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BID is cleaved by caspase-8 within a native complex on the mitochondrial membrane

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Abstract

Caspase-8 stably inserts into the mitochondrial outer membrane during extrinsic apoptosis. Inhibition of caspase-8 enrichment on the mitochondria impairs caspase-8 activation and prevents apoptosis. However, the function of active caspase-8 on the mitochondrial membrane remains unknown. In this study, we have identified a native complex containing caspase-8 and BID on the mitochondrial membrane and showed that death receptor activation by Fas or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced the cleavage of BID (tBID formation) within this complex. tBID then shifted to separate mitochondria-associated complexes which contained other BCL-2 family members, such as BAK and BCL-X_L. We report that cells stabilize active caspase-8 on the mitochondria in order to specifically target mitochondria-associated BID and that BID cleavage on the mitochondria is essential for caspase-8-induced cytochrome c release. Our findings indicate that during extrinsic apoptosis, caspase-8 can specifically target BID where it is mostly needed, on the surface of mitochondria.

Introduction

Programmed cell death or apoptosis is characterized by the activation of a class of proteases called caspases. Caspases are cysteine-dependent aspartic acid-specific proteases which represent the executioners of apoptosis. In mammalian cells, caspases are almost universally expressed as inactive zymogens in the cytosol and are generally classified into two groups, either initiators or effectors. The initiator caspases (caspase-8, -2, -9, -10) are monomeric zymogens and have long pro-domains which aid in their recruitment to multiprotein complexes where they become activated through dimerization and self-cleavage. Typically, active initiator caspases then cleave the effector caspases. The effector caspases (caspase-3, -6, -7) have a short pro-domain and exist as inactive dimers in the cytosol and must undergo proteolysis at an internal aspartate to form an active heterotetrameric caspase. This hierarchy of caspase activation represents one of the hallmarks of apoptotic cell death.

Depending on the death inducing signal, caspase activation and apoptosis can occur via two distinct pathways: intrinsic or extrinsic. Intrinsic apoptosis is triggered by a number of different cellular stresses which induce the release of apoptogenic factors from the mitochondrial intermembrane space into the cytosol, where they activate the caspase cascade.^{1,2} Mitochondrial outer membrane permeabilization (MOMP) is regulated by the interplay between the BCL-2 family of proteins, which is comprised of both pro-apoptotic members that promote apoptosis (i.e. Bax, BAK, BID), and anti-apoptotic members that prevent it (i.e. BCL-2, BCL-X_L, Mcl-1).^{3,4} In contrast to intrinsic apoptosis, extrinsic apoptosis is initiated at the plasma membrane by stimulation of a subgroup of the tumor necrosis factor (TNF) receptor super family which includes TNFR1, Fas, TRAIL-R1, TRAIL-R2 and DR3.⁵ Binding of a death

ligand, such as Fas or TRAIL, triggers receptor trimerization which induces the binding of adaptor molecules.⁶ These adaptor molecules, such as FADD and TRADD, recruit caspase-8 to the receptor complex to form the death-inducing signalling complex (DISC). Pro-caspase-8 (p55) consists of three subunits, the first encompasses two tandem death effector domains (DED; p26) while the other two domains (p18 and p10) make up the active site of the protease (Figure 1a). Caspase-8 exists as a monomer in the cytosol, but upon DISC formation it undergoes dimerization.^{7,8} The dimerization of two caspase-8 monomers (p55/p55) results in the conformational change which exposes the active site of the caspase through a mechanism termed “induced proximity”.^{7,9-11} Dimerization has been demonstrated to be sufficient for activation of caspase-8, but it has been proposed that full activity requires self-cleavage.¹¹⁻¹⁴ Caspase-8 initially cleaves itself between the p18 and p10 domains forming a heterodimer within a heterotetrameric complex (p43-p10/p43-p10). This first cleavage is necessary for caspase-8 to achieve the ability to cleave other substrates such as caspase-3 and BID.^{12,13} p43-p10/p43-p10 can undergo a second cleavage between the p26 domain and the p18 domain which frees the heterotetramer (p18-p10/p18-p10) from the DED domains but does reduce its activity.¹² Extrinsic apoptosis proceeds via two different pathways, type I and type II, depending on the level of caspase-8 activation at the DISC.¹⁵ In type I cells, large amounts of DISC and active caspase-8 are formed which leads to the direct cleavage of effector caspases in the cytosol.¹⁶ In type II cells there is slower DISC assembly and low production of active caspase-8.¹⁵ In this scenario, caspase-8 must recruit the intrinsic (mitochondrial) pathway to amplify the death signal and execute apoptosis. The transition from the extrinsic pathway to the intrinsic pathway is achieved through

caspase-8 processing of BID.^{17,18} Cleaved BID, or tBID, interacts with other BCL-2 family members on the surface of the mitochondria which results in MOMP.

We had previously demonstrated that during type II extrinsic apoptosis caspase-8 becomes enriched on the mitochondrial surface and that by blocking caspase-8 association with the mitochondria we were able to inhibit the formation of p43-p10, prevent BID cleavage and block apoptosis.^{19,20} However, the purpose of the insertion of active caspase-8 into the mitochondrial membrane remains unknown. In this study, we report that caspase-8 and BID form a native macromolecular complex on the mitochondria. Following Fas or TRAIL activation, tBID is formed within this complex by the caspase-8-mediated cleavage of BID and then shifts to a separate complex containing BAK and/or BCL-X_L. By concentrating BID and active caspase-8 on the mitochondrial membrane, type II cells have established a mechanism by which low levels of proteolytically active caspase-8 would be able to specifically target sufficient amounts of BID on the organelle (mitochondria) where it is needed to induce MOMP. These findings further emphasize the important role of compartmentalization in the activation of caspases, such as is seen with the apoptosome and inflammasome.^{21,22} In summary, we have identified a mitochondria-associated triggering platform, from which type II cells launch the mitochondrial phase of apoptosis.

Results

The generation of caspase-8 point mutants to study mitochondrial-dependent cell death. We hypothesized that point mutations which affect caspase-8 processing and activity might also influence the association of caspase-8 with the mitochondria.

Figure 1a depicts a linear cartoon of caspase-8 showing the main functional domains. Caspase-8 is cleaved into three distinct fragments (DED, p18 and p10) whose boundaries and molecular weights are labelled in Figure 1a. Four different mutant constructs were created which specifically targeted aspartate residues in the regions of the first and second cleavage sites of caspase-8 (Figure 1a). All constructs were tagged with GFP in order to examine localization and to distinguish exogenous from endogenous caspase-8. Six different GFP fusion proteins of caspase-8 were constructed: wild type (wt) caspase-8-GFP (C8-GFP), double mutant on second cleavage site (DM2-GFP), triple mutant on second cleavage site (TM2-GFP), single mutant on first cleavage site (SM1-GFP) and double mutant on first cleavage site (DM1-GFP) (Figure 1a). In addition, caspase activity is dependent upon a conserved cysteine residue within the active site of the caspase.²³ C360 serves this function in human caspase-8 and was mutated to serine (C360S-GFP) to form an inactive caspase-8.

We initially explored the cleavage patterns of the caspase-8-GFP point mutants. For that reason, HeLa cells which stably expressed an shRNA against endogenous caspase-8 (Supplementary Figure S1) were transfected with the different caspase-8-GFP mutants. The formation of both p43 and p18 was evident in cells transfected with wt caspase-8 (C8-GFP), or with caspase-8 mutated at the second cleavage site (DM2-GFP or TM2-GFP) (Figure 1b, lanes 3-5) but not in cells transfected with caspase-8 mutated at the first cleavage site (SM1-GFP and DM1-GFP, lanes 6-7). Furthermore, the inactive caspase-8 mutant, C360S-GFP, displayed a strong inhibition of p43 and p18 formation suggesting a defect in self cleavage (Figure 1b, lane 8).

Given the observed alterations in the cleavage pattern of the caspase-8 point mutants we next sought to analyze their effects on the induction of cell death. Surprisingly, all caspase-8 mutants were capable of inducing cell death in control HeLa cells expressing a non-targeting shRNA (shCont) to a level similar to Fas treatment (Figure 1c, black bars) despite the defects in autoprocessing noted in Figure 1B. It should be noted that, in the absence of death ligand, exogenous expression of caspase-8 is able to induce its dimerization and activation through a process termed “induced-proximity”^{7,10,13}. Studies have shown that the first cleavage of caspase-8 between p18 and p10 can occur by inter-dimer cleavage between two separate dimers of caspase-8.^{10,13} Importantly, caspase-8 was not highly overexpressed and most transfected cells displayed a ~2-fold increase in the total amount of caspase-8 (Supplementary Figure S2a). For these reasons, it is likely that dimerization occurs between exogenously-expressed inactive caspase-8 mutants (DM1-GFP and C360S-GFP) and endogenous caspase-8. Indeed, the second cleavage product of caspase-8, p18, was observed in shCont cells transfected with the different active and inactive caspase-8 mutants, but was absent in shCaspase-8 cells which were transfected with the inactive forms of caspase-8 (Supplementary Figure S2b). Of note, less p18 formation was observed in control cells transfected with DM1-GFP, which cannot undergo the first cleavage on its own (Supplementary Figure S2b, lane 7, top panel). This is due to the fact that, unlike all the other caspase-8 constructs, the p18 formation in cells transfected with this mutant is fully dependent on the cleavage of endogenous caspase-8. Next, the effect of the different caspase-8 mutants on viability was assessed in shCaspase-8 HeLa cells. Indeed, these cells were protected from Fas-induced apoptosis (Figure 1c, gray bars). However, the expression of C8-GFP, DM2-GFP, TM2-GFP or SM1-GFP caused levels of cell death similar to that in shCont

HeLa (Figure 1c) indicating that these exogenously-expressed caspase-8 constructs are fully capable of inducing apoptosis. In contrast, the expression of DM1-GFP or C360S-GFP did not induce cell death without the presence of endogenous caspase-8 (Figure 1c).

