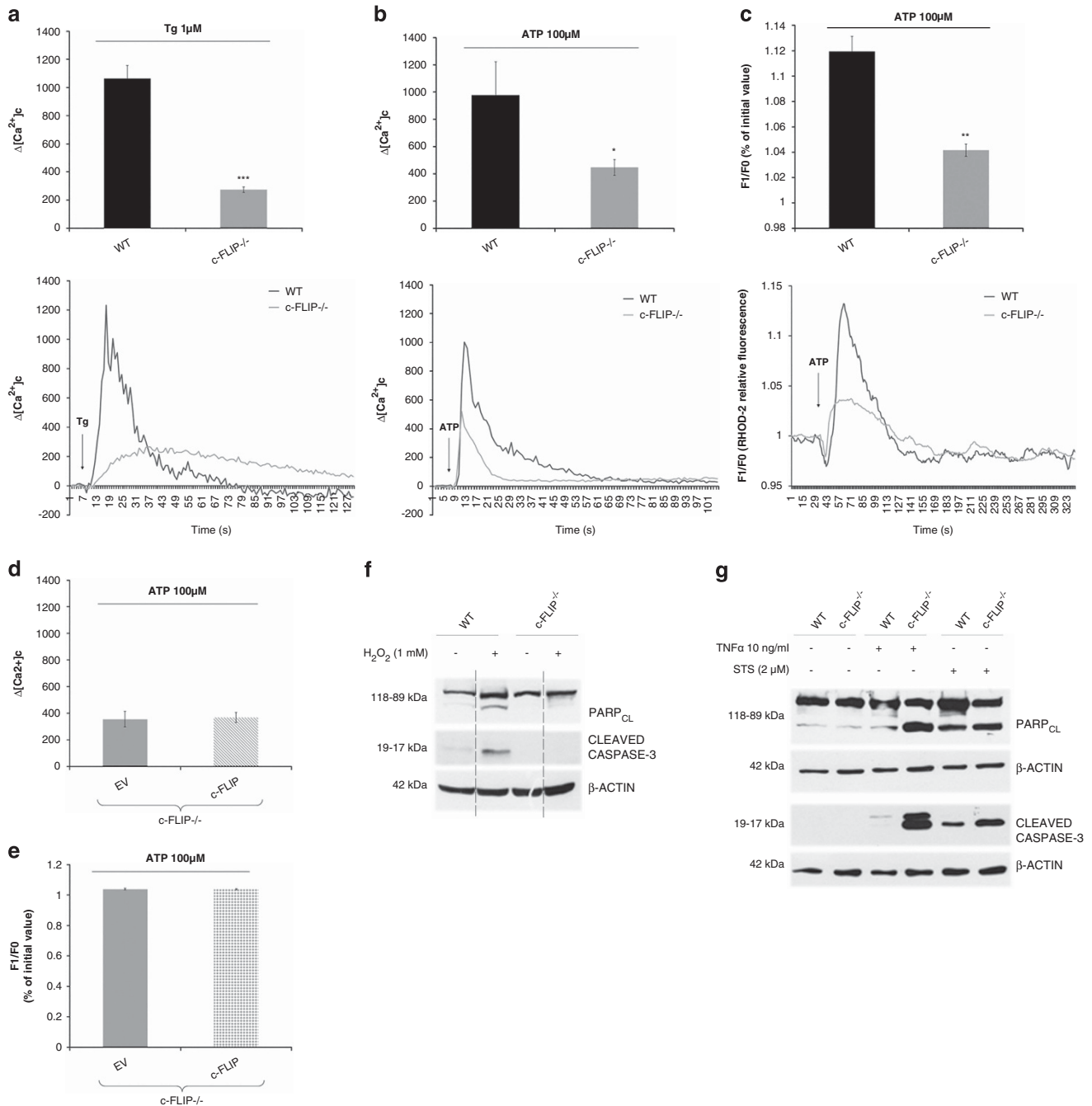
**Calcium chelation in WT MEFs induces ER changes.** TG-induced ER Ca<sup>2+</sup> release is an indirect measure of ER Ca<sup>2+</sup> content at the steady state. According to our data suggesting diminished Ca<sup>2+</sup> levels and Ca<sup>2+</sup> exchange in cells lacking c-FLIP, we speculated that these differences could directly contribute to the observed ER defects. We therefore tested whether the chelator of free, cytosolic calcium 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) could modify the overall ER structure in WT and c-FLIP<sup>-/-</sup> MEF. Unexpectedly,

