Apoptosis induction by Bid requires unconventional ubiquitination and degradation of its N-terminal fragment

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Apoptosis induction by Bid requires unconventional ubiquitination and degradation of its amino-terminal fragment

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Bcl-2 family member Bid is subject to auto-inhibition: in absence of stimuli, its N-terminal region sequesters the pro-apoptotic BH3 domain. Upon proteolytic cleavage in its unstructured loop, Bid is activated, although structural data reveal no apparent resulting conformational change. We report that upon Bid cleavage, the N-terminal fragment (tBid-N) is ubiquitinated and degraded, thus freeing the BH3 domain in the C-terminal fragment (tBid-C). Ubiquitination of tBid-N is unconventional, since acceptor sites are neither lysines nor the amino-terminus. Chemical approaches implicated thio-ester and hydroxy-ester linkage of ubiquitin and mutagenesis implicated serine and possibly threonine as acceptor residues in addition to cysteine. Acceptor sites reside predominantly, but not exclusively in helix 1, which is required for ubiquitination and degradation of tBid-N. Rescue of tBid-N from degradation blocked Bid’s ability to induce mitochondrial outer membrane permeability, but not mitochondrial translocation of the cleaved complex. We conclude that unconventional ubiquitination and proteasome-dependent degradation of tBid-N is required to unleash the pro-apoptotic activity of tBid-C.

Introduction

In mammalian cells, mitochondria activate the apoptotic execution machinery. Apoptotic stimuli induce mitochondrial outer membrane permeability and release of pro-apoptotic proteins such as cytochrome c (Cyt c) into the cytosol. The collective action of these molecules results in effector caspase activation and apoptotic cell death [1]. Mitochondrial permeabilization is controlled by the Bcl-2 protein family, which comprises the anti-apoptotic Bcl-2 subfamily and the pro-apoptotic Bax/Bak and BH3 domain-only subfamilies [2]. Their functional activity relies on homo- and heterodimerization, which proceeds by interaction of the BH3 domain α helix with a groove, formed by BH1 and BH2 domains [3]. The BH3 domain-only subfamily has many members, which specialize in responsiveness to specific apoptotic stimuli [4]. BH3-only proteins require Bax or Bak to induce cell death. They induce assembly of Bax/Bak into homomultimers in the mitochondrial outer membrane [5,6]. These multimers are postulated to either form transmembrane pores themselves, or to facilitate pore formation. Inhibitory Bcl-2 family members sequester BH3-only and/or Bax/Bak proteins, thereby preventing their participation in pro-apoptotic complexes [7]. The BH3 domain-only protein Bid conveys the
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Unconventional ubiquitination allows Bid activity to apoptotic signal from death receptors, such as TNF receptor-1, TRAIL receptors and Fas/CD95, to mitochondria [8,9]. Upon ligand binding, these death receptors induce formation of a complex between the intracellular adaptor FADD and pro-Caspase-8 or -10. This results in increased local concentrations of these inducer caspases, leading to their activation [10]. Active Caspase-8/-10 can cleave and activate Bid, as well as effector caspases. In certain cell types, the mitochondrial route is dispensable for death receptor-induced apoptosis, whereas in others it constitutes an essential amplification loop for effector caspase activation [11].

The three dimensional organisation of full-length Bid has been resolved by NMR [12,13]. Residues 1-12 and 43-77 form unstructured loops. The structured portion consists of eight α helices arranged in a compact fold (Supplementary Figure 1a). Of these, helix 3 constitutes the BH3 domain, which is fixed by hydrophobic contacts with helix 1 and 8. The sites for proteolytic cleavage by Caspase-8 or Granzyme B lie in the second unstructured loop (D60 and D75, respectively). In solution, N- and C-terminal fragments of Bid remain associated upon cleavage by Caspase-8 and the complex undergoes no apparent conformational change [12,14,15]. Cleavage generates an N-terminal glycine in truncated C-terminal Bid (tBid-C) that can be myristoylated. This lipid modification facilitates targeting of tBid-C to mitochondria, but is not required for it [14]. Rather, helices 4, 5, and 6 appeared the critical elements for such targeting, specifically to cardiolipin-enriched domains in the mitochondrial membrane [16].

Making the BH3 domain available for interactions with the BH1/BH2 groove of other Bcl-2 family members is essential for Bid’s pro-apoptotic function. The solution structure of Bid indicates that the BH3 domain must be freed from the structural constraint that is exerted by the tBid-N fragment [12,13]. Indeed, a number of biochemical studies have shown that tBid-N acts as an inhibitor of tBid-C [15,17,18]. Specifically Tan et al. (1999) produced compelling evidence for auto-inhibition of Bid, by interactions between helix 2 and the BH3 domain. A mutant full length Bid protein in which these interactions were abrogated was as effective as tBid-C in inducing apoptosis and binding to Bcl-xL [17]. The natural occurrence of an alternative splice form of Bid that lacks the BH3 domain, but has the inhibitory N-terminal sequences, lends further support to the idea that this is an important mode of tBid-C regulation [18]. We have addressed the question how Bid cleavage can release tBid-C from inhibition by tBid-N. We reveal that this is accomplished by unconventional ubiquitination of the tBid-N fragment, followed by its proteasomal degradation.

Results

tBid-N disappears upon its generation

To follow the fate of tBid-N and tBid-C, Bid was N-terminally Myc-tagged and C-terminally fused to GFP and expressed in MCF-7 breast carcinoma cells, which were examined by confocal laser scanning microscopy (CLSM). In unstimulated cells, Myc and GFP signals indicated a cytoplasmic localization of full length Myc Bid GFP (Figure 1a, panels I-III). After stimulation with TNFα for 8 h, the GFP signal was punctate and colocalized with MitoTracker, indicating translocation of tBid-C GFP to mitochondria (Figure 1a, panels V, VI). Surprisingly, Myc tBid-N was undetectable by CLSM (Figure 1a, panel IV). Full length Myc Bid GFP was readily detectable by both anti-Myc and anti-GFP immunoblotting in unstimulated MCF-7 cells and gradually decreased in amount after stimulation with TNFα (Figure 1b). While the tBid-C GFP fragment could readily be visualized at 4 and 8 h time points, the Myc tBid-N fragment (predicted molecular mass 7 kDa) was undetectable (Figure 1b).