Mitochondrial localization of caspase-8 point mutants. In order to evaluate the link between caspase-8 processing and the subcellular localization, shCont HeLa cells were transfected with the different caspase-8-GFP mutants and assessed by confocal microscopy using the mitochondria-tagged protein Mito-dsRed2 as a marker. zVAD-fmk was added before transfection and kept throughout the experiment to prevent cell death. Exogenously expressed wt caspase-8 was mostly observed on the mitochondria (Figure 2, C8-GFP). Furthermore, we confirmed that the mitochondrial localization of caspase-8 occurred prior to cytochrome c release and was not blocked by BCL-X_L overexpression (Supplementary Figure 3). The disruption of the second cleavage site of caspase-8 still resulted in mitochondrial localization (Figure 2, DM2-GFP and TM2-GFP). In addition, non-mitochondrial punctate structures were consistently observed (Figure 2, white arrows). In contrast, when the first cleavage site of caspase-8 was disrupted, the protein lost its mitochondrial localization and was now diffused in the cytosol (Figure 2, DM1-GFP). This suggested that the formation of the p43-p10 heterodimer may be needed for stable enrichment of caspase-8 on the mitochondria. It is important to mention that even when exogenously-expressed caspase-8 was present on the mitochondria, a significant amount of it was still in its unprocessed form (Figure 1b and 3a-c). The inactive C360S caspase-8 mutant was also observed on the mitochondria (Figure 2, C360S-GFP). This is in line with the fact that unlike DM1-GFP, this mutant is capable of being processed into the p43-p10 form by the

endogenous caspase-8 through intra- or inter-dimer cleavage.¹³ Indeed, C360S-GFP did interact with endogenous caspase-8 on the mitochondria (Figure 3a) and can produce high amounts of mitochondria-associated p43 in shCont HeLa (Figure 3b). The association of the p43 form of C360S-GFP with the mitochondria was completely absent in shCaspase-8 cells demonstrating the need for the endogenous caspase-8 for the first cleavage of this mutant (Figure 3c). Furthermore, unlike the active mutant TM2-GFP, the two inactive mutants DM1-GFP and C360S-GFP could not generate mitochondria-associated caspase-8-like activity (LETDase) without endogenous caspase-8 (Figure 3d). Overall, these results are in line with the observed dependency of these inactive mutants on endogenous caspase-8 for cell death induction (Figure 1c).

BID and caspase-8 form a native complex on the mitochondrial membrane. Our data so far has suggested that the mitochondrial localization of active caspase-8 may be needed to specifically target a protein(s) on the mitochondria to execute apoptosis. Since it was previously shown that type II extrinsic apoptosis is dependent on BID cleavage by caspase-8 we sought to determine if caspase-8 forms a complex with BID on the mitochondria. We initially attempted to use mitochondria isolated from HeLa which co-expressed fluorescently-tagged BID and caspase-8. However, overexpression of these two death inducing proteins caused massive apoptosis. To circumvent this, mitochondria were isolated from shCont HeLa cells which had been transfected with either BID-mCherry or C360S-mCherry. The pan-caspase inhibitor, zVAD-fmk, was added to the C360S-mcherry transfected cells to prevent cell death. The mitochondria were solubilized in the mild non-ionic detergent, dodecyl- β -D-

maltoside (DDM), and the lysates (supernatant) were run by clear native electrophoresis (CNE). Blue native electrophoresis (BNE) was not employed because of the roughly 10-20 fold quenching of the fluorescent signal by coomassie blue, which would interfere with fluorescent imaging of the gel.²⁴ Furthermore, CNE is milder than BNE and has been shown to be helpful in maintaining physiological protein complexes.²⁵ Figure 4a clearly showed that both BID-mCherry and C360S-mCherry formed a >200kDa complex which co-migrated to the same position in the first dimension. The BID-mCherry and C360S-mCherry lanes were excised and run in the second dimension by SDS-PAGE to separate the different proteins composing this large complex. Immunoblotting indicated that the majority of mitochondria-associated endogenous BID (and BID-mCherry) was present within the complex (Figure 4b, dashed line). Furthermore, 2D CNE/SDS-PAGE revealed that endogenous caspase-8 co-migrated with BID-mCherry, suggesting that it was part of the native complex. Identical results were obtained from the reciprocal experiment using C360S-mCherry mitochondria and showed that endogenous BID co-migrated with both C360S-mCherry and endogenous caspase-8 in the first dimension (Figure 4c, dashed line). This was the first evidence of caspase-8 and BID forming a native complex on the mitochondrial membrane. Importantly, active caspase-8 (p43) was observed within this large complex in caspase-8 transfected cells (Figure 4d and Supplementary Figure S4). The mitochondria-associated BCL-2 family proteins, BCL-X_L and BAK were not part of the caspase-8/BID complex (Figure 4b and c).

Fas- or TRAIL-activation induced cleavage of BID within a native complex on the mitochondrial membrane. Having identified a native complex of caspase-8 and

BID, we wanted to examine the effects that activation of caspase-8 had on the state of the complex. Initially, we found that the endogenous caspase-8/BID complex was also resident on the mitochondrial membranes of untransfected and untreated HeLa cells (Figure 5a, solid line). This was supported by 1D SDS-PAGE analysis of isolated mitochondria which confirmed the presence of BID and caspase-8 on naïve mitochondria (Figure 5c, lanes 1-2). The localization of full length BID on mitochondria of untreated cells would be somewhat surprising as BID has been reported to be a cytosolic factor that translocates to the mitochondria only after cleavage by caspase-8.^{17,18} Nevertheless, we consistently detected BID on the mitochondria and after subcellular fractionation analysis estimated that ~20% of full-length BID protein was associated with the mitochondria of untreated cells (data not shown). We also investigated if this complex was present in the non-small cell lung carcinoma cell line H460. H460 are type II cells and are sensitive to TRAIL-induced extrinsic apoptosis.²⁶ Similar to HeLa cells, untreated H460 cells contained a native caspase-8/BID complex on the mitochondria (Figure 6a). Taken together these results indicate that both BID (full-length) and pro-caspase-8 (p55) naturally reside on the mitochondria within a large molecular weight complex.

Marked differences in the constitution and migration of the mitochondrial complexes were observed when caspase-8 was activated by TM2-GFP transfection or death receptor ligation (Figures 4d, 5b and 6b). The initiation of apoptosis led to an increase in the amount of activated caspase-8 (p43) on the mitochondria (Figure 6d, compare lanes 3 and 4). Although p43 was detectable in the DDM soluble fraction of the mitochondria, it was found to be more highly enriched in the DDM resistant fraction (Figure 5c, compare lanes 3 and 4). In contrast, BID and pro-caspase-8 were more equally distributed between the fractions while tBID, BAK, BCL-X_L and

VDAC1 were more soluble (Figure 5c and 6c). Figure 4d clearly showed that in TM2-GFP transfected cells p43 was also present in the caspase-8/BID complex. Similar results were obtained in C360S-GFP transfected HeLa cells (Supplementary Figure S4). In agreement, there was a substantial increase in caspase-8 LETDase activity in the DDM soluble fractions of Fas-treated shCont HeLa and TRAIL-treated H460 cells compared to untreated cells (Figure 5c and 6c, lower panel). In parallel with p43 formation, we found that tBID also formed within the native complex with caspase-8 (Figure 5b and 6b, solid line) and shifted to larger molecular weight complexes which co-migrated with both BAK and BCL-X_L (dotted lines). These results suggested that after cleavage of BID within the caspase-8/BID complex, tBID is potentially targeted to a separate set of complexes with the BCL-2 family members BAK and/or BCL-X_L. A plot profile generated from densitometric analysis of the immunoblots in Figure 5b showed that the majority of the various mitochondrial-bound forms of caspase-8 and BID were within a single complex on the mitochondria (Figure 5b, lower panel).

Recombinant caspase-8 is sufficient to induce cytochrome c release from isolated mitochondria. To further characterize the activity of caspase-8 on mitochondria recombinant active caspase-8 protein (rCaspase-8) was used. Initially, we tested whether active caspase-8 was sufficient to cause the permeabilization of isolated mitochondria. The level of caspase-8 activity on mitochondrial membranes after incubation with rCaspase-8 was comparable to mitochondria isolated from Fas-treated HeLa cells as measured by an LETDase activity assay (Supplementary Figure S5). rCaspase-8 caused a dose-dependent release of cytochrome c from mitochondria isolated from HeLa cells (Figure 7a). This was the first indication that incubation of

isolated mitochondria with active caspase-8 is sufficient for MOMP. Since MOMP is regulated by the BCL-2 family of proteins²⁷, we assessed whether the release of cytochrome c induced by rCaspase-8 could be blocked by BCL-X_L. Incubation of mitochondria isolated from BCL-X_L-expressing HeLa cells with rCaspase-8 showed that cytochrome c release was blocked (Figure 7b, compare lane 2 to 5). These results suggested that caspase-8-induced cytochrome c release from isolated mitochondria was dependent on the BCL-2 family of proteins.