BAPTA-AM affected ER shape and RTN-4B<sub>1,2</sub> integrity of the two cell lines. These effects were more evident in WT MEFs (Figure 7a, left panels), leading to ER enlargement and fragmentation and resembling ER defects of c-FLIP<sup>-/-</sup> MEFs, which were only marginally affected by Ca<sup>2+</sup> chelation (Figure 7a, right panels). WB analysis further revealed that BAPTA-AM caused RTN-4B<sup>1,2</sup> processing in WT cells, augmenting fragments formation in also c-FLIP<sup>-/-</sup> cells. Despite these effects, BAPTA-AM did not modulate c-FLIP<sub>L</sub> levels in WT cells, nor induced PARP cleavage (Figure 7b), suggesting a caspase-independent mechanism. Concordantly, whereas zVAD efficaciously diminished the basal RTN-4B<sub>1,2</sub> cleavage in c-FLIP<sup>-/-</sup> MEFs (Figure 7c), it did not abrogate the BAPTA-AM-induced RTN4 processing in either of the two cell lines (respectively, in Figures 7c and d). These data suggest that, in c-FLIP<sup>-/-</sup> cells, ER effects and RTN4 processing could be also directly associated with disturbed calcium levels, besides to the caspase-dependent pathway.

## Discussion

Components of DR signaling as caspase-8 have been identified on ER and mitochondria, suggesting that target specificity may depend on cellular compartments.<sup>5</sup> In this study, we report that a fraction of the caspase-8 inhibitor c-FLIP<sub>L</sub> is enriched at the ER and MAMs. Along with other MAM-proteins, whose ablation affects ER and mitochondrial morphology or their coupling,<sup>18,37,38</sup> here we demonstrate that c-FLIP deficiency in MEFs impacts on ER organization, causing enlargement of ER sheets to the detriment of tubular structures. The relative amount of the ER-shaping proteins RTN/DP1 and CLIMP63 was described to influence the cell-type-specific ratio of sheets to tubules in mammalian cells.<sup>39</sup> Jozsef *et al.*<sup>40</sup> demonstrated that RTN4 deficiency in MEFs is sufficient to convert ER tubules to cisternal structures, without affecting other ER-shaping factors or ER-resident proteins.<sup>40</sup> Interestingly, we found decreased expression of RTN-4A,B<sub>2,1</sub> in c-FLIP<sup>-/-</sup> MEFs, whereas no major differences were observed in other ER-shaping proteins or in ER proteins, confirming that ER defects of c-FLIP<sup>-/-</sup> MEFs were not related to variations in the overall ER content or to excessive CLIMP63 expression or ATL-mediated fusion.<sup>32</sup> As caspase-7 was formerly reported to process the RTN-4B on its cytoplasmic tail on apoptotic induction,<sup>33</sup> we hypothesized that ER defects observed in c-FLIP<sup>-/-</sup> cells could be associated with a deregulated, caspase-dependent processing of RTN4 occurring in cells lacking the inhibitor of caspase-8. In agreement with our hypothesis, here we demonstrate that c-FLIP<sup>-/-</sup> MEFs show an increased basal RTN4 processing, and that TNF $\alpha$  is sufficient to accelerate its cleavage. Interestingly, despite TNF $\alpha$  promptly induced a dose-dependent activation of caspase-8 and caspase-3, we observed only an initial cleavage of RTN4, decreasing at higher doses of TNF $\alpha$  or at longer time points, concomitantly to the accumulation of a higher-weight form of RTN-4B<sub>2,1</sub>. As Cyclin-dependent kinases 1/2 (CDK1/2) phosphorylate RTN4, abrogating its cleavage and inducing the accumulation of a higher-weight phosphorylated form, we hypothesized that the incomplete RTN4 processing could be associated with a CDK-mediated phosphorylation event. In accordance with