Surprisingly, Myc tBid-N was undetectable by CLSM (Figure 1a, panel IV). Full length Myc Bid GFP was readily detectable by both anti-Myc and anti-GFP immunoblotting in unstimulated MCF-7 cells and gradually decreased in amount after stimulation with TNFα (Figure 1b). While the tBid-C GFP fragment could readily be visualized at 4 and 8 h time points, the Myc tBid-N fragment (predicted molecular mass 7 kDa) was undetectable (Figure 1b).

Similar results were obtained with antibody that recognises both N- and C-terminal epitopes on Bid (data not shown). After stimulation with the death receptor ligand TRAIL, a similar picture emerged. At the 8 h time point, full length Myc Bid GFP levels were decreased and tBid-C GFP fragment had been generated. However, no Myc tBid-N was detectable (Figure 1c). Collectively,
these results suggest that the tBid-N fragment that is generated by Caspase-8/-10 is rapidly degraded.

**tBid-N is degraded in a proteasome-dependent manner**

To investigate whether tBid-N was degraded by the proteasome, MCF-7 cells were stimulated with TNFα in presence or absence of proteasome inhibitor MG132. Stimulation with TNFα for 8 h resulted in the appearance of the endogenous tBid-C fragment, but the tBid-N fragment was barely detectable (Figure 2a, left panel). When cells were stimulated in presence of MG132, the amount of endogenous tBid-N was clearly increased. Similar results were obtained with transfected Myc Bid GFP: the Myc tBid-N fragment was only detectable when cells were stimulated in presence of MG132 (Figure 2a, right panel).

To determine whether targeting of tBid-N for proteasomal degradation required a pro-apoptotic stimulus, the tBid-N fragment was expressed in cells. The degradation pathway was constitutively active, since protein synthesis inhibition with cycloheximide (CHX) for 8 h resulted in loss of HA-tagged tBid-N (Figure 2b). Proteasome inhibition with MG132 abrogated this loss and increased the HA tBid-N pool in cells that had not been treated with CHX (Fig. 2b). HA tBid-N expressing cells were pulse labelled for 2 h with $^{35}$S-methionine and -cysteine and the fate of the labelled pool was followed during a 60 min chase period. The half-life of the labelled HA tBid-N pool was about 5 min (Figure 2c). Untagged tBid-N was even more unstable than the HA-tagged version: it was only detectable when cells had been incubated with MG132 (Figure 2d). Collectively, these results indicate that tBid-N, upon its generation by Caspase-8/10, is targeted for proteasome-dependent degradation by a pathway that is not dependent on apoptotic stimulation.

**tBid-N is ubiquitinated in an unconventional manner**

Since tBid-N was targeted to the proteasome, we investigated whether it was ubiquitinated. To this end, tBid-N N-terminally tagged with the HA epitope (sequence YPDVPDYA)
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was expressed in HeLa cells with or without FLAG-tagged ubiquitin. An anti-HA reactive smear, characteristic of polyubiquitination, was associated with immunoprecipitated HA tBid-N (Figure 3a). In addition, we examined ubiquitination of tBid-N as generated from full-length Bid after death receptor stimulation. MCF-7 cells expressing Myc Bid GFP were left untreated or stimulated with TNFα for 8 h in presence of MG132. Lysates were immunoprecipitated with anti-GFP and sequentially with anti-Myc antibody, to recover Myc tBid-N fragment. Immunoblotting for Bid and ubiquitin revealed that ubiquitin was uniquely associated with cleaved, but not full-length Bid and specified that Myc tBid-N contained ubiquitin (Figure 3b). Ubiquitin was also found to be associated with tBid-C, in accordance with published data.

Ubiquitination was surprising, since tBid-N has no lysine residues that conventionally act as ubiquitin acceptor sites (Supplementary Figure 1b). Typically, Ubiquitin can either be linked via a peptide bond to the ε amino group of a lysine residue, or to the α amino group of an N-terminal residue. Since linkage to a lysine was excluded, we investigated whether the N-terminus of tBid-N acted as the Ubiquitin acceptor site. To this end, tBid-N was fused C-terminally to a TAP tag, which contains a Calmodulin binding domain and Protein A sequence, separated by a tobacco etch virus (TEV) protease sensitive site (ENLYFQG). This allows for two-step purification: first on IgG beads and after cleavage by TEV on Calmodulin beads. In one tBid-N TAP construct (7TEV tBid-N TAP), sequences encoding the first 7 amino acids of tBid-N were cloned upstream of the tBid-N coding region (Figure 3c). If tBid-N would indeed be ubiquitinated at its N-terminal residue, TEV cleavage should remove the associated ubiquitin. tBid-N TAP and 7TEV tBid-N TAP were expressed with HA-ubiquitin in HeLa cells.

Figure 2. tBid-N is degraded by the proteasome.
(a) MCF-7 cells that were not transfected (left) or transfected with Myc Bid GFP (right) were stimulated with TNFα alone or in combination with MG132 for 8 h. Total lysates were probed for endogenous Bid (left) or for transfected Myc Bid GFP (right) and cleavage fragments thereof with anti-Bid antibody. Re-probe was performed with anti-Myc antibody. (b) HeLa cells expressing HA-tagged tBid-N were treated for 8 h with CHX and proteasome inhibitor MG132 as indicated. Total cell lysates were probed for HA tBid-N with anti-HA antibody. (c) HeLa cells expressing HA tBid-N were labelled with [35S]-methionine and -cysteine for 2 h, followed by chase with non-radioactive amino acids for the indicated time periods. Bid protein was immunoprecipitated (IP) from detergent lysates with anti-HA mAb and resolved by SDS-PAGE. Radioactive signals were quantified by phosphorimaging (IDV = integrated density value). (d) HeLa cells transfected to express untagged tBid-N were treated or not with MG132 for 16 h. Total lysates were probed for tBid-N with anti-Bid antibody. Blots were re-probed with anti-actin antibody as indicated.
Panel I in Figure 3c shows the position of tBid-N TAP proteins prior to TEV cleavage, as isolated with IgG beads. TEV cleavage was successful as shown by the difference in migration of Bid species recovered on Calmodulin beads (Figure 3c, panel II). After TEV cleavage, Ubiquitin remained attached to digestion product of 7TEV tBid-N TAP (Figure 3c, panel III). TEV protease had efficiently cleaved at the N-terminal site, since the difference in molecular mass between the two Bid species was smaller than prior to TEV cleavage (compare panels I and II). A very small difference remained, because TEV cleaves within the added protease recognition sequence. Ubiquitin was attached to Bid and not to the TAP tag, since the TAP tag alone did not carry ubiquitin upon co-expression with HA Ubiquitin in HeLa cells (Supplementary Figure 2). This experiment argues against the possibility that the N-terminal residue of tBid-N is the Ubiquitin acceptor site. Moreover, analysis of the first tryptic peptide of tBid-N TAP by MALDI ToF/ToF mass spectrometry determined that the N-terminus of Bid is acetylated and thereby not available for ubiquitin modification (results not shown). These data indicate that tBid-N is ubiquitinated at a site that is neither a lysine nor the N-terminus.
Helix 1 in Bid is critical for ubiquitination

