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THE N-END RULE LIGASE UBR2 INTERACTS WITH THE N-TERMINAL FRAGMENT OF CASPASE-CLEAVED BID AND TARGETS IT FOR UBIQUITINATION

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ABSTRACT

The pro-apoptotic BH3-only protein Bid is activated by proteolytic cleavage. Hereafter, the N-terminal fragment (tBidN) is ubiquitinated and degraded by the proteasome. This event allows the C-terminal fragment (tBidC) to expose its BH3 domain and to transmit the apoptotic signal. tBidN has no lysines, and ubiquitination of tBidN is unconventional, because ubiquitin is conjugated via thio-ester and hydroxyl-ester bonds to cysteine and serine/threonine residues. Ubiquitination via esterification is not confined to tBidN, but has been reported for other mammalian proteins as well. To understand the biology of this modification, it is essential to know the identity of the ubiquitinating enzymes that mediate the conjugation. Here, we use an RNAi-based screening approach and a mass spectrometry-based interactome analysis to identify the enzyme that ubiquitinates tBidN. We identified the N-end rule ligase UBR2 as the E3 that targets tBidN for ubiquitination. The acceptor site(s) still need to be defined. UBR2-tBidN interaction involved a non-N-terminal region in tBidN that interacted with the type 2 binding site in the N-domain of UBR2. These data link N-end rule signaling to apoptosis signaling. In addition, they suggest that N-end rule ligases can mediate both conventional and unconventional ubiquitination, which significantly broadens the list of potential N-end rule substrates.
INTRODUCTION

The Bcl-2 protein Bid acts in the mitochondrial pathway of apoptosis signaling. Mitochondria harbor pro-apoptotic molecules in the space between the inner and outer membrane, that are released into the cytosol upon mitochondrial outer membrane permeabilization (MOMP). This will activate the Caspases, a protease family that cleaves a specific subset of substrates, and thereby executes apoptotic cell death. MOMP is regulated by the Bcl-2 family of proteins, that are grouped together because they all possess one or multiple Bcl-2 homology (BH) domains. The Bcl-2 family is composed of three subgroups: the BH3-only proteins, the effectors Bax and Bak and the anti-apoptotic Bcl-2 family members. The BH3-only proteins are the ‘sensors’ of apoptotic stimuli, because they are either transcriptionally or post-translationally activated by different stress signaling pathways. They subsequently induce multimerization of Bax/Bak at the outer mitochondrial membrane, which causes MOMP. Both the BH3-only proteins and Bax/Bak can be sequestered, and thereby neutralized, by the anti-apoptotic Bcl-2 family members.

The BH3-only family member Bid is a 22 kDa cytosolic protein, that is composed of a central core of two hydrophobic alpha-helices, surrounded by six amphipatic alpha-helices. It transmits the signal from death receptors such as TNF receptor 1, Fas/CD95 and TNF-related apoptosis-inducing ligand (TRAIL) receptors to the mitochondria. Upon death receptor engagement, a signaling platform is assembled that recruits Caspase-8, which subsequently becomes active and cleaves Bid C-terminal of aspartate 60, in a loop between helix 2 and helix 3. This generates two Bid fragments: the 7 kDa N-terminal fragment (tBidN) and the 15 kDa C-terminal fragment (tBidC). tBidC contains the BH3-domain and therefore the pro-apoptotic capacity. After cleavage, Bid accumulates at the mitochondria, where it interacts via its BH3 domain with Bax/Bak and the anti-apoptotic Bcl-2 family members. Bid-interaction with Bax/Bak induces their multimerization, followed by MOMP and cell death.

In solution, tBidN and tBidC remain associated after Caspase-8-mediated cleavage through intramolecular interactions. This interaction is reversible, even at submicromolar concentrations, indicating that it is specific and not induced by potentially high concentrations of isolated protein. Moreover, it has been demonstrated by biochemical and cellular assays that this interaction with tBidN inhibits the pro-apoptotic activity of tBidC, most likely by preventing exposure of its BH3-domain. To liberate the BH3-domain and relieve this inhibition, tBidN is targeted for ubiquitination and for degradation by the proteasome.

Ubiquitin conjugation to a substrate is mediated by a three-step enzymatic cascade involving an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugase and an E3 ubiquitin ligase. In most cases, it is the E2 that is catalytically active and transfers the ubiquitin to the substrate, but it is always the E3 that recognizes the substrate. So the specificity increases downstream in the cascade, which is reflected by the variety of enzymes involved: the human proteome contains two E1 enzymes, at least 38 E2 conjugases but more than 600 E3 ligases.

Normally, ubiquitin is conjugated to a substrate via an isopeptide bond between the C-terminus of ubiquitin and the ε-amino-group of a lysine side chain, or in some cases the α-amino-group of the N-terminal residue. However, tBidN does not contain any lysine
residues, and N-terminal ubiquitination was excluded on basis of biochemical experiments and the fact that tBidN is N-terminally acetylated.\textsuperscript{13,18} Rather, biochemical and mutagenesis data demonstrated ubiquitin conjugation to cysteine and serine/threonine residues, by thio-ester and hydroxyl-ester bonds respectively.\textsuperscript{13} At that moment, tBidN was the first substrate demonstrated to be ubiquitinated on unconventional residues by mammalian ubiquitinating enzymes.

By now, it has been demonstrated that unconventional ubiquitination in mammalian cells is not confined to tBidN alone.\textsuperscript{19-23} To understand the biology of this modification, it is crucial to identify the enzymes mediating unconventional ubiquitination. We therefore aimed to identify the tBidN-specific E2 conjugase, E3 ligase and possible de-ubiquitinating enzymes (DUBs). This would generate insight in ubiquitin signaling, but also in apoptosis signaling, as the activity of these enzymes is expected to regulate the apoptotic threshold. By using a combination of three different methods, \textit{i.e.} candidate testing, RNAi-based screening and Mass Spectrometry-based interactome analysis, we identify the N-end rule ligase UBR2 to interact with and ubiquitinate tBidN.