BID is necessary for caspase-8 induced cytochrome c release from isolated mitochondria. Figure 7b suggested that caspase-8-induced cytochrome c release is regulated by BCL-2 pro-apoptotic proteins. BID is known to be a substrate of caspase-8 after death receptor activation,^{28,29} and we identified a native mitochondrial complex containing caspase-8 and BID (Figures 4-6). Therefore we examined if caspase-8 could cleave mitochondria-associated BID and if this was sufficient to induce MOMP. Indeed, tBID formation was observed in mitochondria treated with rCaspase-8 (Figure 7c, lane 2). Although mitochondria-associated BID was cleaved by caspase-8, there may be other targets of caspase-8 on the mitochondrial membrane which are necessary for MOMP. To test the dependence of caspase-8 on BID to induce MOMP, we transfected HeLa cells with either a non-targeting control siRNA (siCont) or an siRNA against BID (siBID) prior to mitochondria isolation. rCaspase-8 induced the release of the majority of cytochrome c from the mitochondria isolated from siCont cells (Figure 7c, lane 2). In contrast, mitochondria isolated from siBID transfected cells displayed a significant reduction in the amount of cytochrome c release and tBID formation (Figure 7c, lane 4). These results indicated that BID was

required for rCaspase-8-induced cytochrome c release and that the amount of BID present on the mitochondrial membranes is sufficient to support MOMP. In addition, Figure 7c indicated that caspase-8 association with the mitochondria is not dependent on the availability of BID on the mitochondrial membrane (compare lanes 2 and 4). In addition, we found that TRAIL activation of H460 cells resulted in significant decreases in the levels of mitochondrial-bound BID as compared to cytosolic BID, suggesting that the mitochondria may be the main site of caspase-8-induced cleavage of BID (Figure 6d).

Discussion

In Figure 8 we present a model for the biochemical events associated with Fas- or TRAIL-induced extrinsic apoptosis. The first step entails the initial activation of apoptosis by Fas or TRAIL and the formation of the DISC. The next step is the stable insertion of active caspase-8 (p43-p10) into the mitochondrial membrane. In the third step, active caspase-8 cleaves BID within the native complex on the mitochondrial membrane which results in the formation of tBID. Interestingly, after cleavage, tBID gains the ability to dissociate from the caspase-8/BID complex. Cleavage of BID exposes the BH3-domain and may be the driving force behind tBID dissociation from the caspase-8/BID complex.²⁸ tBID then shifts to separate complexes on the mitochondrial membrane where it interacts with BAK and/or BCL-X_L to activate MOMP. However, our results do not directly address the exact mechanism of tBID activity during MOMP, a process which remains unclear.⁴ Apoptogenic factors, such as cytochrome c and Smac/DIABLO, are released from the mitochondria, which leads to the activation of effector caspases and cell death.

Numerous studies have reported that it is extremely difficult to detect active caspase-8 (p43-p10) at the DISC in type II cells.^{15,30} We have now shown that, after Fas- or TRAIL-activation, p43-p10 is readily detectable on the mitochondrial membrane in multiple cells lines. Given that a native caspase-8 population is already present on the mitochondrial membrane, it is tempting to speculate that caspase-8 can undergo dimerization and self-cleavage/activation directly upon the mitochondrial membrane. Alternatively, the presence of non-mitochondrial punctuated structures (Figure 2, white arrows) could suggest that p43-p10 is formed at the DISC and subsequently translocates to the mitochondria. Mitochondrial targeting of active caspase-8 may be needed for the activation of the native caspase-8/BID complexes on the outer membrane. If this scenario were true, one would expect to see p43-p10 enrichment on the mitochondrial membranes of type I cells. Indeed, we did observe tBID formation within the caspase-8/BID complex in cells which underwent a “type-I-like” mitochondrial-independent apoptosis (Supplementary Figure S6). We are currently investigating the mechanism and location of caspase-8 activation in more detail. Nevertheless, the properties and behaviour of the inactive mutant of caspase-8, C360S-GFP, have suggested that it may prove to be a valuable tool for probing how caspase-8 interacts with the mitochondria after death receptor activation.

A number of independent studies have observed the association of caspase-8 with the mitochondria, but the purpose of its localized production on the mitochondrial outer membrane has remained unknown.^{19,31-35} It is plausible that the formation of a death-inducing activation platform on the outer mitochondrial membrane is the mechanism by which type II cells manage to effectively target sufficient amounts of BID to induce MOMP and hence bypass the problem of weak caspase-8 activation at the DISC. We had previously demonstrated that caspase-8

autoprocessing after Fas activation was reliant on an association of caspase-8 with the mitochondrial membrane and that this association was dependent on the presence of mature cardiolipin; a result which further suggests that caspase-8 is processed directly on the mitochondria.¹⁹ In this study, we have identified a macromolecular complex of caspase-8 and BID on the mitochondrial membrane within which the cleavage of BID (tBID formation) occurs. Together, our results support the premise that Fas- or TRAIL-induced production of active caspase-8 on the mitochondria is necessary to target mitochondria-associated BID. Importantly, we have shown four key points to support this idea, namely that caspase-8 was found in a complex with both its substrate (BID) and its product (tBID) (Figures 5b and 6b), a larger proportion of mitochondrial-bound BID was cleaved by caspase-8 compared to cytosolic BID (Figure 6d), incubation of isolated mitochondria with active caspase-8 was sufficient to induce MOMP (Figure 7a) and the amount of BID on the mitochondrial membrane was sufficient to support MOMP (Figure 7c). Overall, these results might explain why tBID is found exclusively on the mitochondrial membrane and why it, like p43-p10, has an affinity for cardiolipin. It should be noted that both full-length BID and tBID preferentially bound cardiolipin-containing liposomes in an *in vitro* assay (Supplementary Figure S7). In addition, we have also found that cells deficient in mature cardiolipin contain less mitochondrial-bound BID (data not shown).¹⁹ These data suggested, in addition to the previously described model in which cardiolipin provides an activation platform for caspase-8, that cardiolipin is also necessary for the function and stabilization of the caspase-8/BID complex. Recently, the presence of cardiolipin-enriched microdomains in the mitochondrial membrane has been described.³⁶ In those experiments, Triton X-100 was used to solubilise the membranes, a detergent often used in the isolation of lipid rafts.³⁷ Interestingly, we

found that the caspase-8/BID complex was not detectable by 2D CNE/SDS-PAGE when Triton X-100 was used to solubilise the mitochondria (Supplementary Figure S8). Mitochondrial-bound caspase-8 and BID were found solely in the Triton X-100-resistant membrane fraction (Supplementary Figure S8), a result which was consistent with the data presented in Figures 5 and 6, where higher amounts of active caspase-8 were found in DDM-resistant mitochondrial membranes. Collectively these data would suggest that the caspase-8/BID complex localizes to mitochondrial membrane microdomains and that disruption of these microdomains (by depletion of mature cardiolipin, for example) inhibits complex formation and caspase-8 activation. However, the presence of mitochondrial lipid rafts is still a matter of debate and characterization of the caspase-8/BID complex and the role of cardiolipin in its function requires further clarification.³⁸ In summation, this novel caspase-8/BID mitochondrial complex concentrates the enzyme (caspase-8) and its substrate (BID) on the organelle where it is needed (mitochondria), thus providing a means by which the low levels of proteolytically active caspase-8 produced during type II apoptosis are able to efficiently engage the mitochondrial pathway.

Materials and methods

Plasmids and expression vectors. The fluorescently-tagged caspase-8 expression constructs were generated by using human *caspase-8* cDNA ligated into the KpnI and BamHI sites of pEGFP-N1 or pmCherry-N1 which expressed C-terminally tagged caspase-8. Caspase-8 point mutations were created by using a Site Directed Mutagenesis Kit (Stratagene). Primer sequences are available upon request. Mito-dsRed2 was a kind gift from Dr. K. Yee. The sequence targeted by siBID was GAAGACATCATCCGGAATATT. Non-targeting siRNA pool (Dharmacon) was used as control. The hairpin sequence for shCaspase8 was CCGGGAATCACAGACTTTGGACAAACTCGAGTTTGCCAAAGTCTGTGATTCTTTT (Open Biosystems; Thermo Scientific). shCont HeLa and BCL-X_L HeLa were described previously.¹⁹

Cell lines and reagents. The cervical carcinoma HeLa cell lines were cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. Transfection of HeLa cells was performed using Lipofectamine 2000 (Invitrogen). BCL-X_L, shCaspase-8 and shCont stable HeLa cell lines were generated by transfection with pcDNA3/BCL-xL, pLKO.1/shCaspase-8 or pSUPER/shCont, respectively, and selected in G418 (0.5 mg/ml), except for shCaspase-8 (puromycin; 3µg/ml). For siRNA transfection, unless otherwise stated, HeLa cells were transfected with 50 nM of the indicated siRNA and the efficiency of the knockdown was validated by western blotting 48 hours post-transfection. Recombinant human caspase-8 (R&D) is the fully active form which forms a stable tetramer composed of two dimers of the two subunits p18 and p10. Antibodies used for immunoblot analyses were: anti-β-actin (Sigma), anti-αtubulin (Sigma), anti-BCL-xL serum (a gift from Dr. Craig Thompson, University of

Pennsylvania, Philadelphia, PA, USA), anti-BID (Cell Signaling), anti-caspase-8 p18 domain (Alexis Biochemicals), anti-cleaved caspase-8 (Cell Signaling), anti-cytochrome c (BD Pharmingen), anti-complex III (Reiske iron-sulfur protein) (Molecular Probes), anti-complex I (ND6 protein) (Molecular Probes), anti-VDAC1 (Molecular Probes), anti-BAK (Upstate Biotechnology), anti-GFP (Clontech).