To identify regions in tBid-N that were required for ubiquitination and concomitant proteasome-mediated degradation, we made progressive N-terminal deletions. Deletion mutants of tBid-N GFP were expressed in HeLa cells, which were treated or not with CHX for 8 h and stability was assessed by immunoblotting of total cell lysates. While tBid-N GFP remained highly unstable upon deletion of up to 13 N-terminal residues, deletion of 15 or more (17, 23, 33) N-terminal amino acids dramatically increased its half-life (Figure 4a). To assess whether stabilization correlated with decreased ubiquitination, wild-type and Δ15 tBid-N GFP were expressed in HeLa cells together with FLAG-tagged Ubiquitin. Anti-Bid immunoblotting of total cell lysates showed that wild-type and Δ15 tBid-N GFP were expressed to a similar extent (Figure 4b). Ubiquitinated tBid-N GFP species were immunoprecipitated with anti-FLAG antibody and identified by anti-Bid immunoblotting. This analysis showed that wild-type tBid-N GFP was polyubiquitinated, whereas the Δ15 tBid-N GFP mutant bore virtually no Ubiquitin (Figure 4b). FLAG Ubiquitin expression was comparable in all transfectants (data not shown). The combined findings in the stability and ubiquitination assays lead to the conclusion that residues 14 and/or 15 contain information that is critical

Figure 4. Helix 1 is critical for tBid-N ubiquitination and degradation. (a) Wild-type (wt) tBid-N GFP and deletion (Δ) mutants lacking 7, 13, 17, 23 or 33 N-terminal amino acids were expressed in HeLa cells, which were treated (+) or not (-) with CHX for 8 h. The tBid-N GFP proteins were detected in total lysates by immunoblotting with anti-Bid antibody. (b) GFP, wt and Δ15 tBid-N GFP were expressed in HeLa cells together with FLAG-tagged ubiquitin. Total cell lysates (TCL) were probed with anti-Bid antibody to assess tBid-N expression levels. Ubiquitinated protein species were isolated with anti-FLAG mAb (IP) and probed with anti-Bid antibody. Single asterisk indicates heavy and light chains of the anti-FLAG mAb, double asterisk endogenous full length Bid. (c) FLAG-tagged ubiquitin was co-expressed in HeLa cells with HA tBid-N versions with a TEV protease cleavage site at position 10, 30 or 43. HA tBid-N molecules were isolated, incubated with (+) or without (-) TEV protease, run on gel and probed by immunoblotting for Bid and ubiquitin (anti-FLAG). In case of HA tBid-N TEV10, the HA-tagged cleavage fragment repeatedly did not resolve on gel, but remaining undigested HA tBid-N TEV10 and weakly ubiquitinated species of the cleavage fragment (arrows) are clearly visible. All experiments in this figure were performed three times with similar results.
for ubiquitination and destabilization of tBid-N. These are the first residues that take part in the α helix 1 of Bid (see Supplementary Figure 1b). Their deletion is expected to disrupt this structure and may therefore result in loss of ubiquitination site(s), and/or loss of a binding site for the Ubiquitin ligase.

To ascertain whether helix 1 contained ubiquitination site(s), we performed mapping experiments. Cleavage sites for the TEV protease were introduced in HA tBid-N after residues 10, 14, 30 or 43 and these recombinant proteins were expressed in presence of FLAG Ubiquitin. They were isolated with anti-HA antibody, digested with TEV protease and probed for the presence of Ubiquitin and Bid. In case of the HA tBid-N TEV43 and -30 proteins, cleavage with TEV protease resulted in a shift in molecular mass of both Bid and the Ubiquitin signal. There was no apparent loss of the Ubiquitin signal (Figure 4c). This proves that tBid-N is directly ubiquitinated and indicates that predominant Ubiquitin acceptor sites are amongst residues 1-30 of tBid-N. The HA tBid-N TEV14 protein did not cleave with TEV protease (data not shown). Upon TEV digestion of HA tBid-N TEV10, the Ubiquitin signal was strongly reduced (Figure 4c). The HA tBid-N 1-10 fragment did not resolve on gel, but weakly ubiquitinated species of this fragment are visible (arrows). The remaining Ubiquitin was mainly associated with the undigested HA tBid-N TEV10. We conclude that tBid-N is predominantly ubiquitinated on sites in between residues 10 to 30, but also to a certain extent on sites in between residues 1 to 10. The deletion and mapping experiments collectively indicate that helix 1 (residues 14-29, Supplementary Figure 1b) is critical for ubiquitination to occur.

**tBid-N is likely ubiquitinated on cysteine, serine and/or threonine residues**

Lack of lysine residues is a common feature of tBid-N in different species (human, chimpanzee, mouse, rat, cow, pig; Supplementary Figure 1c). We surmised that tBid-N might be ubiquitinated on cysteine residues. Three such sites are available among residues 1-30 of human tBid-N: C3, C15 and C28. Ubiquitin linkage to cysteine is not uncommon, since the E2 and E3 enzymes are charged with Ubiquitin in this manner. In such case, the carboxyl group of the C-terminal glycine in Ubiquitin forms a thioester bond with the sulfhydryl group in cysteine, which is easily reducible. To address this possibility, ubiquitinated HA tBid-N was isolated and subjected to reduction with dithiothreitol (DTT) under denaturing conditions. HA tBid-N was re-precipitated with anti-HA antibody to separate it from possibly dissociated Ubiquitin moieties. As a positive control, we used *in vitro* ubiquitinated E2-25K, an E2 that is monoubiquitin-charged through a thioester bond in its active site [22]. Indeed, DTT treatment resulted in loss of Ubiquitin from E2-25K species (Figure 5a). Importantly, the same treatment diminished the amount of Ubiquitin appended to HA tBid-N (Figure 5a). This experiment indicates that cysteine(s) is/are among the Ubiquitin acceptor sites in tBid-N. However, we found highly reproducibly that Ubiquitin was partially retained on tBid-N upon reduction, arguing that other residues play a role as acceptor sites as well. Point mutation of C15 or C28 either alone, or in conjunction (Table 1) did not stabilize tBid-N (Supplementary Figure 3). It also did not reproducibly reduce ubiquitination of the 1-30 fragment of HA tBid-N TEV 30 (Fig. 5b). Additional mutation of C3 made HA tBid-N TEV30 very unstable, such that it could only be expressed at lower levels than the wild-type protein (Figure 5b and Supplementary Figure 3). However, it could clearly be demonstrated that the cysteine-less (C3;C15;C28) mutant of HA tBid-N TEV30 still carried Ubiquitin after TEV digestion (Figure 5b). This ties in with the partial reducibility of the ubiquitination and leads to the important conclusion that residues 1-30 of tBid-N harbour residues other than cysteine to which Ubiquitin can be conjugated.