**Figure 6** c-FLIP absence alters Ca<sup>2+</sup> homeostasis and Ca<sup>2+</sup>-dependent apoptosis. **(a)** Fura-2AM recordings of cytosolic Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>c</sub>) after passive discharge of ER Ca<sup>2+</sup> stores induced by 1  $\mu$ M TG in MEFs of the indicated genotype. **Upper panel:** maximum Ca<sup>2+</sup> concentrations in bar charts. Mean  $\pm$  S.E.M. of  $n=70-100$  cells. **Lower panel:** changes in Ca<sup>2+</sup> concentrations shown as representative traces. **(b)** Fura-2AM recordings of cytosolic Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>c</sub>) after active ER Ca<sup>2+</sup> release induced by 100 mM ATP in MEFs of the indicated genotype. **Upper panel:** maximum Ca<sup>2+</sup> concentrations in bar charts. Mean  $\pm$  S.E.M. of  $n=70-100$  cells. **Lower panel:** changes in Ca<sup>2+</sup> concentrations shown as representative traces. **(c)** Rhod-2AM recordings of mitochondrial Ca<sup>2+</sup> entry (F<sub>1</sub>/F<sub>0</sub>) after active ER-to-cytosol Ca<sup>2+</sup> release induced by 100 mM ATP in MEFs of the indicated genotype. **Upper panel:** maximum Ca<sup>2+</sup> concentrations in bar charts. Mean  $\pm$  S.E.M. of  $n=70-100$  cells. **Lower panel:** changes in Ca<sup>2+</sup> concentrations. In **a-c** \* $P \leq 0.05$ ; \*\* $P \leq 0.001$ ; \*\*\* $P \leq 0.0001$ . **(d)** Fura-2AM recordings of cytosolic Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>c</sub>) after active ER Ca<sup>2+</sup> release induced by 100 mM ATP in c-FLIP<sup>-/-</sup> MEFs transfected with the indicated plasmids. **(e)** Rhod-2AM recordings of mitochondrial Ca<sup>2+</sup> entry (F<sub>1</sub>/F<sub>0</sub>) after active ER-to-cytosol Ca<sup>2+</sup> release induced by 100 mM ATP in c-FLIP<sup>-/-</sup> MEFs transfected with the indicated plasmids. **(f)** Representative WB analysis of total lysates (40  $\mu$ g) from MEFs of the indicated genotype, on treatment with H<sub>2</sub>O<sub>2</sub> 1 mM for 3 h. Apoptosis was investigated evaluating cleavage of PARP and caspase-3. **(g)** Representative WB analysis of total lysates (40  $\mu$ g) from MEFs of the indicated genotype, on treatment with STS (2  $\mu$ M) for 3h and TNF $\alpha$  10 ng/ml for 2 h. Apoptosis was investigated evaluating cleavage of PARP and caspase-3. ( $n=5$  for H<sub>2</sub>O<sub>2</sub> treatment;  $n=3$  for STS and TNF $\alpha$  treatments)



