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Nrdp1-mediated degradation of the gigantic IAP, BRUCE, is a novel pathway for triggering apoptosis

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Degradation of certain inhibitor of apoptosis proteins (IAPs) appears to be critical in the initiation of apoptosis, but the factors that regulate their degradation in mammalian cells are unknown. Nrdp1/FLRF is a RING finger-containing ubiquitin ligase that catalyzes degradation of the EGF receptor family member, ErbB3. We show here that Nrdp1 associates with BRUCE/apollon, a 530 kDa membrane-associated IAP, which contains a ubiquitin-carrier protein (E2) domain. In the presence of an exogenous E2, UbcH5c, purified Nrdp1 catalyzes BRUCE ubiquitination. *In vivo*, overexpression of Nrdp1 promotes ubiquitination and proteasomal degradation of BRUCE. In many cell types, apoptotic stimuli induce proteasomal degradation of BRUCE (but not of XIAP or c-IAP1), and decreasing Nrdp1 levels by RNA interference reduces this loss of BRUCE. Furthermore, decreasing BRUCE content by RNA interference or overexpression of Nrdp1 promotes apoptosis. Thus, BRUCE normally inhibits apoptosis, and Nrdp1 can be important in the initiation of apoptosis by catalyzing ubiquitination and degradation of BRUCE.

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Introduction

The primary mediators of apoptosis (or programmed cell death) are caspases, cytosolic cysteine proteases that, once activated, initiate an irreversible cascade of events resulting in rapid cell death (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). The only known cellular caspase inhibitors are the inhibitor of apoptosis proteins (IAPs), which are characterized by the presence of one to three tandem baculoviral IAP repeats (BIRs). Overexpression of almost all the known IAPs suppresses apoptosis. Several human IAPs (e.g., XIAP, c-IAP1, c-IAP2, and NAIP) and *Drosophila* IAP1 directly bind and inhibit caspases (Kaiser *et al*, 1998; Hawkins *et al*, 1999; Salvesen and Duckett, 2002; Wilson *et al*, 2002; Shiozaki *et al*, 2003).

In addition to BIR domain, certain IAPs contain a domain that is implicated in ubiquitination. Ubiquitination is the primary mechanism for targeting proteins for rapid degradation by the 26S proteasome (Coux *et al*, 1996; Hershko and Ciechanover, 1998). Conjugation of a chain of ubiquitin (Ub) molecules to the substrate involves the sequential actions of the Ub-activating enzyme (E1), a Ub-carrier protein (E2), and a Ub-protein ligase (E3). The E3 binds the substrate and catalyzes the transfer of the activated Ub from a specific E2 to the substrate. Several IAPs, including c-IAP1, XIAP, and *Drosophila* IAP1, contain a RING finger domain, a characteristic feature of a large family of E3s (Joazeiro and Weissman, 2000). In certain cells, these IAPs catalyze ubiquitination and degradation of themselves and other proteins important for apoptosis, such as caspase 3 and TRAF2 (Yang *et al*, 2000; Suzuki *et al*, 2001b; Li *et al*, 2002; Palaga and Osborne, 2002). In addition, certain IAPs can promote ubiquitination of the RHG motif-containing proteins (e.g., Rpr in *Drosophila* or Smac/DIABLO in mammals), which interact with and inhibit several IAPs (Du *et al*, 2000; Salvesen and Duckett, 2002; Hu and Yang, 2003; Olson *et al*, 2003).

BRUCE/apollon is a huge membrane-associated protein (530 kDa) that bears one BIR domain at its N-terminal region. It is exceptional in containing a C-terminal E2 motif, which can form thioester bonds with Ub (Hauser *et al*, 1998; Chen *et al*, 1999). BRUCE has also been proposed to function as an E3, since some E3s can form thioester bonds with Ub (Hauser *et al*, 1998; Jesenberger and Jentsch, 2002). Interestingly, BRUCE is upregulated in certain brain cancers (gliomas) that are resistant to certain DNA-damaging agents, and antisense oligonucleotides against BRUCE enhance the extent of apoptosis induced by these agents (Chen *et al*, 1999). It remains uncertain whether BRUCE reduces the damage to DNA or inhibits apoptosis. However, overexpression of *Drosophila* BRUCE has been shown to suppress cell death induced by the proapoptotic factors, Rpr and Grim (Vernooy *et al*, 2002).