The potential alternative ubiquitination sites in tBid-N (1-30) are serine and threonine residues, since the carboxyl group of the C-terminal glycine in Ubiquitin can theoretically form an ester bond with the hydroxyl group in the side
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Figure 5. Ubiquitin is partially reducible, hydrolysable and affected by combined mutation of S, T and C residues.
(a) HA tBid-N was co-expressed with FLAG Ubiquitin in HeLa cells. Anti-HA immunoprecipitates were reduced (+) or not (-) as described in Experimental Procedures and re-precipitated with anti-HA mAb. Re-precipitates were probed for Bid (anti-HA) and ubiquitin (anti-FLAG). Recombinant E2-25K as a positive control for cysteine ubiquitination was ubiquitinated in vitro, reduced or not, and probed with mAb to E2-25K or ubiquitin. Asterisk indicates free recombinant ubiquitin. IDV = integrated density values, showing comparable tBid-N loading. (b) Alanine substitution mutations were generated in HA tBid-N TEV30. All mutants listed in Table I were tested for ubiquitination and part of the results are shown here. Wild-type and mutant proteins were expressed in HeLa cells together with FLAG Ubiquitin, isolated with anti-HA mAb and treated (+) or not (-) with TEV protease. Anti-HA immunoblotting was performed to assess Bid expression and cleavage and anti-FLAG immunoblotting to examine ubiquitination. Arrows indicate positions of the ubiquitinated TEV cleavage fragment. (c) tBid-N TAP was co-expressed with HA Ubiquitin in HeLa cells. Cell lysates were treated at pH 7.4 or pH 12 as indicated in Experimental Procedures and tBid-N TAP was purified on the TAP tag. Calmodulin-bound protein was separated by SDS-PAGE and probed for Bid (anti-Bid) and ubiquitin (anti-HA). All experiments in this figure were performed three times with similar results.

chain of these amino acids. There is recent biological precedent for this type of Ubiquitin conjugation by viral Ubiquitin ligases [23]. To test this possibility, we investigated whether the Ubiquitin linkage was susceptible to alkaline hydrolysis. HeLa cells expressing tBid-N TAP and HA Ubiquitin were lysed at either pH 7.4 or pH 12, heated for 10 min at 70°C, diluted out in buffer with neutral pH and subjected to TAP purification. Isolates were probed for Bid and Ubiquitin. Equal amounts of tBid-N TAP were recovered from both lysates, but after treatment at pH 12, the amount of Ubiquitin associated with tBid-N was greatly diminished (Figure 5c). Thioester bonds are very easily hydrolysable, but the dramatic loss of ubiquitination after
hydrolysis was compatible with the notion that hydroxyl ester bonds were involved as well. To verify that the hydrolysis procedure left conventional lysine-linked Ubiquitin moieties unaffected, we tested its effect on a complex of purified, in vitro ubiquitinated Ring1b and Bmi1 [23]. After the ubiquitination reaction, the complex was incubated at pH 7.4 or at pH12, exactly as done for tBid-N TAP. In addition, it was heated in presence of β-mercaptoethanol to test the effect of reduction. The conditions were neutralized, the complex was recovered by virtue of His tags, and examined for extent of ubiquitination by immunoblotting. None of the treatments affected the ubiquitination status of Ring1b (Supplementary Figure 4).

For mutation analysis of serines and threonines, we focused on the residues in helix 1. Among these, T17 is completely conserved between species (Supplementary Figure 1c). However, alanine substitution of T17 alone did not stabilize tBid-N, or affect ubiquitination of the 1-30 fragment of tBid-N (not shown). Additional mutation of S27, C28 and S29, or mutation of all serines, threonines and cysteines in helix 1 did not stabilize tBid-N either (Supplementary Figure 3). It reproducibly reduced ubiquitination of the 1-30 fragment to a certain extent, but did not abrogate it (Figure 5b). Additional mutation of C3 rendered HA tBid-N TEV30 highly unstable (Supplementary Figure 3), but we could reveal ubiquitination on the 1-30 fragment, supporting the conclusion that other residues than cysteines are acceptor sites (Figure 5b). This result pointed to a possible role for S9 and/or S10. However, alanine substitution of these residues either alone, or in conjunction with the potential acceptor sites in helix 1 did not stabilize tBid-N (Supplementary Figure 3). Importantly, ubiquitination of the 1-30 fragment was virtually abrogated when alanine substitution of S9 and S10 was combined with alanine substitution of cysteine, threonine and serine residues in helix 1 (Figure 5b). This result indicates that one or more of these residues is a ubiquitination site.

We conclude from the combined biochemical and mutation analyses that tBid-N is ubiquitinated on cysteine residues, but not exclusively so. There are ubiquitination sites other than cysteine amongst residues 1-30. We postulate that these are serine and/or threonine residues, given the hydrolysability of the ubiquitination and the results of mutation analysis. Unambiguously pinpointing the ubiquitination sites by mutation analysis is hampered by effects of mutation on protein stability and by the fact that the ubiquitination machinery is promiscuous and ubiquitinates multiple sites, including alternative residues when the primary target sites are not available due to mutation.