and worsened RTN-4B<sub>2,1</sub> fragmentation in c-FLIP<sup>-/-</sup> MEFs. These effects were more pronounced in WT than in c-FLIP<sup>-/-</sup> cells, probably as a consequence of the different Ca<sup>2+</sup> content of the two cell lines. To better investigate whether modulation of Ca<sup>2+</sup> levels could modify the ER organization independently of c-FLIP and caspases, we evaluated c-FLIP<sub>L</sub> levels and PARP cleavage as a measure of caspase activation. Interestingly, BAPTA-AM neither modulated c-FLIP<sub>L</sub> in WT MEFs, nor induced RTN-4B<sub>2,1</sub> cleavage via caspases. Nevertheless, although caspase activation was not described as necessary to ER remodeling during apoptosis,<sup>41</sup> we cannot exclude that the increased caspase-8 activity observed in c-FLIP<sup>-/-</sup> cells at basal conditions could impinge on RTN4 cleavage and ER integrity, in turn emphasizing the role of caspase-mediated processing of RTN4. To summarize, in addition to showing c-FLIP<sub>L</sub> at the ER and MAMs, in this paper we demonstrate its relevance in modulating ER morphology and tethering to mitochondria. We also provide evidence of a new role for caspases in ER structure modulation via processing of RTN4. Although the importance of RTNs transmembrane domains in ER tubulation was firstly described,<sup>20,21</sup> modifications in the intrinsically unstructured, cytosolic regions of RTN4<sup>42</sup> have been reported to influence the variable functions of RTNs or specific interactions.<sup>20,40</sup> We can therefore speculate that caspases, acting on exposed regions of RTN4, might disturb the localization<sup>43</sup> or the ability of ER-shaping proteins to form complexes and interact with cytoskeletal components or organelles.<sup>44</sup> Concluding, the present study highlights additional non-apoptotic roles for the death-receptor pathway, a frame in which c-FLIP<sub>L</sub> and caspases emerge as new factors in the maintenance of ER homeostasis.

## Materials and Methods

**Cell cultures and reagents.** Wild type (WT) and c-FLIP<sup>-/-</sup> MEFs were a generous gift of Tak W. Mak (Amgen Institute, Toronto, Canada). MEFs were cultured in DMEM, enriched with 10% fetal bovine serum, glutamine (2 mmol/l), sodium pyruvate (1 mmol/l) and non-essential amino acids, in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml). The Insolution Caspase-8 inhibitor II, indicated as Z-IE(OMe)TD(OMe)-FMK (zIETD), was purchased from Calbiochem (San Diego, CA, USA). Cells were treated for 24 h with zIETD 40 μM or with the vehicle DMSO. The *N*-Benzoyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (zVAD-FMK) was purchased from Sigma-Aldrich (St Louis, MO, USA). Cells were treated for 24 h with zVAD 40 μM or with the vehicle DMSO. Where indicated, JNJ-7706621 was added to cell cultures. After 1 h pre-treatment, TNF $\alpha$  was added. JNJ-7706621 was synthesized by the Cancer Therapeutics Research Team (Johnson & Johnson Pharmaceutical Research and Development, LLC). Transfections with pDsRed2-Mito (Clontech laboratories INC—Palo Alto, CA, USA), pEGFP-ER (Clontech) and pBluescript or c-FLIP<sub>L</sub> plasmid (kindly provided by M. Peter (The Ben May Institute for Cancer Research, University of Chicago, Chicago, IL USA) were performed using Transfectin reagent (BioRad, Hercules, CA, USA) at 2 μg/μl, or Lipofectamin Reagent (Invitrogen, San Giuliano Milanese, Italy) at 4 μg/μl, according to the manufacturer's instructions. Where indicated, BAPTA-AM (Sigma-Aldrich) 5 μM or the vehicle DMSO was added to cell cultures for 3 h at 37 °C and ER morphology and WB analysis of RTN4 were performed.

**Subcellular fractionation.** Whole mouse liver from adult CD1 mice or WT and c-FLIP<sup>-/-</sup> MEFs obtained from four, confluent, 500 cm<sup>2</sup> petri dishes was washed twice with PBS, re-suspended in isolation buffer (IB, 200 mM sucrose, 1 mM EGTA-Tris, and 10 mM Tris-MOPS, pH 7.4), and then broken up by dounce homogenization. Purified fractions were obtained according to Frezza *et al.*<sup>45</sup> Protein concentration was determined by micro BCA assay (Pierce Rockford, USA) and 20 μg of protein extract was separated by SDS-PAGE and immunoblotted for the indicated antibodies. All animal procedures were conducted in compliance with the Italian Ministry for Health guidelines.