Cell fractionation. HeLa cells were washed twice in cold PBS. Cells were then scraped into ice cold PBS and spun at 600 x *g* for 10 minutes. Pellets were resuspended in Mitochondrial Isolation Buffer (MIB) (200 mM Mannitol, 70 mM Sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.4) supplemented with 1mM dithiothreitol and 1X protease inhibitor cocktail (Sigma) and incubated on ice for 5 minutes. All subsequent steps were performed on ice. The cells were homogenized in a tight-fitting potter and an RZR 2051 overhead stirrer (Heidolph) at 1600 rpm. The nuclei and cell debris were separated and discarded by centrifugation at 600 x *g* for 10 minutes. The remaining supernatant was centrifuged at 10 000 × *g* for 10 minutes and the mitochondria-enriched fraction was washed once in MIB and then resuspended in a minimal volume of MIB. The mitochondrial and cytosolic protein concentrations were assessed using the Protein Assay Reagent (Bio-Rad).

Confocal microscopy. In general, cells were grown in 6-well plates on glass coverslips. Cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature. Coverslips were washed in PBS and mounted in immunofluorescence mounting medium (Vectashield; Vector Laboratories). Alternatively, cells were permeabilized in Triton X-100, blocked with 10% FBS and incubated overnight with

anti-cytochrome c (BD Pharmingen) in 5% BSA. Coverslips were incubated with Alexa Fluor 488 anti-mouse IgG (Molecular Probes) in 5% BSA, washed in PBS and mounted immunofluorescence mounting medium containing DAPI. Specimens were analyzed at room temperature using a confocal microscope (Leica SP2 (DMIRBE) laser scanning confocal with Leica SP2 software) equipped with a 63x/1.32.na oil HCX PL APO lens (Leica).

Immunoprecipitation from isolated mitochondria. Isolated mitochondria were washed once by resuspending in 500 μ l of MIB followed by centrifugation at 10 000 x g for 10 minutes. Pellets were resuspended in mitochondrial assay buffer (MAB) (120 mM KCl, 1 mM KH_2PO_4 , 0.1 mM EGTA, 1 mM EDTA, 20 mM HEPES pH 7.4). After resuspension, 1% CHAPS was added and the mitochondrial membranes were incubated on ice for 5 minutes. Detergent solubilized mitochondrial membranes were spun at 10 000 x g for 10 minutes. Supernatants were diluted to 0.5% CHAPS and incubated for 2 hours at 4°C with anti-GFP agarose beads (MBL International). Beads were washed 3 times in MAB + 0.5% CHAPS and resuspended in SDS sample buffer before western blot analysis.

Caspase-8 activity measurements. Isolated mitochondria were washed once in MIB followed by centrifugation at 10 000 x g for 10 minutes. Pellets were resuspended in MIB and incubated for 1 hour at room temperature in the dark with Caspase-Glo 8 Reagent (Promega). The relative light unit (RLU) produced by caspase-8 cleavage of benzyloxycarbonyl-Leu-Glu-Thr-Asp (zLETD)-aminoluciferin was recorded using GLOMAX software on a Veritas Microplate Luminometer (Turner Biosystems).

Cell death assays and caspase-8 processing. Twenty-four hours post transfection cells were harvested and washed in PBS. Pellets were resuspended in PBS containing 2 µg/ml propidium iodide (PI) (Molecular Probes, Invitrogen). The number of GFP positive/PI positive cells was quantified by flow cytometry using a BD FACSCalibur system with CellQuest software (Becton Dickinson). Alternatively, pellets were resuspended directly in SDS sample buffer for analysis by SDS-PAGE.

In vitro cytochrome c release assays. Isolated mitochondria were resuspended in reaction buffer (125 mM KCl, 1 mM KH₂PO₄, 2.5 mM MgCl₂, 0.2 mM EGTA, 5 mM HEPES, 0.1% BSA; pH 7.4) supplemented with 1X protease inhibitor cocktail (Sigma). Mitochondria were incubated for 5 minutes at room temperature with 10 mM succinate and 10 µM rotenone. Recombinant protein was added at the stated concentration and incubated for 15 minutes at 37°C. Mitochondria were collected by centrifugation at 10 000 x g for 10 minutes. Supernatants and pellets were solubilised in SDS sample buffer and analyzed by western blotting. Alternatively, pellets were washed and resuspended in reaction buffer and incubated for 1 hour at room temperature in the dark with Caspase-Glo 8 Reagent (Promega) to measure LETDase activity.

2D clear native electrophoresis (CNE)/SDS-PAGE. Isolated mitochondria were washed once by resuspending in 500 µl of MIB followed by centrifugation at 10 000 x g for 10 minutes. Pellets were resuspended in mitochondrial resuspension buffer

(MRB) (50 mM NaCl, 50 mM imidazole/HCl, 1 mM EDTA, pH 7.1). 2.5 g/g dodecyl- β -D-maltoside (Invitrogen) or 3.0 g/g Triton X-100 (Sigma) was added and solubilization was allowed to proceed for 5-10 minutes on ice. Samples were spun at 100 000 x *g* for 17 minutes at 4°C using an Optima MAX-XP ultracentrifuge (Beckman Coulter). The supernatant was removed and 4X sample buffer (Invitrogen) added. Pellets were solubilized directly in SDS-PAGE sample buffer. The solubilized proteins were loaded onto a 4-16% Native-PAGE pre-cast gel (Invitrogen) and run at 4°C. Where appropriate, fluorescence was measured using the Odyssey Infrared Imaging System (Li-Cor Biosciences) and Odyssey V1.2 software. The gel lanes were excised and denatured in LDS sample buffer supplemented with 4% β -mercaptoethanol before being loaded onto a 4-12% Bis-Tris ZOOM gel (Invitrogen). Proteins were then electroblotted from the gel onto PVDF membranes for immunoblotting.

Liposome preparation and analysis. To analyze the binding of full-length BID and tBID to cardiolipin, these proteins were incubated with artificial liposomes either containing or lacking cardiolipin. The lipid composition of liposomes was based on the composition of mitochondrial contact sites as previously reported.³⁹ Liposomes containing cardiolipin consisted of 41 mol% PC, 22 mol% PE, 8 mol% PI, 9 mol% cholesterol and 20 mol% cardiolipin. In the liposomes lacking cardiolipin, we increased both the PC and PE content to compensate for the lack of cardiolipin, resulting in a composition of 53 mol% PC, 30 mol% PE, 8 mol% PI and 9 mol% cholesterol. Bovine liver PC, PE and PI were from Avanti Polar Lipids, bovine heart cardiolipin and cholesterol were purchased from Sigma. For the preparation of the liposomes, lipids were dissolved in chloroform, dried under a stream of nitrogen and

resuspended in binding buffer (100 mM NaCl, 2 mM MgCl₂, 10 mM Tris, pH 7.1). The lipid mixture was passed 11 times through a 400 nm polycarbonate membrane of a Mini-Extruder (Avanti Polar Lipids) to form large unilamellar vesicles. Following precipitation and wash of liposomes at 100 000 x g for 15 minutes at 4°C the presence or absence of cardiolipin was confirmed by MS analysis. The indicated proteins were incubated with the liposomes in binding buffer at 37°C for 30 minutes and liposomes were then centrifuged, washed and lysed for protein analysis.

Acknowledgement

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Conflict of interest.

The authors declare no conflict of interest.

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Titles and legends to figures

Figure 1 Analysis of the autoprocessing of exogenously expressed caspase-8-GFP mutants in shCaspase-8 HeLa cells **(a)** Linear scheme depicting the location and molecular masses of the three functional domains of caspase-8. The sites of the first and second cleavage are labelled. p26 contains the DED domains and p43 is made up of the DED domains and p18 domain. The amino acid substitutions of the caspase-8-GFP mutants are indicated in the table. **(b)** Immunoblot analysis of whole cell lysates from shCaspase-8 HeLa cells transfected with the caspase-8-GFP point mutants. Fas-treated cells were incubated in 0.5 µg/ml anti-Fas for 14 hours. **(c)** shCont (black bars) and shCaspase-8 (gray bars) HeLa cells were transfected with the various caspase-8-GFP mutants and assayed for cell death by flow cytometry. Only GFP positive cells were counted and cell death was measured by propidium iodide (PI) staining. Fas conditions were the same as in (a). Error bars represent standard deviation of three or more experiments done in triplicate.

Figure 2 Caspase-8-GFP translocation to the mitochondria is dependent on the formation of p43-p10. Confocal images of shCont HeLa transfected with caspase-8-GFP mutants. z-VAD-fmk, a cell-permeable pan-caspase inhibitor, was added prior to transfection and kept throughout the experiment to prevent cell death. Expression of Mito-dsRed2 was used as a mitochondrial marker. White bars = 10 µm. White arrows mark the presence of punctate non-mitochondrial caspase-8-GFP.

Figure 3 p43 formation is necessary for translocation and activity of caspase-8 on the mitochondria. **(a)** Mitochondrial enriched fractions were generated from C360S-GFP or pEGFP-N1 transfected shCont HeLa cells. Mitochondrial lysates were pelleted and the supernatant was then immunoprecipitated with anti-GFP and immunoblotted for caspase-8, GFP and VDAC1. **(b-c)** Mitochondria were isolated from pEGFP-N1, TM2-GFP, DM1-GFP and C360S-GFP shCont or shCaspase-8 HeLa cells. Blots were probed with anti-cleaved caspase-8 (D374), anti-GFP and anti-TOM20. Note the lack of p10-GFP formation and low levels of p43 on DM1-GFP mitochondria. All lanes in **(c)** were taken from the same exposure. **(d)** The mitochondria used in **(c)** were used to assess the activity of mitochondrial-bound caspase-8. The level of caspase-8-like activity (zLETD-aminoluciferin cleavage) is displayed relative to the GFP control (defined as zero) in units of luminescence (RLU). Note the similar levels of caspase-8 activity in DM1-GFP and C360S-GFP cells. Error bars represent standard deviation ($n = 3$).