Proteasome-dependent degradation of tBid-N is critical for its membrane-permeabilizing activity

To investigate whether degradation of tBid-N was required for tBid-C function, we determined the impact of proteasome inhibition on tBid-C translocation. MCF-7 cells expressing Myc Bid GFP were stimulated with TNFα for 8 h in presence or absence of MG132 and examined by CLSM for the distribution of Myc tBid-N and tBid-C GFP cleavage fragments. In accordance with the biochemical data (Figure 2a), Myc tBid-N was rescued from degradation by MG132 (Figure 6a, panel IV). Despite this, tBid-C GFP translocated to mitochondria (panel V), where it partially co-localized with Myc tBid-N (panel VI). This result indicates that degradation of tBid-N is not required for tBid-C translocation.

In line with a previous study [14], it also suggests that the cleaved, but still associated tBid-N/tBid-C complex can translocate to mitochondria. Next, we investigated whether proteasome inhibition affected the capacity of tBid-C to permeabilize the mitochondrial outer membrane. Untransfected MCF-7 cells were stimulated with TNFα in the presence or absence of MG132 and analyzed by flow cytometry for Cyt C content. The dramatic release of Cyt C induced by TNFα stimulation was greatly reduced upon co-incubation with MG132 (Figure 6b), suggesting that degradation of tBid-N is required for tBid-C function.

Since the proteasome may have other targets in this pathway, we aimed to test a Bid
molecule harbouring tBid-N that could escape from proteasomal degradation. Unfortunately, the stabilizing N-terminal deletion mutation Δ15 unleashed constitutive mitochondrion localisation and permeabilizing activity in full length Bid (data not shown) and was therefore not useful. In the course of our work, though, we had noted that addition of a bulky group such as GFP or a repetitive Myc tag to the N-terminus of tBid-N greatly increased its stability. Therefore, we compared the functional activity of GFP Bid VSV, containing a stabilized GFP tBid-N, with that of Myc Bid GFP, containing the wild-type, unstable Myc tBid-N.

To create a cell line that exclusively expressed stabilized tBid-N, endogenous Bid was constitutively downregulated in MCF-7 cells by RNA interference (RNAi) (Figure 7a). To express stabilized GFP Bid VSV and control (unstable) Myc Bid GFP into these cells, a silent mutation was introduced into the Bid sequence that was not targeted by the siRNA. This allowed appropriate expression of the Bid constructs upon transient transfection (Figure 7a,b). Processing of Myc Bid GFP and GFP Bid VSV by Caspase-8/-10, as well as the half-life of the Bid fragments was followed kinetically after stimulation of the reconstituted MCF-7 cells with TNFα. In case of Myc Bid GFP, the tBid-C GFP cleavage product was visible from the 2 h time point onward and its signal increased in time. The Myc tBid-N fragment was detected at the 2 h and 4 h time points, but not at the 8 h time point (Figure 7b). Processing of GFP Bid VSV was also visible at 2 h, but in this case not only the tBid-C VSV fragment remained detectable in the 8 h time frame, but also the GFP tBid-N fragment (Figure 7b). We conclude that the GFP Bid VSV protein is properly processed by Caspase-8/10 and that the GFP tBid-N fragment is long-lived, whereas the Myc tBid-N fragment has a short half-life.

The fate of stabilized GFP tBid-N was also followed by CLSM. In unstimulated cells, GFP and VSV signals were consistent with a cytoplasmic localization of full length GFP Bid VSV (Figure 7c, panels I, II). At 8 h after stimulation, the VSV signal was punctate and colocalized with MitoTracker, indicating mitochondrial localization of tBid-C VSV (Panel

**Figure 6. Proteasome inhibition prohibits tBid-C function.** (a) MCF-7 cells expressing Myc Bid GFP were stimulated with TNFα with or without MG132 for 8 h. Stimulation resulted in translocation of tBid-C GFP (green) to mitochondria (panels II, III). N- and C terminal epitopes partially colocalized at mitochondria (panel VI). (b) TNFα-induced Cyt c release in presence or absence of MG132 was tested by flow cytometric analysis in untransfected MCF-7 cells after 8 h of stimulation. Results are means plus SD from three independent experiments. Values are corrected for Cyt c release in absence of stimulus (15 ± 5%). Asterisk indicates statistically significant difference (p<0.05 according to Student’s t test).
V). The GFP signal was still present at this time point (Panel IV) and partially colocalized with VSV and MitoTracker signals (Panel VI). This indicated long-term stabilisation of GFP tBid-N (compare with disappearance of Myc tBid-N, Figure 1a). As in the case of proteasome inhibition (Figure 6a), tBid-N stabilization did not interfere with mitochondrial translocation of tBid-C and both fragments partially colocalized at mitochondria. This suggests that tBid-N and tBid-C may remain associated after cleavage of full-length Bid and translocate to mitochondria as a complex.

Next, Cyt C release upon TNFa stimulation was compared in wild-type MCF-7 and Bid RNAi cells, before and after reconstitution with unstable or stabilized Bid versions. Bid RNAi effectively blocked TNFa-induced Cyt C release (Figure 7d). Importantly, introduction of GFP Bid VSV, bearing stabilized tBid-N, did not restore the ability of TNFa to induce Cyt C release, whereas introduction of Myc Bid GFP,
bearing unstable tBid-N did (Figure 7d). Taken together, these results indicate that degradation of tBid-N regulates the pro-apoptotic function of tBid-C. Based on these data, we propose a model in which selective removal of tBid-N by proteasome-mediate degradation liberates tBid-C to induce mitochondrial membrane permeabilization.

**Discussion**

Sequences in Bid’s N-terminal fragment sequester the pro-apoptotic BH3 domain. Through this autoinhibitory mechanism, full length Bid is kept in an inactive state [12,17]. Bid can be converted to its active form by cleavage in its second unstructured loop, such as at D60 by Caspase-8/-10 and at D75 by Granzyme B [8,25,26]. Mechanistically, it was unclear why cleavage should lead to Bid activation. Upon Bid cleavage *in vitro*, the N-terminal and C-terminal fragments remain associated according to both NMR and biochemical analyses [6,12,14]. Moreover, several investigators have demonstrated that the tBid-N-fragment, when expressed independently, can block the pro-apoptotic activity of the tBid-C fragment [15,17,18]. Collectively, these data suggest a critical inhibitory effect of the Bid N-terminus upon the C-terminal pro-apoptotic function both before and after Bid cleavage. Based on our results, we propose that cleavage of Bid acts as a pro-apoptotic activation signal by allowing ubiquitination and subsequent proteasomal destruction of the inhibitory N-terminal fragment.