Nrdp1 (referred to as FLRF in mice) is a Ub ligase that promotes ubiquitination and proteasomal degradation of ErbB3, a member of the epidermal growth factor (EGF) receptor family (Qiu and Goldberg, 2002). Nrdp1 may play a role in the regulation of growth or development, since it is differentially expressed in mouse fetal and adult hematopoietic stem cells and progenitors (Abdullah *et al*, 2001). While the C-terminal half of Nrdp1 associates with ErbB3, its N-terminal half contains a variant of the RING finger domain (C3HC3D) that is essential for its E3 activity (Qiu and Goldberg, 2002). It seems likely that Nrdp1 targets other substrates for degradation, since an ortholog of Nrdp1 is present in *Drosophila*, which lacks ErbB3. In a search for other substrates in mammalian cells, we have found that the C-terminal half of Nrdp1 also binds BRUCE. Here we present

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evidence (1) that Nrdp1 catalyzes ubiquitination and proteasomal degradation of BRUCE and promotes apoptosis, (2) that, in response to apoptotic stimuli, BRUCE is degraded in all cell types tested, while XIAP and c-IAP1 are degraded only in certain cell types, and (3) that decreasing BRUCE content by RNA interference (RNAi) can induce apoptosis. Thus, BRUCE, unlike other mammalian IAPs, is essential for viability in various cell types, and Nrdp1-mediated degradation of BRUCE represents a novel mechanism for apoptosis.

Results

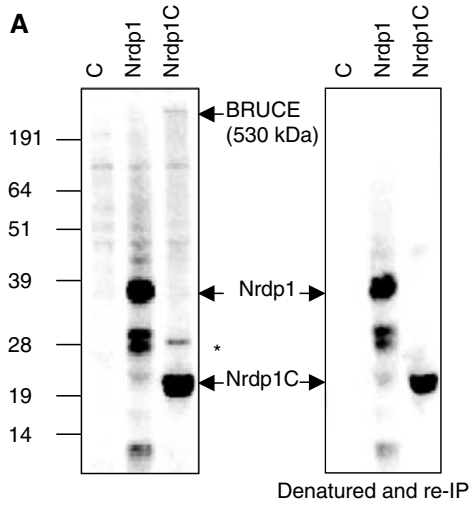
Nrdp1 associates with BRUCE in cells

In order to identify additional substrate(s) of Nrdp1, we first used co-immunoprecipitation and mass spectrometry to isolate and identify proteins that associate with Nrdp1 in 293T cells, which do not express ErbB3 (the only previously known Nrdp1 substrate). The C-terminal half of Nrdp1 (Nrdp1C, amino acids 134–317) was also used to isolate potential substrates because it contains the region that binds ErbB3, but cannot catalyze ubiquitination since it lacks the RING finger domain (Qiu and Goldberg, 2002). Following transfection with either Nrdp1 or Nrdp1C, both of which carried a FLAG tag on their C-termini, 293T cell proteins were metabolically labeled with [^{35}S]methionine. Nrdp1 or Nrdp1C and several other polypeptides were precipitated from the cell lysates by the anti-FLAG antibody (Figure 1A). All the major polypeptides that were precipitated from the lysates of the cells transfected with full-length Nrdp1 seemed to be bound directly to the antibody rather than to Nrdp1, because when the immunoprecipitates were denatured and re-dissolved in SDS sample buffer, these polypeptides could be re-precipitated with the same antibody (Figure 1A, right panel). Thus, our attempts to co-immunoprecipitate substrates with full-length Nrdp1 brought down insufficient amounts for detection, probably due to the rapid degradation of the substrates following ubiquitination by Nrdp1. However, immunoprecipitation of the enzymatically inactive C-terminal half of Nrdp1 brought down significant amounts of an unusually large protein of 530 kDa (Figure 1A). Mass spectrometry identified 25 peptides from this band, which matched sequences from various parts of BRUCE (Figure 1B). Following immunoprecipitation of either the full-length or the C-terminal half of Nrdp1, BRUCE was also detected in the precipitates by Western blotting using an anti-BRUCE antibody (Figure 1C and Supplementary Figure 1-s).

Thus, BRUCE, like ErbB3, associates with the C-terminal half of Nrdp1 *in vivo*.

Nrdp1 promotes proteasomal degradation of BRUCE

This association with the substrate-binding region of Nrdp1 raised the possibility that BRUCE might also be a substrate for Nrdp1. Indeed, transfection of 293T cells with Nrdp1 markedly reduced the levels of BRUCE, but did not affect the levels of two other IAP family members, c-IAP1 and XIAP



B

Sequences of 25 peptides from the 500-kDa band match BRUCE

Position in BRUCE	Sequence
202–213	SIASAIVNELKK
231–248	LSYLLPSARPELGVGPGR
549–561	SPATSPISSNSHR
808–817	IVTLEEEPIK
866–885	TSDISTLGHLVITTQGGYVK
1191–1197	SFLIHVK
1228–1237	GYSLASLLAK
2150–2165	LLDYVATVEDEAAAAK
2273–2283	SNLDTEVTTAK
2526–2542	NGSQTVSVSVSQALDAR
2787–2795	LIHILSTER
2796–2810	GAFQTGQGPLDAQVK
2916–2931	VSVTTNTTDSVSDEEK
3104–3111	FLDSGPNK
3259–3269	DASTLGLSQIK
3557–3569	KQDLSSSLTDDSK
3570–3596	NAQAPLALTESHLATLASSSQSPEAIK
3829–3844	TLHLPVSTTLSDVLDR
3845–3854	VSDTPSITAK
3876–3893	AENGFQDNYSVVVASGLK
4191–4207	SLFSTTPLTTDDGVLLR
4264–4284	GTGFGTGSTASGWDVEQALTK
4432–4455	TGVKPDASDQEPEGLTLLVPDIQK
4456–4468	TAEIVYAATTSLR
4550–4572	LAQEAVTLSTSLPLSSSSSVFVR