Figure 4 Caspase-8 and BID form a high molecular weight complex on the mitochondrial membrane. **(a)** shCont HeLa were transfected with BID-mCherry or C360S-mCherry. 50 μ M zVAD-fmk was added to C360S-mCherry transfected cells to prevent cell death. Isolated mitochondria were solubilised in DDM and run on a 4-16% native gel. The gel was scanned for direct comparison of fluorescent signals. **(b-c)** Lanes from the gel in **(a)** were excised, denatured and run in the second dimension by SDS-PAGE. Membranes were immunoblotted for caspase-8 and the BCL-2 family proteins BID, BAK, BCL-X_L. Complex III was used as a control. **(d)** Mitochondria isolated from shCont HeLa transfected with TM2-GFP were treated as in **(a)**. The presence of p43 was clearly detected on the 200 μ g immunoblot. 100 μ g of

mitochondria were used for all subsequent 2D CNE./SDS-PAGE experiments for clarity purposes.

Figure 5 Fas-activation induces the cleavage of BID within the caspase-8/BID complex. **(a)** Mitochondria from untreated shCont HeLa were subjected to 2D CNE/SDS-PAGE. Caspase-8 and BID form a native complex on the mitochondrial membrane (solid line). **(b)** Fas-activation led to appearance of tBID in the caspase-8/BID complex (solid line). tBID also co-migrated with BAK and/or BCL-X_L, suggesting the formation of other complexes on the mitochondrial membrane (dotted lines). A plot profile was generated from the immunoblots shown in the top panel using the software program, ImageJ. Note that the majority of caspase-8, BID and tBID on the mitochondrial membrane is within a single complex. Additionally, there were large decreases in the levels of full-length BID on the mitochondrial membrane after Fas-treatment (compare BID panel of (a) to that of (b)). **(c)** DDM-resistant fractions from (a) and (b) were analyzed by SDS-PAGE after solubilising the pellet in 1% SDS. p43, and a substantial proportion of p55 and BID, were preferentially retained in the pellet compared to the other mitochondrial proteins examined. Again, there were large decreases in the levels of full-length BID on the mitochondrial membrane after Fas-treatment (compare lanes 1-2 to 3-4). *Lower panel*, The LETDase activity of the DDM soluble fractions from untreated or α Fas-treated shCont HeLa mitochondria was measured as described in Materials and Methods. A substantial increase in caspase-8 activity was seen in the DDM soluble fraction from α Fas-treated compared to untreated cells.

Figure 6 TRAIL-activation induces the cleavage of BID within the caspase-8/BID complex. **(a-b)** Mitochondria from untreated H460 cells were subjected to 2D CNE/SDS-PAGE. As seen in HeLa cells, caspase-8 and BID form a native complex on the mitochondrial membrane (solid line). **(b)** TRAIL-activation led to appearance of tBID in the caspase-8/BID complex (solid line). tBID also co-migrated with BAK and/or BCL-X_L (dotted lines). **(c)** The DDM-resistant fraction from the mitochondria was analyzed for the presence of active (p43) caspase-8. Similar to HeLa cells, a substantial proportion of p55 and p43 were present in the detergent-resistant fraction. Also, note that tBID was detectable in the soluble fraction. *Lower panel*, The LETDase activity of the DDM soluble fractions from untreated or TRAIL-treated H460 mitochondria was measured as in Figure 5c. A substantial increase in caspase-8 activity was seen in the DDM soluble fraction from TRAIL-treated compared to untreated cells. **(d)** Subcellular fractionation of H460 cells ± TRAIL revealed that p43 is specifically enriched on the mitochondria of H460 cells after TRAIL activation (compare lanes 3 and 4) and that BID on the mitochondria was preferentially processed by caspase-8 in comparison to cytosolic BID.

Figure 7 Caspase-8 specifically targets BID on the mitochondrial membrane to induce MOMP. **(a-b)** Mitochondria isolated from shCont HeLa (or BCL-X_L HeLa) by subcellular fractionation were incubated with different concentrations of recombinant active caspase-8 (rC8). Mitochondria were separated from the supernatant by centrifugation and both fractions were immunoblotted for cytochrome c release. The Triton X-100 lane represents the total amount of cytochrome c in the purified mitochondria **(c)** Mitochondria were isolated from shCont cells transfected with a

non-targeting siRNA (siCont) or an siRNA against BID (siBID). Mitochondria were treated as described in (a).

Figure 8 Schematic cartoon depicting a model for type II extrinsic apoptosis. Activation of the DISC leads to the production of caspase-8 (p43) on the outer mitochondrial membrane. Caspase-8 (C8) and BID form a native complex on the mitochondrial membrane. Activated caspase-8 cleaves mitochondrial-bound BID in a cardiolipin-dependent process. tBID shifts from the caspase-8/BID complex to interact and activate BAK and/or inhibit BCL-X_L which in turn induces mitochondrial outer membrane permeabilization.

Figure S1 Three different HeLa cell clones were used in this study. BCL-X_L cells which overexpress BCL-X_L. shCont HeLa express a non-targeting shRNA and shCaspase-8 stably express an shRNA against caspase-8. See Materials and Methods for sequences.

Figure S2 Analysis of the expression and autoprocessing of exogenously expressed caspase-8-GFP mutants in shCont and shCaspase-8 HeLa cells. (a) shCont cells were transfected with the caspase-8 constructs listed in Figure 1a and whole cell lysates were analyzed by immunoblotting. The immunoblot revealed that the caspase-8-GFP constructs were not massively overexpressed. It should be noted that cells were subjected to flow cytometry prior to lysis to determine the transfection efficiency (% of GFP positive cells). The graph represents densitometric analysis (ImageJ) of the immunoblot shown in the top panel. The data show that most caspase-8 construct

increased the total amount of caspase-8 per cell by 1.7- to 3-fold, however, this was sufficient to activate apoptosis. **(b)** Immunoblot analysis of the p18 subdomain of caspase-8 from whole cell lysates of shCont and shCaspase-8 HeLa which had been treated with Fas or transfected with the caspase-8-GFP mutants. Note the significant inhibition of p18 formation in lanes 2, 7 and 8 in shCaspase-8 HeLa relative to lanes 3-6.

Figure S3 Caspase-8 translocation to the mitochondria was not dependent on the the release of cytochrome c and was not blocked by the expression of BCL-X_L. Confocal images of shCont HeLa and BCL-X_L HeLa transfected with mCherry-tagged caspase-8 mutants (pseudocolored green). 50 μ M z-VAD-fmk was added prior to transfection and kept throughout the experiment to prevent cell death. Samples were immunostained for cytochrome c (pseudocolored red). The presence of non-mitochondrial punctuated structures were consistently observed in all cells. White bars = 10 μ m. White arrow represents a cell in which cytochrome c was released while caspase-8 remained peri-nuclear and punctuated. Nuclei were labelled with DAPI.

Figure S4 shCont HeLa were transfected with C360S-GFP in the presence of 50 μ M zVAD-fmk. 200 μ g of isolated mitochondria were solubilised in DDM and run on a 4-16% native gel. The lane was excised from the gel, denatured and run in the second dimension by SDS-PAGE. Membranes were immunoblotted for caspase-8 (anti-p18) and BID..

Figure S5 Untreated mitochondria were incubated with rCaspase-8 and then subjected to one or two washes. Caspase-8 activity was determined using a LETDase assay. Results showed that 200ng of rCaspase-8 within the range of 8-16 hours of anti-Fas treatment in terms of the activity of caspase-8 on the mitochondria. Note that the activity of caspase-8 was barely affected by the washing, suggesting that active caspase-8 stably inserts into the mitochondrial membrane.

Figure S6 Transfection of BCL-X_L HeLa with C8-GFP, DM2-GFP, TM2-GFP or SM1-GFP induced a “type-I-like” (mitochondrial-independent) cell death. **(a)** BCL-X_L HeLa cells were transfected with the various caspase-8-GFP mutants and assayed for cell death by flow cytometry. Only GFP positive cells were counted and cell death was measured by propidium iodide (PI) staining. Fas conditions were the same as in (Figure 1a). BCL-X_L expression inhibits Fas-induced cell death (dotted line). Interestingly, BCL-X_L expression prevented cell death in those cells (DM1-GFP and C360S-GFP) in which low levels of active caspase-8 are formed. Error bars represent standard deviation of three or more experiments done in triplicate. **(b)** TM2-GFP shCont cells were subjected to 2D CNE/SDS-PAGE and immunoblotted for caspase-8, BID, BAK, BCL-X_L and VDAC1. Although TM2-GFP induces a “type-I-like” mitochondrial-independent cell death, the caspase-8/BID complex still becomes activated and tBID is produced in these cells. These results suggest that the caspase-8/BID complex may still function in type I cells.

Figure S7 Cardiolipin-containing or -deficient liposomes were either left untreated or incubated with recombinant full-length BID with or without recombinant active

caspase-8. Following precipitation of the liposomes, Western blot analyses were used to detect the full-length and truncated BID proteins. In lane 7 BID and active caspase-8 were incubated in the buffer without liposomes. Note that both BID and tBID seem to be dependent on the presence of cardiolipin for association with membranes.

Figure S8 Mitochondrial-bound caspase-8 and BID are completely insoluble in Triton X-100. The caspase-8/BID complex was not detected by 2D CNE/SDS-PAGE analysis of Triton X-100 solubilized shCont HeLa mitochondria. However, caspase-8 and BID were found in the detergent resistant membrane fraction. These results suggest that the caspase-8/BID complex is present in detergent resistant mitochondrial membrane microdomains. Of note, we now found that BAK and BCL-X_L co-migrated in the first dimension, which is in contrast to the DDM solubilised mitochondria. However, it has been shown that Triton X-100 has the ability to induce the activation of certain BCL-2 family members.⁴⁰ Most likely Triton X-100 artificially induced BAK activation and in the absence of tBID, BCL-X_L was able to bind and inhibit BAK.