While originally recognized as a signal for the steady state destruction of aged and misfolded proteins, ubiquitination is now known to play an essential regulatory role in cellular signaling. Various apoptosis signaling molecules are regulated by proteasomal destruction [27-29]. These include inhibitory Bcl-2 family members Bcl-2 [30], Mcl-1 [31,32] and A1 [33], pro-apoptotic Bax [25], as well as BH3-only proteins Bim [34,35] and Bik [36] and the C-terminal fragment of Bid [30]. Polyubiquitination and degradation generally negatively regulate the function of the target protein by its complete elimination. The case of tBid-N ubiquitination is different, since it represents a form of positive regulation: it is required for the pro-apoptotic function of Bid. In the non-canonical NF-κB pathway, polyubiquitination and partial protein degradation of p105/p100 also acts as an activating principle [29]. Both in case of the NF-κB precursor and in the case of Bid, a portion of the molecule escapes from degradation and is released to perform its function.

Mutation analysis specifically implicated 5 out of 13 lysines in Mcl-1 in its destruction [32], while mutation of all 4 lysine residues in tBid-C was shown to increase its half-life [19]. In case of the other Bcl-2 family members, no specific target sites have been identified. In case of Bid, ubiquitination occurred with certainty on the Bid fragment encompassing residues 1-30. Indeed, the TEV protease mapping experiments indicate that tBid-N can be ubiquitinated on sites among amino acid 11-30, as well as to a lesser extent on sites among amino acids 1-10. This classifies the ubiquitination as unconventional, since it did not concern a peptide bond to an ε amino group of lysine or an α amino group at the N-terminus of the Bid protein. Following reduction, ubiquitination was partially, but not completely lost. This indicates that cysteine residues are among the Ubiquitin acceptor sites. A thioester linkage is not unusual for Ubiquitin, since the ubiquitin conjugating (E2) and Ubiquitin ligase (E3) enzymes bear Ubiquitin in such a manner. However, to our knowledge, cysteine ubiquitination of a target other than E2 or E3 proteins has only one precedent in the existing literature. This concerns the cytoplasmic tail of MHC class I that contains no lysine residues, but was ubiquitinated in a reducible manner by an E3 encoded by a herpesvirus [36]. Ubiquitination of tBid-N was to a significant degree maintained upon reduction (Figure 5b) consistent with the possibility that threonine and serine residues are primary targets of the ubiquitination machinery. The fact that a tBid-N mutant lacking all three cysteine residues was still ubiquitinated on fragment 1-30 proves...
that residues other than cysteine are ubiquitin acceptors. This is consistent with the fact that cysteines are not fully conserved in tBid-N. In fact, rat tBid-N does not contain any cysteines (Supplementary Figure 1c).

Our extensive mutation analysis has not pinpointed the ubiquitination sites. These experiments are hampered by the fact that Ubiquitin is likely appended to other candidate sites when the original sites are not available, including residues outside region 1-30. Mutation of all cysteines, threonines and serines in tBid-N made it much more unstable than the wild-type protein, such that it was barely detectable even in the presence of proteasome inhibitor (not shown). In this case, the protein likely bears little structural resemblance to the wild-type tBid-N and results are difficult to interpret. Thus far, attempts to map the ubiquitination sites by mass spectrometry have not been successful. Lability of the (thio)ester bonds is the most likely limiting factor in this analysis, particularly after tryptic digestion which converts the polyubiquitin chain into a reactive Gly Gly amine. Theoretically, Ubiquitin can link to serine and threonine by the formation of an ester bond between the carboxyl group of the C-terminal glycine and the hydroxyl group in the side chain of these amino acids. The first evidence for such linkage in a biological system has been reported when this manuscript was in final stage of preparation. It again concerned the cytoplasmic tail of MHC class I, which was ubiquitinated by another herpesvirus-derived E3 than the one responsible for cysteine ubiquitination [23]. Our work provides additional evidence for such linkage and adds that it can occur in a mammalian system.

While ∆13 tBid-N was as unstable as the wild-type version, the ∆15 mutant was fully stabilized. C15 is a good candidate Ubiquitin acceptor site, since it lies at the border of helix 1 and is accessible in the Bid structure [12,13]. However, point mutation of C15 did not affect ubiquitination. In stark contrast, ubiquitination was almost completely lost from tBid-N GFP upon deletion of residues 1-15. This suggests that these residues, and in particular residues 14 and 15 (given the instability of the ∆13 mutant) are critical for binding of the (undefined) ubiquitination machinery to tBid-N.

In our CLSM experiments, we found tBid-N together with tBid-C at the mitochondrial membrane after TNFα stimulation, when tBid-N degradation was blocked by proteasome inhibition (Figure 6a) or GFP fusion (Figure 7c). It is therefore clear that tBid-N degradation is not required for mitochondrial translocation of tBid-C. We suggest that the stabilized tBid-N fragment inhibits the function of tBid-C at the mitochondria. In time course experiments with wild-type Myc Bid GFP, we also found a degree of colocalization of Myc and GFP signals at mitochondria at 2 h after TNFα stimulation (Supplementary Figure 5). These findings tie in with the in vitro observation that the cleaved complex stays intact [12] and a previous study showing association of the cleaved Bid complex with mitochondria [14]. These data suggest that tBid-N and tBid-C may remain associated during mitochondrial translocation. Further experiments are required to resolve whether tBid-N ubiquitination takes place at the mitochondrial membrane and whether this event determines its dissociation from tBid-C.

We have shown here that Bid’s pro-apoptotic function is activated through cleavage-inducible ubiquitination and proteasomal degradation of tBid-N. Freeing of the BH3 domain by this mechanism may apply to other Bcl-2 family members as well. For instance, BimEL is cleaved by caspases at an early stage during apoptosis induction, rendering it more pro-apoptotic [37]. Moreover, Bcl-2, Bfl-1 and Bcl-xL can be converted into pro-apoptotic proteins following cleavage by caspases or Calpain [7,33,38]. Perhaps in these cases a similar mechanism operates as we have shown here for Bid activation.