**Immunoblotting.** Cells were collected and lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) in the presence of complete protease-inhibitor mixture (Sigma-Aldrich). Protein concentration was determined by micro BCA assay (Pierce, Rockford, IL, USA) and the indicated proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF, BioRad) or nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ, USA). Membranes were probed using the following antibodies: anti- $\beta$ -actin (1:10 000, Sigma-Aldrich); anti-calnexin (1:2000, Abcam, Cambridge, UK); anti-Tom20 (1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-lactate dehydrogenase (LDH) (1:2000, Rockland); anti-long-chain fatty-acid CoA synthases (FACL4) (1:500, Santa Cruz Biotechnology); anti-calreticulin (CRT) (1:1000, Abcam); anti-c-FLIP Dave II clone (1:800, Alexis, Lausen, Switzerland); anti-RTN4 (1:1000, Abcam); anti-CLIMP63 (1:1000, Proteintech Group, Chicago, IL, USA); anti-cleaved caspase-3 (1:500, Cell Signaling Technology) and anti-PARP (1:1000; Cell Signaling Technology).

**Imaging.** For confocal imaging and analysis of the morphology and tethering of ER and mitochondria, cells seeded on 24-mm round glass coverslips and incubated in Hanks balanced salt solution (HBSS), supplemented with HEPES 10 mM, were placed on the stage of a Nikon Eclipse TE300 inverted microscope equipped with a spinning-disk PerkinElmer Ultraview LCI confocal system, a piezoelectric z-axis motorized stage (Pifoc, Physik Instrumente, Germany) and an Orca ER 12-bit charge-coupled device camera (Hamamatsu Photonics, Japan). Cells expressing ERYFP and/or mRFP were excited using the 488 nm or the 543 nm line of the HeNe laser (PerkinElmer, Waltham, MA, USA) by using a  $\times$ 60 1.4 NA Plan Apo objective (Nikon Corporation, Japan). For confocal z-axis stacks, images separated by 0.2 μm along the z-axis were acquired. To evaluate ER morphology and ER-mitochondria tethering on zVAD or BAPTA treatment a Leica confocal microscope (Laser Scanning TCS SP2, Leica Microsystems Heidelberg GmbH, Mannheim, Germany) equipped with Ar/ArKr and He/Ne lasers and with a  $\times$ 40 oil 1.40 plan Apo immersion objective was utilized. Analysis of the interactions between ER and mitochondria was performed with the software ImageJ. Deconvolution, 2D- and 3D-reconstruction and surface rendering were realized using the VolumeJ plugin of ImageJ. Analysis of the interaction between ER and mitochondria was performed using Manders' colocalization coefficient.<sup>31</sup>

**Fluorescence recovery after photobleaching.** Cells were plated and transfected with ERYFP as previously described. After 48 h they were incubated in HBSS and placed on the stage of a laser scanning microscope (TCS SP5, Leica). Using the LasAF software (Leica), 16-μm<sup>2</sup> regions were manually defined to be bleached. To bleach the YFP fluorescence, one z-plane was bleached for a total of 3 s using 100% laser power of the 514 nm laser line with a  $\times$ 63, 1.4NA objective. The post-bleaching images were taken at 0.937 s intervals for a total of 120 s. Intensities of the photobleached regions were measured and normalized to the intensities of the same region before photobleaching.