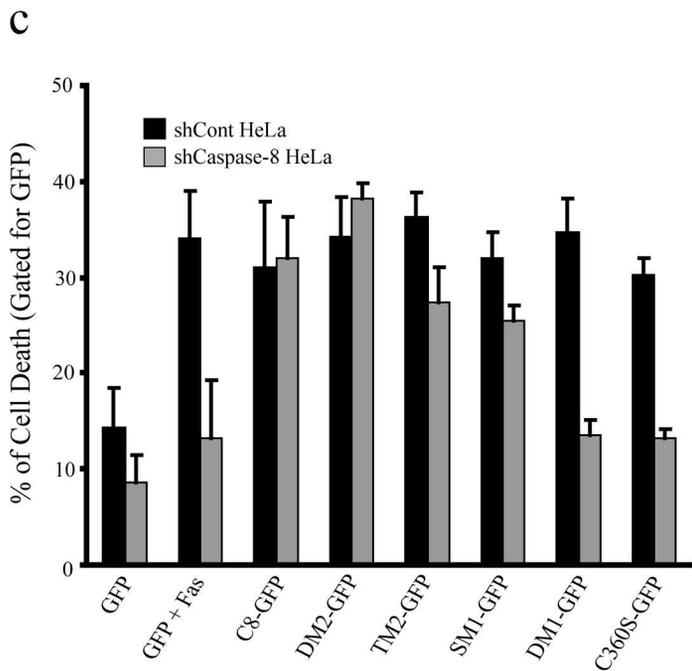
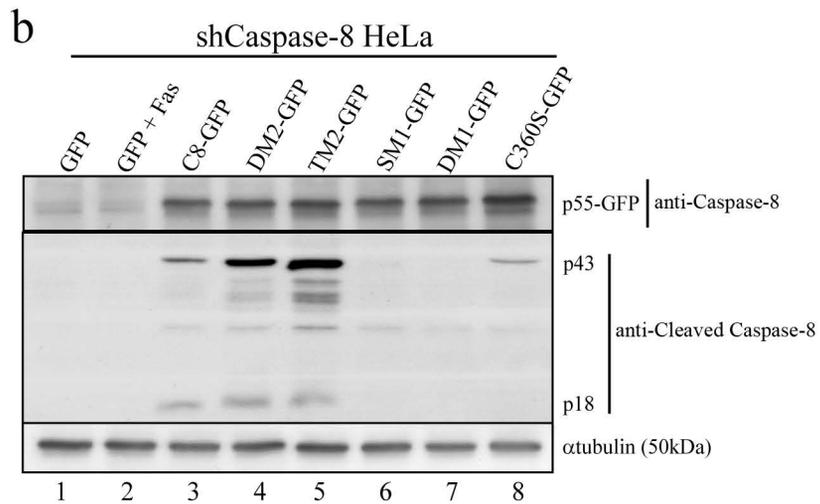
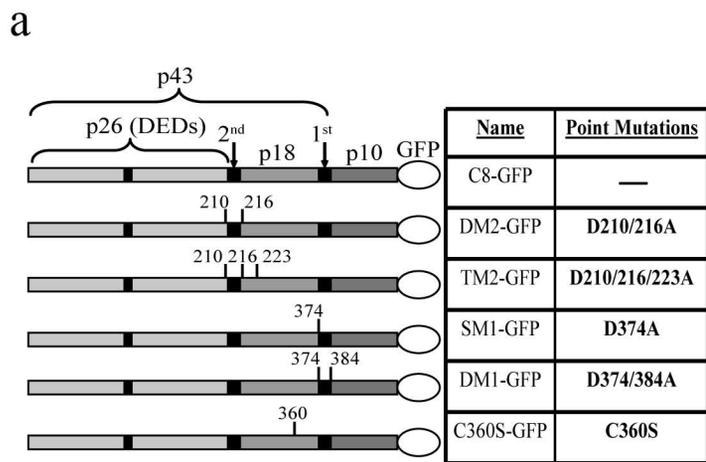