**Acknowledgements**

We are grateful to Gretel Buchwald, Puck Knipscheer and Titia Sixma for help with the in vitro ubiquitination experiments and
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Western blotting, metabolic labelling and immunoprecipitation

For immunoprecipitation, all cells (adherent and floating) were harvested, washed twice in PBS and lysed by sonication in Nonidet P-40 (NP-40) buffer, consisting of 50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM PMSF and Complete EDTA-free Protease Inhibitors (Roche Molecular Biochemicals). Cell lysates were clarified by centrifugation for 15 min at 13,000 g and protein content was measured by Bio-Rad protein assay. All SDS-PAGE was done on pre-cast 4-12% NuPAGE minigels, according to the manufacturer’s protocol (Invitrogen). In this procedure, samples are heated for 10 min at 70°C in reducing (100 mM DTT) sample buffer prior to electrophoresis. Equal amounts of protein per lane (20 µg) were separated by SDS-PAGE and transferred to nitrocellulose sheets. Immunoblotting and enhanced chemiluminescence were performed as described [40]. For metabolic labelling, cells (1 x 10⁶ cells in a 10 cm dish) were starved in methionine- and cysteine-free DMEM for 2 h, radiolabelled with [35S]-methionine and -cysteine (13.25 MBq per dish, Amersham) for 2 h in the same medium. For the chase, cells were incubated for the indicated time in complete DMEM medium, supplemented with unlabelled methionine and cysteine (2.5 mM). Immunoprecipitated proteins were resolved by SDS-PAGE, detected by autoradiography and quantified by phosphorimaging (Fuji Film FLA-3000 and AIDA software).

Tandem affinity purification

Cells (1x10⁶) were lysed in NP-40 buffer. Lysates were clarified by centrifugation and incubated with rabbit 1G9 agarose (Sigma) for 2 h at 4°C. Agarose beads were washed in NP-40 buffer and in TEV cleavage buffer (10 mM Tris pH 8, 0.1% NP-40, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT) and incubated with 10 units TEV protease (Invitrogen) in cleavage buffer overnight at 4°C. Next, supernatant was diluted 3-fold in Calmodulin-binding buffer (10 mM Tris-HCl pH 8, 0.1% NP-40, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM CaCl₂) and incubated with calmodulin-sepharose beads (Stratagene) at 4°C for 1 h. Calmodulin-bound proteins were separated by SDS-PAGE and detected by Western blotting. For alkaline hydrolysis experiments, cells were lysed in PBS pH 7.4 or pH 12, 1% NP-40 and Complete EDTA-free Protease Inhibitors. Lysates were clarified by centrifugation, heated at 70°C for 10 min, diluted out 100-fold in NP-40 buffer and subjected to TAP purification.

Treatment of immunoprecipitates

For TEV protease treatment, HA Bid immunoprecipitates were resuspended in 300 µl TEV cleavage buffer and incubated with or without 2 µl TEV protease overnight at 4°C. Beads were spun down, washed three times and taken up in sample buffer for SDS-PAGE. For reduction, HA Bid immunoprecipitates were suspended in 10 µl NP-40 buffer. To this, 15 µl of 100 mM Tris-HCl pH 6.8, 4% SDS, 8 M urea, 20% glycerol was added, with or without 600 mM fresh DTT. Control samples without DTT were kept on ice, while samples with DTT were heated at 95°C for 10 min. Next, samples were diluted out 80 times in NP-40 buffer with 10 mM iodoacetamide and 50 µg/ml RNase A (as carrier protein). Beads were spun down and supernatants
were subjected to re-precipitation with anti-HA antibody. For the sequential immunoprecipitation shown in Fig. 3b, following immunoprecipitation with anti-GFP antibody, the lysate was incubated 3 times with Protein G sepharose beads followed by immunoprecipitation with anti-Myc antibody.

**In vitro ubiquitination**

To ubiquitinate the recombinant E2 core domain of E2-25K (22), recombinant human ubiquitin activating enzyme (E1) (120 ng; 0.2 µM), E2-25K-I151 (1.5 µg; 17 µM) and ubiquitin (2 µg; 47 µM) were co-incubated in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol with 5 mM ATP and 5 mM MgCl2, for 2 min at 23 °C. Reactions were terminated by addition of 1:1 dilution in sample buffer (100 mM Tris pH 6.8, 8 M urea, 4% SDS, 20% glycerol, with or without 600 mM DTT) and samples were kept on ice or heated at 95 °C as described above prior to electrophoresis. A complex of His-tagged Ring1b (residues 1-331) and full-length His-tagged Bmi1 were co-expressed in SF9 insect cells by simultaneous infection with separate recombinant baculoviruses. The complex was purified on Ni-agarose (Hitrap column, Pharmacia) and further purified as described [24]. Ring1b/Bmi1 was incubated at 6 µM, together with human E1 (220 mM), human UbcH5c (2 µM), ubiquitin (20 µM) and ATP (3 mM) in a total volume of 50 µl ligase buffer, consisting of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 µM ZnCl2, 1 mM DTT at 30°C for 1 h. For the control sample, the ubiquitination reaction was stopped by addition of LDS sample buffer (NuPAGE®, Invitrogen). Samples for immunoprecipitation (IP) were treated as follows: 1) incubated with 12.5-fold excess of ligase buffer as control, 2) heated for 10 min at 70 °C, 3) incubated with 12.5-fold excess PBS pH 12 and heated for 10 min with 200 µg/ml digitonin in PBS, 150 mM KCl, 1 mM EDTA for 10 min on ice. Cells were washed once in PBS, fixed in 4% SDS, 20% glycerol, with or without 600 mM DTT) and samples were kept on ice or heated at 95°C as described above prior to electrophoresis. A complex of His-tagged Ring1b (residues 1-331) and full-length His-tagged Bmi1 were co-expressed in SF9 insect cells by simultaneous infection with separate recombinant baculoviruses. The complex was purified on Ni-agarose (Hitrap column, Pharmacia) and further purified as described [24]. Ring1b/Bmi1 was incubated at 6 µM, together with human E1 (220 mM), human UbcH5c (2 µM), ubiquitin (20 µM) and ATP (3 mM) in a total volume of 50 µl ligase buffer, consisting of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 µM ZnCl2, 1 mM DTT at 30°C for 1 h. For the control sample, the ubiquitination reaction was stopped by addition of LDS sample buffer (NuPAGE®, Invitrogen). Samples for immunoprecipitation (IP) were treated as follows: 1) incubated with 12.5-fold excess of ligase buffer as control, 2) heated for 8 min at 95°C in presence of 5% β-mercaptoethanol, after which 12.5-fold excess ligase buffer was added, 3) incubated with 12.5-fold excess PBS pH 7.4 and heated for 10 min at 70 °C, 4) incubated with 12.5-fold excess PBS pH 12 and heated for 10 min at 70°C. Next, samples were diluted with NP-40 buffer (see above) to a total volume of 10 ml and subjected to immunoprecipitation with Ni-agarose beads (Qiagen). Reaction products were separated by NuPAGE® in 4-12% Bis-Tris precast gels (Invitrogen), blotted to nitrocellulose and probed independently with PDI1 antibody directed to ubiquitin and anti-Ring1b (H. Koseki, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan).