**RESULTS**

**Ubiquitination of tBidN is not mediated by candidate ubiquitin ligases Itch and MARCH-5**

As a first approach to identify the E3 ubiquitin ligase targeting tBidN for ubiquitination, we tested candidate ligases derived from literature. We considered the ubiquitin ligase Itch as a candidate, because it has been demonstrated to target tBidC for ubiquitination.\textsuperscript{24} To test this hypothesis, we studied tBidN ubiquitination in HeLa cells transfected with either a control vector or Itch targeting shRNAs, together with HA-tBidN and FLAG-ubiquitin cDNA. Three days, later cells were treated with proteasome inhibitor MG132 to accumulate ubiquitinated protein, tBidN was isolated by α-HA immunoprecipitation (IP) and the precipitate was analyzed by immunoblotting.

A ubiquitin smear co-precipitated with tBidN, indicating the presence of poly-ubiquitinated tBidN species (Fig. 1A). Importantly, the levels of ubiquitinated tBidN did not change upon co-transfection of an Itch targeting shRNA (Fig. 1A). To validate whether the shRNAs silenced Itch expression, HeLa cells were retrovirally transduced to generate cell lines stably expressing either of the Itch shRNAs, and Itch transcript levels were analyzed by quantitative Real-Time (qRT) PCR. In the cells transfected with Itch shRNA18, Itch expression was silenced to 40% of the expression level in control cells (Fig. 1B). These data suggest that either a more than two-fold decrease in Itch expression is not sufficient to affect tBidN ubiquitination, or that Itch does not ubiquitinate tBidN.

Next, to assess the role of Itch in tBidN stability, we analyzed the degradation kinetics of tBidN in control and Itch knockdown cell lines. HA-tBidN was expressed in these cell lines, followed by treatment with the translation inhibitor cycloheximide (CHX) for different time periods. Within 60 minutes after CHX addition, the total pool of HA-tBidN was degraded, confirming previous results (Fig. 1C).\textsuperscript{13} This degradation was likely mediated...
by the proteasome, because it was blocked by co-treatment with the proteasome inhibitor MG132 (Fig. 1C). The degradation kinetics in control and Itch knockdown cell lines were identical (Fig. 1C), indicating that Itch is not required for tBidN degradation. All together, these data suggest that Itch is not the ubiquitin ligase targeting tBidN.

As a second candidate ligase we studied membrane-associated RING-CH protein 5 (MARCH-5) that is considered a candidate ligase for two reasons: its localization at the mitochondria and its atypical RING domain.\textsuperscript{25} With regards to the first reason, we have observed that upon death receptor induction tBidN localizes at the mitochondrial surface.\textsuperscript{13} This indicates that either tBidN and tBidC translocate after cleavage as an associated complex from the cytosol to the mitochondria, or Bid gets cleaved at the mitochondrial surface, which has been suggested to occur by Schug et al.\textsuperscript{26} In both situations, it is likely that tBidN is targeted for ubiquitination at the mitochondria, where MARCH-5 resides. Second, the RING domain of MARCH-5, that binds the E2 conjugase and is essential for ubiquitin transfer to the substrate, has an atypical order of the zinc-binding cysteine and histidine residues.\textsuperscript{25} This atypical RING domain is shared by viral homologues of MARCH-5 that ubiquitinate their substrates on cysteine and serine/threonine residues.\textsuperscript{27-28}

To test whether MARCH-5 ubiquitinated tBidN, we obtained the cDNA of wild-type (WT) FLAG-tagged MARCH-5, and of a ubiquitination-defective RING mutant (RM).\textsuperscript{29} HeLa cells were transfected to express these MARCH-5 variants, together with tBidN-GFP and HA-ubiquitin, followed by isolation of tBidN. Immunoblotting of the tBidN-GFP isolate revealed the presence of ubiquitinated tBidN, but importantly, the levels of ubiquitinated tBidN did not change upon expression of WT MARCH-5 or RM MARCH-5 (Fig. 1D).

Possibly, this specific experimental setting was too artificial to detect a subtle phenotype induced by changed MARCH-5 expression. We therefore assessed stability of tBidN directly after its generation from Caspase-8 cleaved full-length Bid, which resembles what occurs during apoptosis signaling. For this experiment, we used MCF-7 cells that do not express Caspase-3 and are therefore highly resistant to death receptor-induced apoptosis. To exclude signal from endogenous Bid, we used MCF-7 Bid knock-down (KD) cells, that were transfected with RNAi-resistant Myc-Bid-GFP, together with either a control vector or a MARCH-5-targeting shRNA. Next, these cells were treated with the death ligand TNF-related apoptosis-inducing ligand (TRAIL) for various time periods to induce Caspase-8 activity and therefore Bid cleavage.