**Ratiometric measurement of cytosolic Ca<sup>2+</sup> concentration.** A total of 15 $\times$ 10<sup>4</sup> WT and c-FLIP<sup>-/-</sup> MEFs were seeded on 35 mm Petri dishes. Twenty-four hours after seeding, cells were incubated for 45 min at 37 °C in culture medium containing Fura-2AM 3.5 μM (Invitrogen), which is a ratiometric fluorescent dye binding to free intracellular calcium. Cells were then washed free of Fura-2AM and Hanks balanced solution (HBSS, 130 mM NaCl, 5.2 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM glucose, 10 mM HEPES, pH 7.4) was added. EGTA was added to all solutions. Dishes were placed into a culture chamber at 37 °C on the stage of an inverted fluorescence microscope (Nikon TE2000E) and connected to a cooled CCD camera (512B Cascade; Roper Scientific, Princeton Instruments, Trenton, NJ, USA). Samples were illuminated alternately at 340 and 380 nm using a random access monochromator (Photon Technology International, Birmingham, NJ, USA) and emission was detected using a 510 nm emission filter. Images were acquired (1-ratio image/s) using Metafluor software (Universal Imaging Corporation, Downingtown, PA, USA). Calibration was obtained at the end of each experiment by maximally increasing intracellular Ca<sup>2+</sup>-dependent Fura-2AM fluorescence with ionomycin salt solution (Sigma-Aldrich) 5 μM, followed by recording minimal fluorescence in a Ca<sup>2+</sup>-free medium. Ca<sup>2+</sup> concentration was calculated according to the previously described formulas.<sup>46</sup> Where indicated, c-FLIP<sup>-/-</sup> cells were transiently transfected with pBluescript or c-FLIP<sub>L</sub> plasmids and ER-to-cytosol Ca<sup>2+</sup>-fluxes were measured in Fura-2AM-loaded cells.

**Measurement of mitochondrial Ca<sup>2+</sup> concentration.** A total of 15 × 10<sup>4</sup> WT and c-FLIP<sup>-/-</sup> MEFs were seeded on 35 mm, high, glass bottom Petri dishes. Twenty-four hours after seeding, cells were incubated for 1 h at 37 °C in culture medium containing Rhod2-AM 6 μM (Invitrogen). The ester forms of this calcium indicator are cationic, resulting in potential-driven uptake into mitochondria. Cells were then washed with standard medium and EGTA-containing HBSS at room temperature. Single-cell fluorescence was excited at 545 nm using a random access monochromator (Photon Technology International) and emission was detected using a 580 nm emission filter. Images of the emitted fluorescence, obtained with a ×60 oil objective, were collected using Metafluor software (Universal Imaging Corporation). Where indicated, c-FLIP<sup>-/-</sup> cells were transiently transfected with pBluescript or c-FLIP<sub>L</sub> plasmids and mitochondrial Ca<sup>2+</sup> entry were measured in Rhod2-AM-loaded cells.

**Evaluation of caspase-8 activity.** Caspase-8 activity was measured by the previously tested<sup>47</sup> colorimetric FLICE/Caspase-8 Assay kit (BioVision/Vinci-Biochem, Firenze, Italy) according to the manufacturer's protocol. Levels of caspase-8 activation were normalized by total protein concentration.

**Transmission electron microscopy.** For ultrastructural observation, cell monolayers were fixed in 2.5% glutaraldehyde (Electron Microscopy Science, Pennsylvania, USA) and in 0.1% cacodylate buffer (Electron Microscopy Science), postfixed in 1% osmium tetroxide (Electron Microscopy Science) and then treated with 1% tannic acid (Acros Organics, New Jersey, NJ, USA). Dehydrated in ethanol (Carlo Erba), cells were detached from the plastic dish by a brief treatment in 1% toluene (Carlo Erba). The pelleted monolayer was then incubated in a solution of 50% toluene and 50% EPON 812 (Electron Microscopy Science) and finally processed for conventional EPON 812 embedding. Ultra-thin sections were cut in a Leica Ultracut R ultramicrotome, contrasted for 10 min in 1% acid tannic and successively in 1% lead hydroxide (Società Italiana Chimici, Rome, Italy) and then viewed in a Hitachi 7000 transmission electron microscope.

**Statistical analysis.** Values are expressed as mean ± S.E. Statistical evaluation was performed by Student's *t*-test analysis and a value of *P* ≤ 0.05 was considered statistically significant.

### Conflict of Interest

The authors declare no conflict of interest.

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### Author contributions

E.S.M. designed, performed experiments and wrote the paper, S.C. performed apoptosis experiments, C.G. and S.P. assisted in interpreting results and writing the manuscript, L.S. and E.Z. conceived the overall design and wrote the manuscript with input from A.F.

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