Figure 1

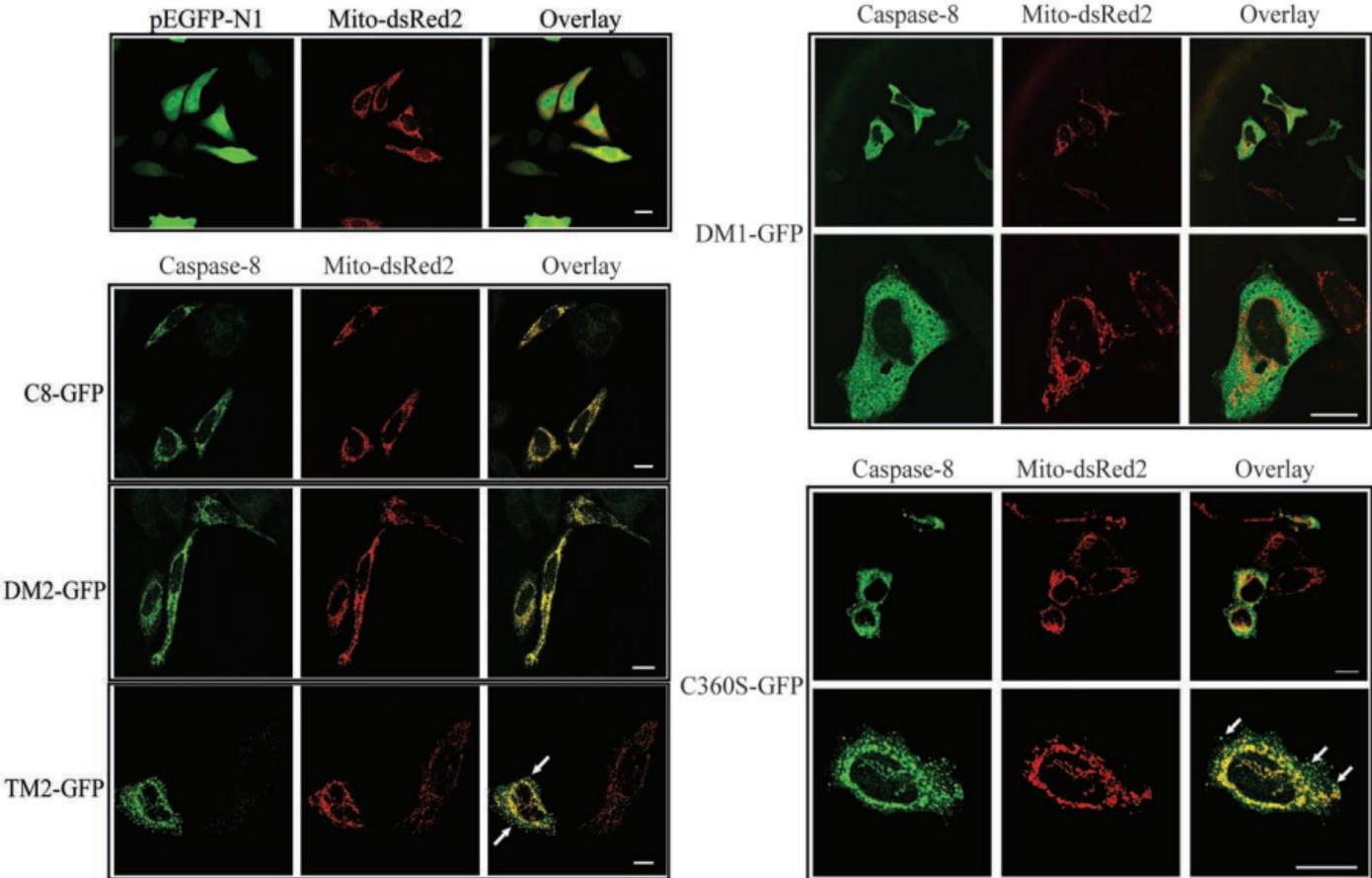


Figure 2

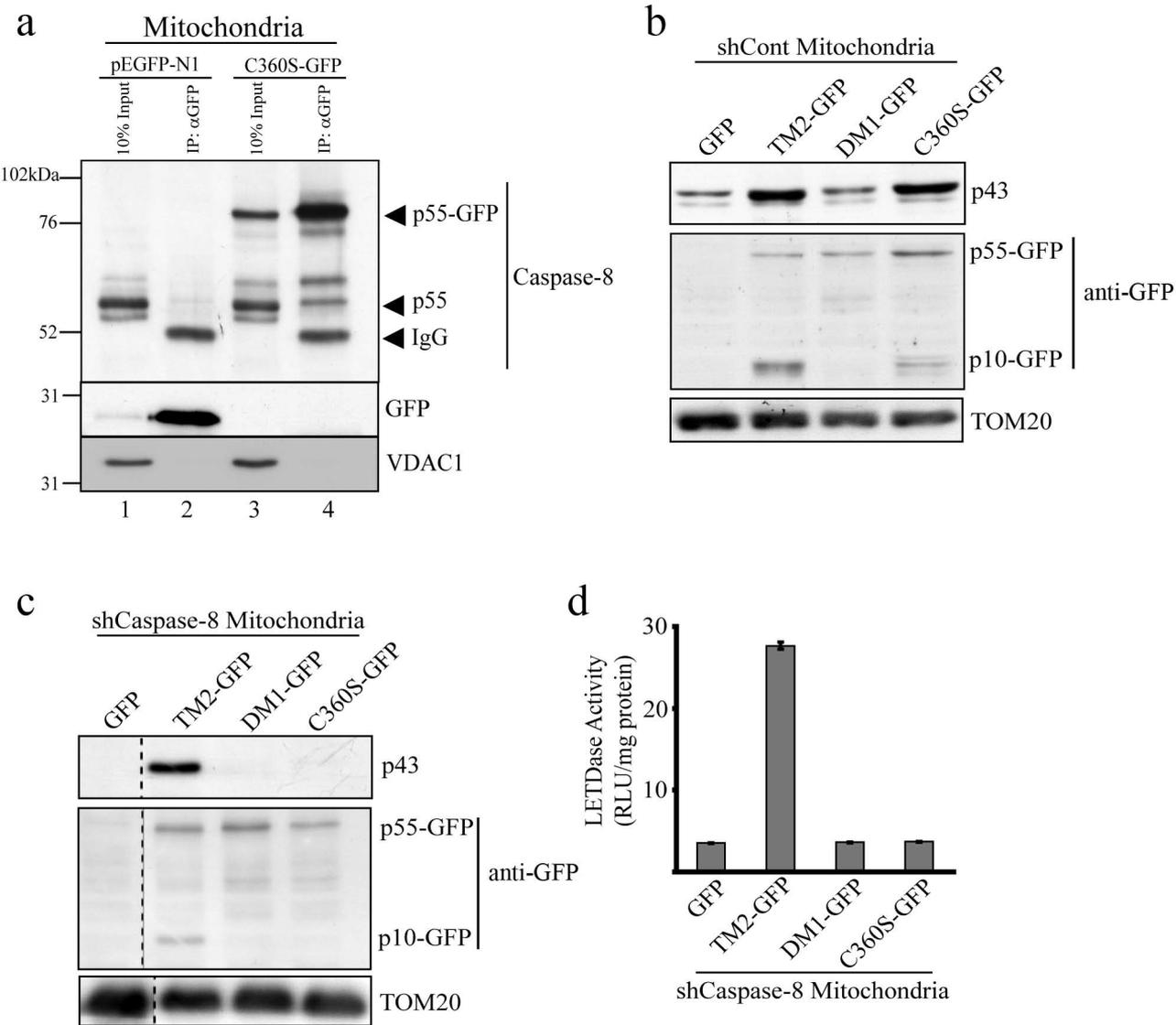


Figure 3

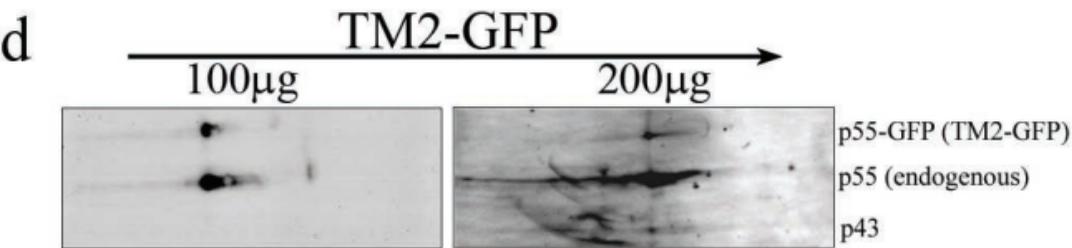
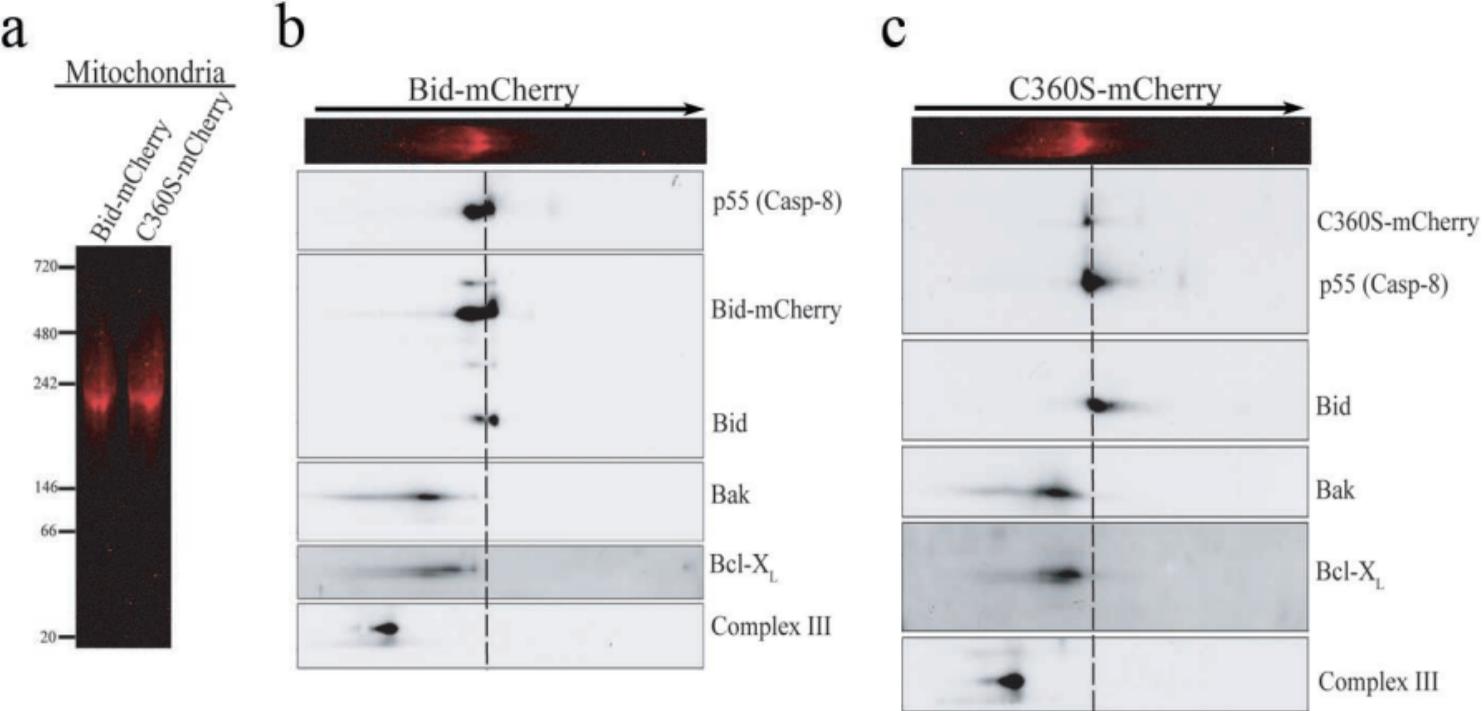