Within several hours, Myc-tBidN and tBidC-GFP fragments were generated by cleavage of full-length Bid (Fig. 1E). However, the Myc-tBidN fragments disappeared several hours after their generation, while the tBidC-GFP fragments remained (Fig. 1E). This disappearance was due to proteasomal degradation of Myc-tBidN, since it was prevented by blocking proteasome function with MG132 (Fig. 1E). Importantly, Myc-tBidN was still degraded in the MARCH-5 shRNA expressing cells (Fig. 1E). The MARCH-5 shRNA efficiently silenced MARCH-5 expression, as revealed by transcript analysis of cell lines stably expressing the shRNA construct (Fig. 1F). These and above data suggest that MARCH-5 is not involved in ubiquitination or degradation of tBidN.
Figure 1. The ubiquitin ligases Itch and MARCH-5 do not ubiquitinate tBidN. (A) HeLa cells were transfected with HA-tBidN and FLAG-ubiquitin cDNA, together with either a control vector or an Itch targeting shRNA. Three days later, cells were lysed and tBidN was isolated from the lysate by IP with anti (α)-HA mAb, followed by immunoblotting of the precipitate with α-FLAG or α-HA mAb. (B) HeLa cells were retrovirally transduced with either a control vector or an Itch-targeting shRNA, followed by selection of transduced cells with puromycin. Total RNA was isolated and reverse transcribed, followed by qRT-PCR of Itch transcript. Itch transcript levels were corrected for GAPDH transcript levels, and value in control cells was set to 100. Depicted is mean and SD of an experiment performed with three different cDNA dilutions in triplicate. (C) Control and Itch KD cell lines described in (B) were transfected to express HA-tBidN and treated with either cycloheximide (CHX; 10 μg/ml) alone, or together with MG132 (10 μM) for indicated time periods. Cells were harvested, followed by immunoblot (IB) analysis of the total cell lysates with α-HA mAb. (D) HeLa cells were transfected to express tBidN-GFP together with HA-ubiquitin, and either a wild-type (WT) or RING mutant (RM) FLAG-MARCH-5. Next, tBidN was isolated by IP with α-GFP antibody, and...
A high throughput screen identifies candidate ubiquitin ligases targeting tBidN

Because the candidate ligases did not mediate tBidN ubiquitination, we decided to switch to a screening approach. We designed an RNAi-based screen with tBidN protein levels as a read-out (Fig. 2A). A tBidN-GFP fusion construct was generated to facilitate high throughput analysis of protein levels by flow cytometric measurement of GFP fluorescence intensity. We previously demonstrated that tBidN is still targeted for ubiquitination and proteasomal degradation when C-terminally fused to GFP.13

The osteosarcoma cell line U2OS was retrovirally transduced to generate cells stably expressing tBidN-GFP (Fig. 2A). This same cell line was transduced to express the two other Bcl-2 family members Bcl-B and Noxa, fused to red (RFP) and blue (BFP) fluorescent proteins respectively (Fig. 2A). Both Bcl-B and Noxa are targeted for ubiquitination, but the enzymes regulating their ubiquitination are unknown.30-31 Expressing three substrates in one cell line improves efficiency of the screen but also specificity, because specific changes in substrate levels can be distinguished from global changes in protein levels. The resulting U2OS cell line was transfected in a 96-wells format with siRNA smartpool libraries targeting 625 different E3 ubiquitin ligases and E2 ubiquitin conjugases, and 128 different DUBs. Three days later, GFP, RFP and BFP content was analyzed by flow cytometry reflecting tBidN, Bcl-B and Noxa levels respectively.

The result of the screen for tBidN is depicted as Z-scores for each individual well in figure 2B. A hit was defined as the target of a siRNA smartpool for which transfection resulted in an above threshold increase (ligase/conjugase) or below threshold decrease (DUB) of tBidN levels (Fig. 2B, C). The ligase/conjugase screen revealed a total of 24 hits, of which 15 were specific for tBidN (Fig. 2C). Unfortunately, no DUB hits were revealed (Fig. 2B). However, a strong increase in tBidN, Bcl-B and Noxa protein levels was observed upon transfection of an siRNA smartpool targeting the gene **PSMD14** (data not shown). This gene encodes for the proteasome-associated DUB Poh1, which is essential for proteasome function.32-33 The identification of Poh1 therefore provides a proof-of-principle hit, validating the screening approach. The hitlist of our screen therefore represents a pool of candidate ligases that target tBidN for ubiquitination and degradation.

A proteomic approach identifies the N-end rule ligase UBR2 as a tBidN interactor

We reasoned that a rapid way to validate the ligase hits derived from the RNAi-based screen would be to test whether any of the ligases would interact with tBidN. We therefore set out

- the precipitate and total cell lysate (TCL) were immunoblotted with α-FLAG, anti-GFP or α-HA antibodies.
- (E) MCF-7 cells in which endogenous Bid expression was silenced were transfected with Myc-Bid-GFP cDNA, together with either a control vector or a MARCH-5-targeting shRNA. The cells were treated with TRAIL (100 ng/ml) together with CHX (10 μg/ml) for indicated time periods, either alone or in combination with MG132 (50 μM). Total cell lysates were analyzed by immunoblotting with α-Bid antibody.
- (F) MCF-7 cells were retrovirally transduced with either a control vector or a MARCH-5-targeting shRNA, followed by selection of transduced cells with puromycin. Total RNA was isolated and reverse transcribed, followed by qRT-PCR of MARCH-5 transcript. MARCH-5 transcript levels were corrected for Actin-β transcript level, and value in control cells was set to 100. Depicted is the mean of an experiment performed in duplicate.
1. Cell line co-expressing three substrate-fusion proteins
2. Transfect with siRNA library targeting 625 E3 ligases and 128 DUBs
3. Read-out fluorescence by flow cytometry

A

Figure 2. High throughput siRNA-based screen identifies potential E3 ligases and E2 conjugases targeting tBidN for ubiquitination (A) Experimental set-up of the high throughput RNAi-based screen. (B) Result of the DUB (left panel) and ligase (right panel) screen for tBidN. Depicted are the Z-scores ((mean in each well - average per plate)/SD of plate) for each individual siRNA transfected well. Intermittent lines depict threshold Z-score of -2 (DUBs) and 2 (ligases), used to define hits. (C) Hitlist of the ligase screen for tBidN. In bold are the hits that are unique for tBidN.