**RNA interference**

A short interfering (si) oligonucleotide targeting human Bid (nt 238-256, 5’ gaagacatcataaggaata 3’) was cloned into pSUPER and pRETO-SUPER [42]. Stable expression of Bid siRNA was achieved by retroviral transduction of cell lines of interest with the pRETO-SUPER construct. For rescue experiments, two silent mutations were introduced in the appropriate Bid sequence (gaagacatcataaggaata), using the QuikChange site-directed mutagenesis kit, in order to avoid siRNA-mediated silencing.

**Transfection and retroviral transduction**

Cells were transfected with FuGENE 6 according to manufacturer’s instructions (Roche Molecular Biochemicals). Unless otherwise indicated, cells were manipulated 20 h after transfection. Production of amphotropic retrovirus was done in ΦNX-Ampho cells as described [39]. For transduction, virus-containing packaging cell supernatant was incubated on ice for 10 min with 10 µg/ml DOTAP (Roche) and subsequently incubated with target cells overnight. Cells were selected with puromycin (1 µg/ml) 48 h after transduction.

**Cyt c release assay**

Cells were analyzed for Cyt c release essentially as described [43]. All cells were collected, washed in PBS and permeabilized with 200 µg/ml digitonin in PBS, 150 mM KCl, 1 mM EDTA for 10 min on ice. Cells were washed once in PBS, fixed in 4% paraformaldehyde, incubated in blocking buffer (2% BSA in PBS) for 30 min at room temperature and incubated with anti-Cyt c mAb 6H2.B4 at 1:200 dilution in blocking buffer overnight at 4°C. Cells were washed in blocking buffer and then incubated with a Cy5-conjugated goat anti-mouse IgG (Invitrogen). Next, cells were washed in PBS-Tween and mounted on slides using Vectashield (Vector Laboratories). All treatments were done at room temperature. CLSM was performed using a Leica TCS SP2 system (Leica Microsystems, Heidelberg, Germany) using a Leica 63x 1.32 NA oil immersion objective and Leica Confocal Software. Images were processed (cropping, level adjustment) using Adobe Photoshop software. To minimise spectral leak-through, images were obtained by sequential scanning.

**References**

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control of the pro-apoptotic activity of BH3-only proteins. Cell Death Diff. 9, 505-512.


Supplementary Information

Table S1. Wild-type and Bid mutants tested for stabilization and ubiquitination

<table>
<thead>
<tr>
<th>HA tBid-N TEV 30</th>
<th>C28A</th>
<th>C15A;C28A</th>
<th>C15A;T17A;S27A;C28A;S29A</th>
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<th>C3A;C15A;C28A</th>
<th>C3A;C15A;T17A;S27A;C28A;S29A</th>
</tr>
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</table>

Supplementary Figure 1. Bid structure and sequence. (a) The three dimensional structure of full length human Bid, as determined by NMR (Chou et al., 1999; McDonnell et al., 1999). Cleavage by Caspase-8 at D60 results in the tBid-N fragment (residues 1-60) indicated in blue and the tBid-C fragment indicated in red, which in vitro remain associated in a complex with the same structure as uncleaved Bid [Chou et al., 1999]. The pro-apoptotic BH3 domain (helix 3) is indicated in yellow. (b) Amino acid sequence of full length human Bid. Amino acids of tBid-N are numbered. Helices (H) 1-8 are annotated based on Chou et al. [1999]. The Caspase-8 cleavage site is indicated with the large arrowhead. (c) Amino acid sequence of tBid-N in various species. Cysteine residues are indicated in red, the conserved T17 is underlined.
Supplementary Figure 2. The Bid sequence is target for ubiquitination. HA ubiquitin was co-expressed in HeLa cells with the TAP empty vector, tBid-N TAP and tBid-N TAP lacking the first 23 amino acids of Bid (Δ23). Proteins were purified on the TAP tag, separated by SDS-PAGE and probed by immunoblotting for ubiquitin and Bid with anti-HA and anti-Bid antibodies, respectively. Data show that ubiquitination is specific for Bid, since the TAP tag alone does not carry ubiquitin and deletion mutation of Bid abrogates ubiquitination.

Supplementary Figure 3. Effects of point mutations on tBid-N stability. Alanine substitutions mutations were generated in HA tBid-N TEV30. Wild-type and mutant proteins were expressed in HeLa cells, which were incubated for the indicated time periods with CHX with or without MG132. Total cell lysates (TCL) were examined by anti-HA immunoblotting for levels of HA tBid-N TEV30. Lines indicate samples tested together in one experiment.
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**Supplementary Figure 4. Control for hydrolysis and reduction of ubiquitination.** A complex of Ring1b and Bmi1 was subjected to *in vitro* ubiquitination. Ubiquitin is attached to lysine on Ring1b, as shown by mass spectrometry (Buchwald et al. 2006). The *in vitro* ubiquitinated sample without further treatment is the control (C) shown in the first lane. The other lanes show material that was recovered by precipitation with Ni agarose beads (isolate) after it had been exposed to the indicated conditions: buffer (C), reduction with β-mercaptoethanol (βME), mild hydrolysis at pH12 and incubation at pH7.4 as control. The precise procedures are outlined in the Materials and Methods section. Recovered materials were resolved by NuPAGE and immunoblotted as indicated to detect Ring1b and its ubiquitinated species. The arrow denotes the non-ubiquitinated Ring 1b protein. Lower MW species (bracket) are Ring1b breakdown products.

**Supplementary Figure 5. Time course of tBid-N disappearance.** MCF-7 Bid RNAi cells expressing Myc Bid GFP were stimulated with TNFα for 2, 4 or 8 h or not (control), stained to detect mitochondria (MitoTracker) and the N-terminal Myc tag and examined by CLSM. The 4 h time point represents cells in which the GFP signal localized at mitochondria (about 40% of the population). At 8 h, the frequency of cells with such localisation was about 80%. In the other cells, localisation was as depicted for the 2 h time point.