Figure 4

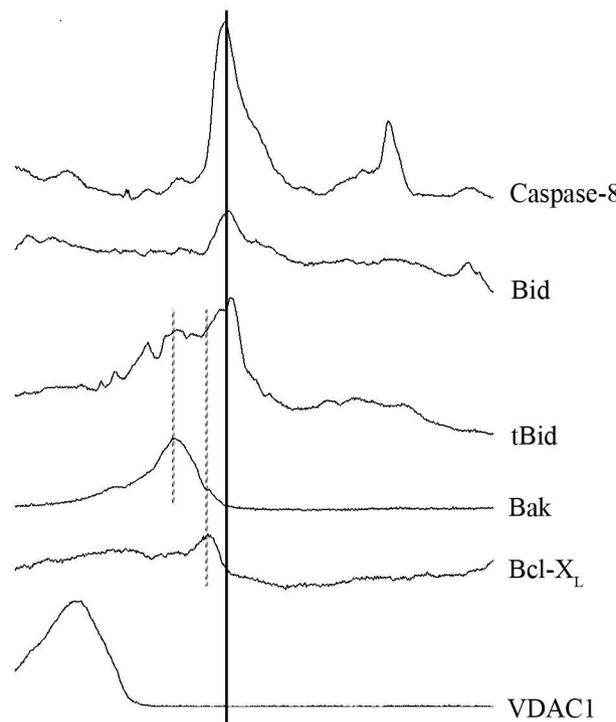
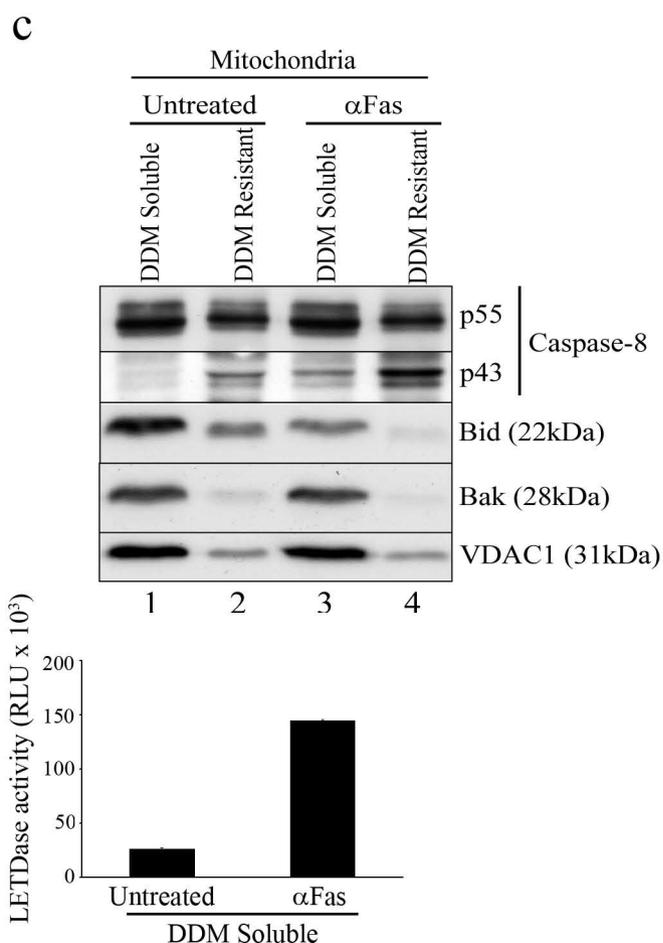
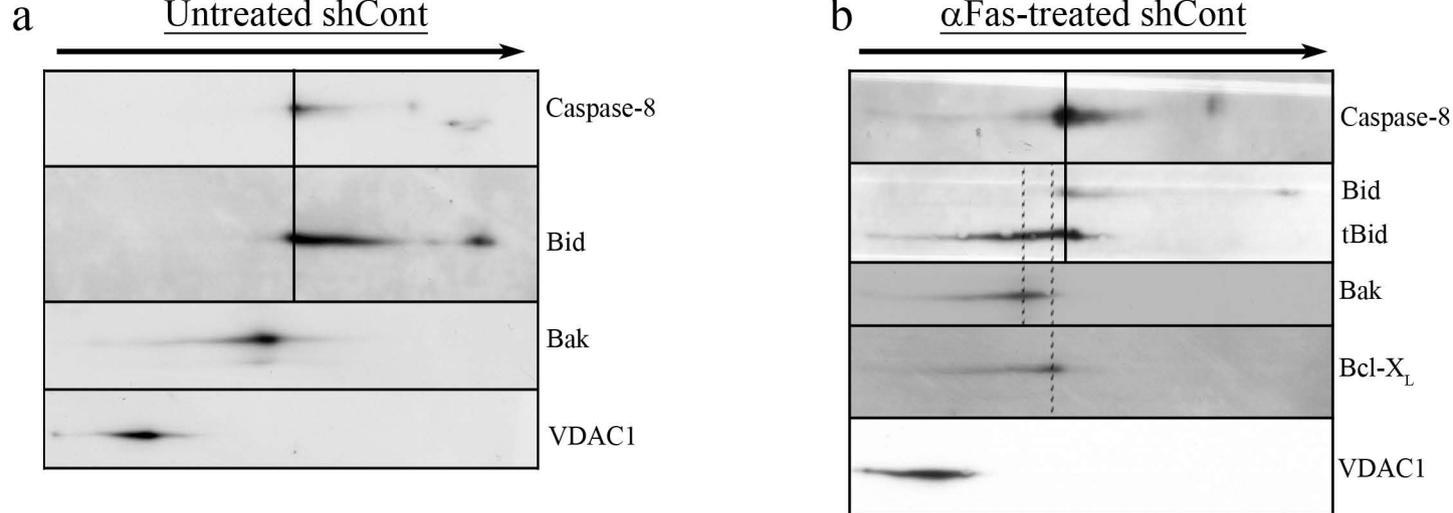
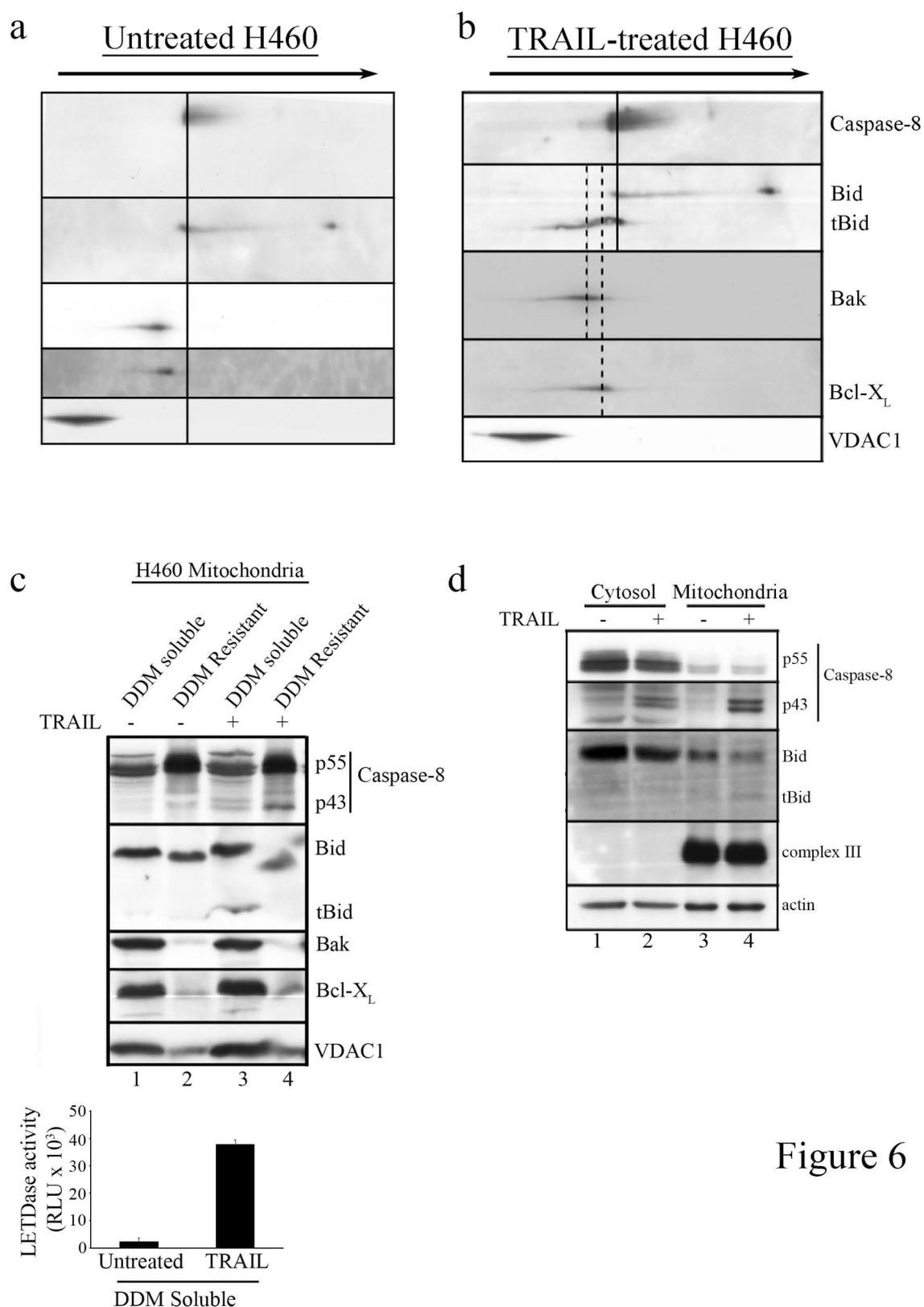


Figure 5



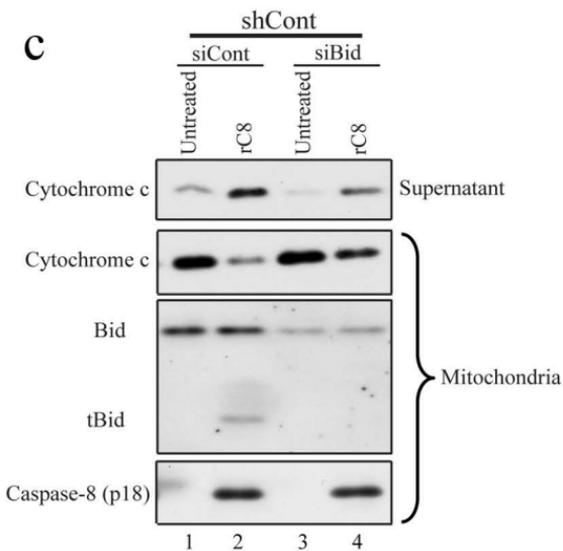
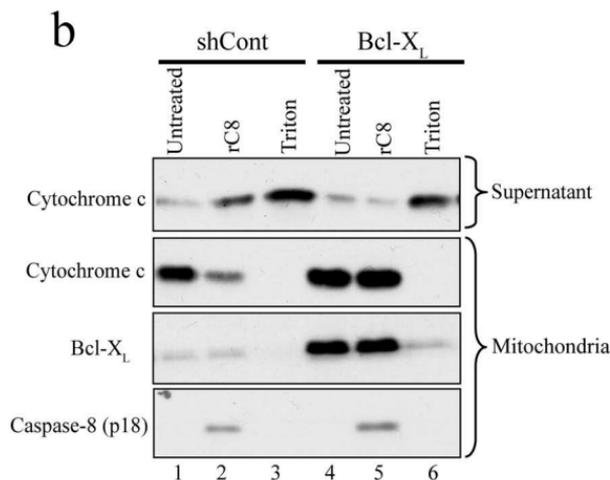
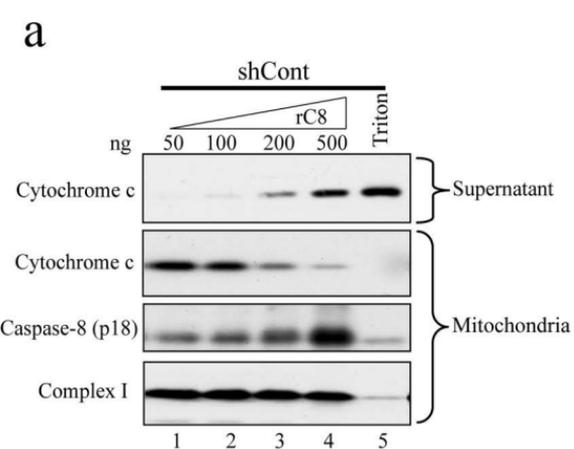


Figure 7