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<th>Noxa Z-score</th>
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to purify tBidN from cells and analyze the interacting proteins by Mass Spectrometry (MS). To this end, we generated a tBidN construct with a C-terminal tandem affinity purification (TAP) tag, consisting of a calmodulin binding domain and a Protein A (ProtA) sequence, separated by a tobacco etch virus (TEV) cleavage site (Fig. 3A). Purified protein can be obtained using this TAP-tag by a two-step isolation procedure involving IP with IgG beads, followed by TEV-protease mediated cleavage and re-IP with calmodulin beads.

HeLa cells were transfected to express tBidN-TAP together with HA-ubiquitin, and tBidN was isolated by the TAP-protocol. Coomassie-stainable amounts of tBidN were obtained (Fig. 3B), and immunoblotting of a fraction of the isolate revealed the presence of tBidN-ubiquitin conjugates (Fig. 3B). The complete gel-lane containing all isolated protein content was excised in separate bands and proteins were trypsin-digested, followed by liquid chromatography tandem-MS (LC/MS-MS) analysis. A total of 263 unique proteins were identified, representing the putative tBidN interactome. Among these potential interactors were five ubiquitin ligases and three DUBs (Fig. 3C). Unfortunately, there was no overlap between these ligases and the ligase hits derived from the RNAi-based screen (Fig. 2C and 3C). The putative interactome can therefore be considered as a novel source of candidate tBidN ubiquitin ligases in addition to what was found by the RNAi screen, rather than a validation of the screen hits.

The ubiquitin ligase UBR2 was strongly represented within the putative tBidN interactome, with a total of 79 spectra representing 55 unique UBR2 peptides (Fig. 3C), while the median spectral count for each detected protein was six spectra. UBR proteins are N-end rule ubiquitin ligases that recognize specific amino-terminal residues, called N-degrons. Mammals contain seven members of the UBR family, termed UBR1 to UBR7. Interestingly, two other UBR family members, UBR1 and UBR5, were detected in the putative tBidN interactome (Fig. 3C). Together, this MS experiment identifies UBR2, and possibly more members of the UBR family, as potential tBidN interacting proteins, suggesting that they might mediate ubiquitination of tBidN.

tBidN interacts with UBR2

Next, we aimed to validate the UBR2-tBidN interaction. We obtained the cDNA of FLAG-tagged mouse UBR2 (mUBR2), that shares 90% sequence identity with human UBR2. HeLa cells were transfected to express FLAG-mUBR2 together with tBidN-TAP, and treated with MG132 to accumulate tBidN. Interestingly, in untreated cells, tBidN-TAP was detected as a single band, but after blocking proteasomal degradation with MG132 a second tBidN-TAP species (ΔtBidN-TAP) appeared (Fig. 4A). This ΔtBidN-TAP was about 1-2 kDa lighter than tBidN-TAP and apparently efficiently targeted for proteasomal degradation.

The ΔtBidN-TAP species was also present in the isolate obtained to determine the tBidN interactome (Fig. 3B). To identify the nature of ΔtBidN-TAP, this band was specifically excised and analyzed by MS. All peptides of tBidN and the remainder of the TAP tag were detected except for the two peptides representing the first 20 amino acids (Fig. S1A). Therefore, ΔtBidN-TAP may be a truncated tBidN variant. Since it is not certain that the
two peptides are indeed absent, the alternative is that ΔtBidN-TAP has a reduced mol mass because it lacks a posttranslational modification normally present in tBidN.

When FLAG-mUBR2 was isolated by IP from the cell lysate, tBidN-TAP co-precipitated, validating the interaction suggested by our MS data (Fig. 4A). Notably, it was mainly the ΔtBidN-TAP species that interacted with FLAG-mUBR2 (Fig. 4A). Furthermore, a ladder of α-Bid antibody-reactive proteins was detected, but only when both UBR was ectopically expressed and the proteasome was inhibited with MG132 (Fig. 4A). We speculate that this ladder might represent ubiquitinated tBidN. UBR2 and tBidN also co-precipitated when a differentially tagged tBidN variant, carrying a C-terminal HisHA-tag, was isolated from HeLa cells by α-HA IP (Fig. 4B). This excludes a possible involvement of the TAP-tag in interaction with UBR2. The interaction did not require the RING domain of UBR2, as a ΔRING mutant of FLAG-mUBR2 (ΔR) co-precipitated with tBidN-HisHA (Fig. 4B). Together, from these data and the MS data, we conclude that tBidN interacts with the N-end rule ligase UBR2.
The potential N-degrons in the predicted N-terminus of ΔtBidN are dispensable for interaction with UBR2

To test what regions in tBidN were important for its interaction with UBR2, we generated several tBidN mutants. The mutations were mainly based on the hypothesis that the ΔtBidN-TAP is an N-terminally truncated form of tBidN-TAP, and the newly formed N-terminus is an N-degron that can be recognized by UBR2. The N-end rule ligases can recognize two types of N-degrons, as summarized in figure S1B. The positively charged type 1 residues (R, K and L), can be recognized by the type 1 binding site in the UBR box, a domain shared by all seven different UBR family members.$^{35}$ The hydrophobic type two residues (F, Y, W, I and L) are recognized by a binding site in the N domain, that is found only in UBR1 and UBR2.$^{35}$ Furthermore, several other residues can be arginylated, either directly (D and E) or after deamidation (N and Q) or oxidation (C) reactions, allowing recognition by the type 1 binding site.$^{35}$

The N-terminal truncation of ΔtBidN-TAP was at maximum 20 amino acids, as the rest of tBidN was detected by MS (Fig. 3B and Fig. S1A). Based on the estimated mass difference of 1-2 kDa with tBidN-TAP, the N-terminal truncation of ΔtBidN-TAP was likely at least ten amino acids (Fig. 4A), leaving the residues L11 to V21 as potential N-degrons. Many residues in this region are potentially recognized by UBR2, so we generated a total of five tBidN mutants in which either single residues or a combination of residues was mutated to alanines (Fig. S1A). We made these mutations in tBidN-TAP or in tBidN-GFP that also appears as a doublet consisting of tBidN-GFP and ΔtBidN-GFP, and interacts with FLAG-mUBR2 as demonstrated by co-IP (Fig. 5A).

All tBidN mutants we tested co-precipitated with FLAG-mUBR2 (Fig. 5A, B). We therefore generated an additional tBidN F22A mutant, but this also interacted with UBR2 (Fig. 5C). Mutation of residues in the hydrophobic region between L19 and F22 resulted in a reduction of ΔtBidN protein levels, while tBidN protein levels were increased (Fig. 5D).
This suggests that this region is involved in generating ΔtBidN from tBidN. For these mutants, less ΔtBidN was detected in the UBR2 precipitate, but when corrected for the low levels of ΔtBidN in the total cell lysate, the stoichiometry of interaction appeared similar to that of WT tBidN with UBR2 (Fig. 5A, C). In addition to the mutation analysis, we assessed interaction of full length (fl) Bid with UBR2, but could not detect any co-precipitation (Fig. 5C). This indicates that cleavage of Bid is required to allow recognition of

Figure 5. Potential N-degrons in the region in tBidN spanning amino acid 11 to 22 are dispensable for its interaction with UBR2. (A) HEK 293T cells were transfected to express FLAG-mUBR2, together with either tBidN-GFP WT or indicated mutants. The cells were treated for four hours with MG132 (50 μM) followed by IP with α-FLAG mAb to isolate UBR2 from the cell lysate. TCL and precipitate were analyzed by immunoblotting.

(B) HeLa cells were transfected to express FLAG-mUBR2 together with tBidN-TAP WT or indicated mutants. The cells were left untreated or treated for four hours with MG132 (25 μM) followed by α-FLAG IP to isolate UBR2 from the cell lysate. TCL and precipitate were analyzed by immunoblotting with α-FLAG and α-Bid antibodies.

(C) HEK 293T cells were transfected to express FLAG-mUBR2, together with a WT or F22A mutant of either tBidN-TAP or full length (fl) Bid-GFP. The cells were left untreated or treated for four hours with MG132 (50 μM) followed by α-FLAG IP to isolate UBR2 from the cell lysate. TCL and precipitate were analyzed by immunoblotting with α-FLAG and α-Bid antibodies.
the N-terminal part by UBR2. These data indicate that the UBR2-tBidN interaction does not require any of the potential N-degrons in tBidN in the region spanning amino acid 11 till 22.

**UBR2 recognizes tBidN via its type 2 binding site and targets tBidN for ubiquitination**

As mentioned, UBR2 contains two substrate binding sites: a type 1 binding site in the UBR box recognizes positively charged residues, while a type 2 binding site in the N-domain recognizes bulky hydrophobic residues. To understand the biochemical details of the interaction with tBidN, we aimed to identify to which of these sites tBidN binds. We therefore performed co-IP assays in the presence of different dipeptides, with either an arginine (R) or phenylalanine (F) as N-terminal residue. These dipeptides can bind respectively in the type 1 or type 2 binding site, and can therefore competitively inhibit substrate binding. As a negative control, dipeptides with a N-terminal alanines (A), and the R or F residues on the C-terminus were used.

Interestingly, tBidN interaction with UBR2 was reduced by FA dipeptide, while it was not affected by any of the other peptides (Fig. 6A). The FA peptide inhibited tBidN-UBR2 interaction in a concentration-dependent manner (Fig. 6B). We conclude from these data that tBidN binds in the hydrophobic type 2 binding site of UBR2.

Since we had validated the interaction between tBidN and UBR2, we next examined whether UBR2 could ubiquitinate tBidN. A ladder of α-Bid antibody-reactive species was present when UBR2 was co-expressed and the proteasome was inhibited. This already suggested that UBR2 generated ubiquitin-tBidN conjugates, but it did not formally demonstrate it (Fig. 4A and Fig. 5B). Therefore, tBidN-TAP was expressed in HeLa cells that have low levels of endogenous Bid protein, together with HA-ubiquitin and FLAG-mUBR2. In addition, a FLAG-mUBR2 ΔRING mutant was expressed that had a C-terminal truncation

### Figure 6. tBidN binds to the hydrophobic type 2 binding site of UBR2.

(A) HEK 293T cells were transfected to express tBidN-TAP together with FLAG-mUBR2, followed by treatment of the cells with MG132 (50 μM) for four hours. UBR2 was isolated by α-FLAG IP in the absence or presence of indicated dipeptides (500 μM), and the precipitates were analyzed by immunoblotting with α-FLAG and α-Bid antibodies.

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>α-FLAG</th>
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<tbody>
<tr>
<td>RA AR FA AF</td>
<td>α-FLAG</td>
</tr>
<tr>
<td>ΔtBidN-TAP</td>
<td>α-FLAG</td>
</tr>
</tbody>
</table>

(B) As in (A), but now with different concentration of the dipeptide FA.

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>FA</th>
<th>AR</th>
<th>RA</th>
<th>AF</th>
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<tr>
<td>Dipeptide conc μM</td>
<td>62.5</td>
<td>125</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>FLAG-mUBR2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>tBidN-TAP</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>ΔtBidN-TAP</td>
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<table>
<thead>
<tr>
<th>TCL</th>
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(Fig. 6B)
including the RING domain and the region C-terminal of the RING domain. To distinguish between the functions of these two domains, a FLAG-mUBR2 ΔC mutant was expressed, in which only the domain C-terminal of the RING was absent. After incubation with MG132, tBidN was isolated by α-Bid IP and total cell lysate and isolate were immunoblotted.

When tBidN was precipitated, an α-HA responsive smear was detected, likely representing poly-ubiquitinated tBidN (Fig. 7). In the presence of FLAG-mUBR2, a ladder of specific ubiquitinated tBidN species increased in intensity (Fig. 7), indicating that UBR2 can ubiquitinate tBidN. This ubiquitin ladder was not detected when the ΔC or ΔRING UBR2 mutants were expressed, indicating that at least the C-terminal domain, and possibly also the RING domain are required for UBR2 to ubiquitinate tBidN (Fig. 7). These and all above data demonstrate that the N-end rule ligase UBR2 interacts with tBidN, and targets tBidN-TAP for ubiquitination.

**DISCUSSION**

The N-terminal fragment (tBidN) of Caspase-cleaved Bid gets ubiquitinated on cysteine and serine/threonine residues, which allows full apoptotic activity of the C-terminal fragment (tBidC). This unconventional ubiquitination by esterification is a broad phenomenon in mammalian cells that occurs on more substrates besides tBidN. It is therefore essential to identify the E2 conjugase, E3 ligase and possible DUB that mediate the conjugation or hydrolysis of these unconventional ubiquitin bonds. Here, we aimed to identify the enzymes regulating tBidN ubiquitination, using several approaches including an RNAi-based screen and MS-based analysis of the tBidN interactome.

The RNAi-based screen revealed a hitlist containing 24 conjugases/ligases potentially ubiquitinating tBidN. Importantly, the screen has only been performed once, so it should be...
repeated to allow proper interpretation of the hits. During a preliminary hit analysis however, we noted several different components that can interact in a cullin ring ligase (CRL) complex: SKP1A, RBX1, FBXW2 and FBXL8. CRL complexes get activated by modification with the ubiquitin-like molecule NEDD8, and interestingly the NEDD8-conjugating enzyme UBE2M was also among the hits. It is therefore tempting to speculate that a CRL-complex can be involved in tBidN ubiquitination, but this awaits further validation.

No DUB hits were identified in the RNAi screen. However, the DUB USP9X was among the putative tBidN interactors, as defined by MS-based interactome analysis. Interestingly, USP9X can de-ubiquitinate Pex5p, a substrate of unconventional ubiquitination involved in protein shuttling from the cytosol to the peroxisome. The active site of USP9X is apparently quite flexible towards different bonds, because it could hydrolyze both thio-ester and isopeptide ubiquitin-Pex5p bonds. It would be interesting to further study whether USP9X can de-ubiquitinate tBidN.

The N-end rule ligase UBR2 was a dominant component of the tBidN isolate. Subsequent co-IP studies validated that UBR2 interacts with tBidN. UBR2 predominantly interacted with ΔtBidN, a 1-2 kDa lighter variant of tBidN itself that was very efficiently targeted for proteasomal degradation. It is most likely that ΔtBidN is an N-terminally truncated variant of tBidN, as MS-analysis demonstrated that it is complete from at least residue 20 until the C-terminal residue. When the hydrophobic residues in the region L19-F22 were mutated to alanines, tBidN levels increased while ΔtBidN disappeared. This suggests that ΔtBidN is generated from tBidN, most likely by proteolytic processing, and the region L19-F22 is important for this processing to occur. What the new N-terminal residue of ΔtBidN is, and what protease is responsible for the cleavage event that generates ΔtBidN remains to be investigated.

The UBR family of ligases can recognize specific residues on the N-terminus of proteins. We hypothesized that the N-terminal residue of ΔtBidN, estimated to be one of the residues in the region L11 till F22, was an N-degron, but none of the obvious potential N-degrons in this region were involved in interaction with UBR2. We did not formally exclude the potential N-degron N18 as the UBR2 binding site. However, interaction with UBR2 was inhibited by FA dipeptides, suggesting that tBidN binds to the hydrophobic type 2 binding site in the N-domain of UBR2. Because asparagines would be processed to bind to the type 1 binding site in UBR2, it is unlikely that N18 is involved in tBidN-UBR2 interaction.

So either the N-degron of ΔtBidN is outside of the L11-F22 region, or tBidN binds to UBR2 via an internal (non N-terminal) recognition site. The yeast N-end rule ligase UBR1 recognizes several substrates through internal degrons. The internal degron of the substrate Cup9 binds to a third substrate-binding site in yUBR1, other than the UBR box and N-domain. This third binding site is normally shielded by the C-terminal domain of yUBR1, but becomes exposed when the UBR box and N-domain are occupied by type 1 and type 2 substrates. Recognition of another internal degron, of the substrate Mgt1, probably involves another binding site in yUBR1, because in this case both type 1 and type 2 dipeptide substrates inhibited Mgt1-yUBR1 interaction.

UBR2 interaction with tBidN seems to involve the N-domain of UBR2. No structural data for substrate binding to the N-domain have been obtained, but it is predicted to occur
in a manner similar to how the bacterial protein ClpS recognizes its substrate.\textsuperscript{44} ClpS is a functional homologue of the mammalian N-end rule ligases that recognizes N-terminal bulky, hydrophobic residues. Binding of the N-terminus in the hydrophobic pocket of ClpS involves recognition of the α-amino-group of the substrate.\textsuperscript{45} It is therefore unclear how tBid\textsubscript{N} would bind to the N-domain via an internal degron.

Our data indicated that UBR2 targets tBid\textsubscript{N}-TAP for ubiquitination. In yeast, the N-end rule pathway cooperates with a ubiquitin chain elongating ligase Ufd4.\textsuperscript{40} Interestingly, the mammalian chain elongating ligase \textit{UBE4B} was among the hits derived form the RNAi screen, so possibly these pathways also cooperate in tBid\textsubscript{N} ubiquitination. UBR2 required the region C-terminal of its RING domain to ubiquitinate tBid\textsubscript{N}. This is surprising, since this region is known as the auto-inhibitory domain, because it inhibits yUBR1 binding to Cup9.\textsuperscript{43} In the same article however, the authors mention that expression of a C-terminal truncation mutant of yUBR1 could not rescue the N-end rule pathway in a ΔUBR yeast strain.\textsuperscript{43} Together, these and our data suggest that for several substrates, the C-terminus of the UBR ligases may inhibit substrate recognition and ubiquitination, but in general it is required for UBR function.

The N-end rule pathway regulates apoptosis signaling on multiple levels. It was demonstrated to inhibit apoptosis by targeting the C-terminal fragments of peptidase-cleaved pro-apoptotic proteins for degradation.\textsuperscript{46} Among these pro-apoptotic substrates was Bid, that after calpain-mediated cleavage exposes an arginine N-degron on its C-terminus.\textsuperscript{46-47} In contrast, the N-end rule activates apoptosis signaling in \textit{Drosophila}, by targeting the Caspase-cleaved fragment of the inhibitor of apoptosis protein DIAP1 for degradation.\textsuperscript{48} In the case of tBid\textsubscript{N}, UBR2-mediated degradation releases the pro-apoptotic activity of tBid\textsubscript{C}. The outcome of N-end rule-mediated protein degradation in apoptosis will therefore depend on the exact stimulus and cell-type.

Importantly, the TAP-tag has several lysine residues, of which at least one can be targeted for ubiquitination (B. vd Kooij and J. Borst, unpublished results). This experiment should be repeated therefore with a tBid\textsubscript{N} variant with a lysine-less tag to confirm whether UBR2 can ubiquitinate tBid\textsubscript{N} on unconventional residues. Until now, N-end rule ubiquitination has been defined to require an N-degron, and a surface exposed lysine residue that can be targeted for ubiquitination.\textsuperscript{35} If UBR ligases can mediate ubiquitination by esterification, the subset of potential N-end-rule substrates is broader than anticipated.

One other mammalian E3 ligase has been described to mediate unconventional ubiquitin conjugation. The ER-resident ligase Hrd1 ubiquitinates the Immunoglobulin κ light chain and the T-Cell Receptor α-chain on serine/threonine residues.\textsuperscript{20-21} Hrd1 is involved in ER-associated degradation: it ubiquitinates misfolded ER proteins, and therefore targets them for proteasomal degradation in the cytosol.\textsuperscript{49} The examples of Hrd1, and possibly UBR2, demonstrate that the same ligase can catalyze conventional and unconventional ubiquitination. Both ligases seem to target a rather large and diverse subset of substrates. Possibly, to broaden their substrate repertoire, evolution shaped them in such a way that their activity is not restricted towards lysine residues.
MATERIALS AND METHODS

Constructs
The constructs encoding FLAG-ubiquitin, HA-ubiquitin, HA-tBidN, tBidN-GFP and tBidN-TAP in mammalian expression vectors were previously described. All tBidN-GFP and tBidN-TAP mutants were generated by PCR-mediated mutagenesis. The Itch targeting shRNAs (ITCH 2: AAACATTAAAGTCAAACAATATG, ITCH 18: AAGGAGC AACATCTGGATTATA) in pRetroSuper were a kind gift from M. Hijmans, The Netherlands Cancer Institute, Amsterdam, The Netherlands. pCMV(3xFLAG)MARCH-5 wild-type and ring mutant were a kind gift from dr. Hirose, Tokyo Institute of Technology, Yokohama, Japan and previously described in ref. 29. For the MARCH-5 shRNA 3 (GGAGAGAGCTGATCCTTTA) oligo’s were annealed and ligated in pRSC. For pBabe(PURO)-tRFP-Bcl-B tRFP and Bcl-B cDNA (ImaGenes) were PCR amplified, fused by PCR and ligated in pBabe. For tBFP-Noxa BFP and Noxa were PCR amplified and ligated into LZRS-blasticidin. pcDNA3-FLAG-mUBR2 was a kind gift from dr. Kwon, University of Pittsburgh, Pittsburgh, PE, US, and previously described in ref. 36. The ΔRING and ΔC mUBR2 mutants were generated by PCR mediated mutagenesis.

Cell culture, reagents and antibodies.
The cell lines HeLa, HEK 293T, MCF-7, U2OS, and the retroviral packaging cell line Phoenix-Ampho were cultured in DMEM supplemented with 8% FCS and antibiotics. Transfection was performed either with FuGENE 6 according to manufacturer’s instruction, or with polyethylenemine (PEI). Cells were harvested for analysis 24h after transfection of cDNAs, or 48h-72h after transfection of shRNAs. Retroviral transduction was done as described 50. Three days after transduction cells were treated with selection reagents puromycin (10 μg/ml), blasticidin (Sigma; 10 μg/ml) and zeocin (Invitrogen; 200 μg/ml). MG132 (Calbiochem) and cycloheximide (Sigma-Aldrich) were prepared as stock solutions in DMSO. Isoleucine-Zippered TRAIL was previously described 50. Dipeptides were from AnaSpec.

Western blotting and immunoprecipitation
Cells were lysed in 1% NP-40 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM PMSF, Roche protease inhibitor cocktail, 1mM EDTA). Insoluble material was removed by centrifugation at 14.000 rpm for 15 min. Protein content was determined by Bradford protein assay, and antibody and Prot G sepharose beads were incubated with lysates containting equal amounts of protein for 2 hours while rotating at 4°C. Precipitate was washed 3-5 times with NP-40 buffer, and protein was eluted by boiling at 95°C for 5 min in SDS-sample buffer with DTT. IP and total cell lysate were separated by SDS-PAGE on 4-12% NuPage gels according to manufacturer’s instructions (Invitrogen). Protein was transferred to nitrocellulose either by wet blotting at 70V for 90 min., or by semi-dry blotting using the Trans-Blot Turbo system (Bio-Rad). Primary antibodies used were: rabbit anti-GFP polyclonal 51, anti-HA mAb 12CA5 (free or fluorochrome conjugated), anti-FLAG mAb M2 (free or HRP-conjugated; Sigma-Aldrich) and rabbit anti-Bid polyclonal. 52 Secondary antibodies were either fluorescantly labeled Goat-anti-rabbit Ig or Goat-anti-mouse Ig
Fluorescence signals were visualized and quantified on the Odyssey Imaging System (LI-COR), and chemiluminescence signals (Pierce Biotechnology) by the ChemiDoc imaging system (Bio-Rad) or exposure to film (Kodak).

**qRT-PCR**

RNA was isolated using the RNeasy mini kit (Qiagen) according to manufacturer’s protocol. RNA was reverse transcribed to copy-DNA with SuperScript II RT and random hexamers (Both Invitrogen). The quantitative Real-Time PCR was performed using FAST SYBR Green master mix (Applied Biosystems), and read-out by a Lightcycler 480 system (Roche). Primers used were (5’-3’): Itch FWD: ACAACTTGGTTCATGGGTAGC; Itch REV: CCACCTGGAGCTGTTGTT; MARCH-5 FWD: CAGGTGCAGAGGATCTACAAAAT; MARCH-5 REV: CCAAGACGTAAACCCTGGAC; Actin-β FWD: GCGGGAAATCGTGCCGTAC; Actin-β REV: GATGGAATGGTAGCTTCTG; GAPDH FWD: AAGGTGAAGGGTCCAGTCAA; GAPDH REV: AATGAAGGGGTACATGAG.

**Mass Spectrometry**

HeLa cells (~40*10^6) were transfected with tBidN-TAP and HA-ubiquitin, using FuGENE 6 according to manufacturer’s instruction. A variant of tBidN carrying L11A and R35A mutations was used to optimize peptide length for MS analysis after trypsin and chymotrypsin cleavage. Tandem affinity purification IP was performed as described in ref. 13. Precipitate was eluted by boiling (95°C, 5 min.) in SDS sample buffer without reducing agents. About 1/80th of precipitate was analyzed by western blotting, the rest was divided over two lanes, separated by SDS-PAGE, and the gel was stained using SimplyBlue Safe stain (Life Technologies). Whole lanes, separated in 28 bands, were excised and digested either with trypsin only, or with both trypsin and chymotrypsin. Digestion was performed using the Proteineer DP digestion robot (Bruker). Peptides were extracted from the gel, lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitril/formic acid and subsequently analyzed by on-line nanoHPLC MS/MS using an 1100 HPLC system (Agilent Technologies), as previously described.\(^\text{33}\) Peptides were trapped at 10 µL/min on a 15-mm column (100-µm ID; ReproSil-Pur C18-AQ, 3 µm, Dr. Maisch GmbH) and eluted to a 200 mm column (50-µm ID; ReproSil-Pur C18-AQ, 3 µm) at 150 nL/min. All columns were packed in house. The column was developed with a 30-min gradient from 0 to 50% acetonitrile in 0.1% formic acid. The end of the nanoLC column was drawn to a tip (ID ∼5 µm), from which the eluent was sprayed into a 7-tesla LTQ-FT Ultra mass spectrometer (Thermo Electron). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 3,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion-monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. Selected ions were fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post-
analysis process, raw data were first converted to peak lists using Bioworks Browser software v 3.2 (Thermo Electron), then submitted to the Swissprot database using Mascot v. 2.2.04 (www.matrixscience.com) for protein identification. Mascot searches were with 2 ppm and 0.8 Da deviation for precursor and fragment mass, respectively, and trypsin/chymotrypsin as enzyme. Collision-induced dissociation spectra were manually inspected.

High throughput RNAi-based screen

U2OS cells were retrovirally transduced to express tBidN-GFP, tBFP-Noxa and tRFP-Bcl-B, followed by selection on antibiotics. The cell line was reverse transfected using DharmaFECT 1 (Thermo Scientific) in 96 wells plate with the following siRNA smartpool libraries (Thermo Scientific): G-005615 Human Ubiquitin Conjugation Subset 1 Lot 08119; G-005625 Human Ubiquitin Conjugation Subset 2 Lot 08120; G-005635 Human Ubiquitin Conjugation Subset 3 Lot 08121; Deubiquitinating Enzymes Lot 060323. As controls, scrambled siRNA and GFP siRNA transfected cells, and the untransduced U2OS mother cell line were taken along. Three days later, cells were harvested by trypsinization and fluorescence intensity (FI) for each color was measured by flow cytometry on the LSR Fortessa (BD Biosciences) using a plate reader. Data were analyzed using FlowJo 7.6 software (Tree Star Inc.). Background fluorescence was subtracted from sample fluorescence, and Z-score was calculated with the formula: Z-score = (sample FI - average FI in plate)/Standard Deviation of plate.

ACKNOWLEDGEMENTS

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REFERENCES

SUPPLEMENTARY FIGURE

A

| tBidN | MDCEVNNGSSLRDCEITNLNFVGFLQSCDNSTRITPDHELGVLPQWEGYDELQTD |
| ΔtBidN | VFGFLQSCDNSTRITPDHELGVLPQWEGYDELQTD |

B

Fig. S1 The N-end rule pathway targets tBidN for ubiquitination. (A) The amino acid sequence of tBidN, and the part of ΔtBidN that was detected by mass spectrometry. (B) Schematic representation of the N-terminal residues that can be recognized by the N-end rule ligases of the UBR family. Figure is adjusted from Tasaki et al., Annu Rev Biochem 2012. 81; p. 261-89.