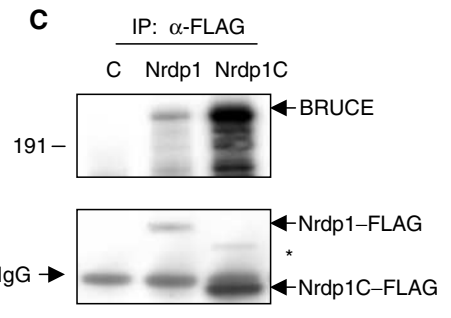


Figure 1 Nrdp1, through its C-terminal half, associates with BRUCE *in vivo*. (**A**) Cellular proteins associated with Nrdp1C. 293T cells were transiently transfected with empty vectors or the vectors encoding Nrdp1-FLAG or Nrdp1C-FLAG. The cells were labeled with [^{35}S] in the presence of 10 μM of MG132 for 3 h. Cell lysates were immunoprecipitated by using anti-FLAG resin and separated on a 4–12% gradient SDS–gel, and proteins on the gel were detected with a PhosphoImager. A re-immunoprecipitation experiment was performed after elution of the initial immunoprecipitate in the SDS sample buffer (right panel). Asterisk denotes a 30-kDa protein co-purified with Nrdp1C. (**B**) Analysis of the 500-kDa band by mass spectrometry. The proteins on the gel were detected by Coomassie staining, and then the 500-kDa band was sliced and subjected to mass spectrometry. (**C**) Detection of BRUCE co-immunoprecipitated with Nrdp1 or Nrdp1C by immunoblotting using anti-BRUCE antibodies. 293T cells were transfected as in (A). Asterisk denotes a nonspecific band.

(Figure 2A). Nrdp1C, presumably by acting as a dominant-negative form of Nrdp1, can markedly elevate the levels of transfected ErbB3 (Qiu and Goldberg, 2002). In contrast, transfection with Nrdp1C did not change the levels of BRUCE, suggesting that BRUCE is relatively stable in normal cells (also see below). Furthermore, when NIH/3T3 cells were transfected with Nrdp1 fused to green fluorescence protein (GFP), BRUCE disappeared from the cells (Figure 2B). Pulse-chase analysis of the 293T cells transfected with Nrdp1 confirmed that Nrdp1 promoted BRUCE degradation (Figure 2C). In the cells transfected with control vectors, BRUCE was quite stable (only about 10% were lost within 4 h). However, in the cells transfected with Nrdp1, BRUCE had a half-life of less than 2 h. In addition, the proteasome inhibitors, MG132 and lactacystin, reduced BRUCE degradation, while the lysosome inhibitor chloroquine had no effect. Thus, Nrdp1 stimulates degradation of BRUCE, apparently by the 26S proteasome.

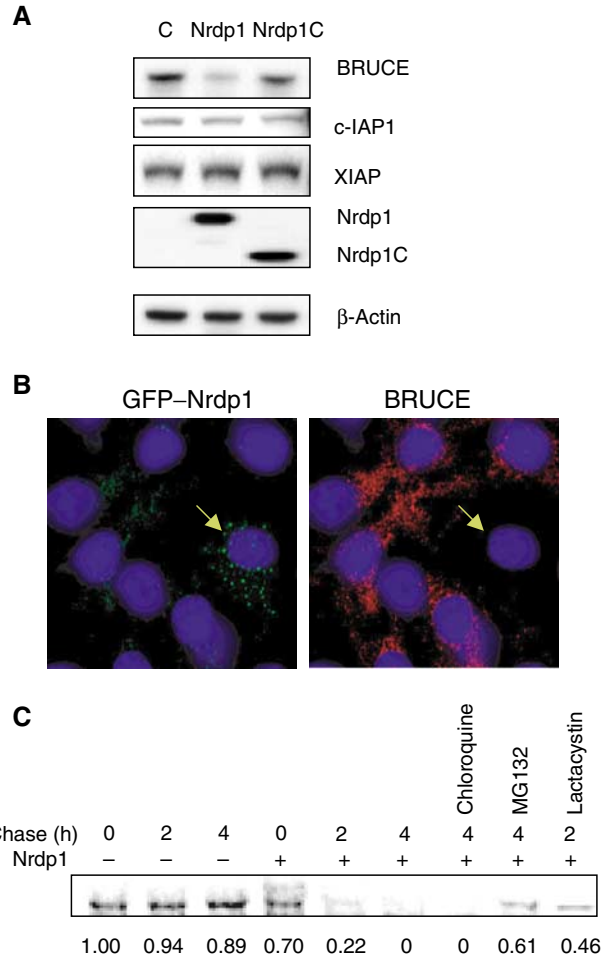


Figure 2 Nrdp1 promotes BRUCE degradation by proteasomes. (**A**) Nrdp1 reduces cellular levels of BRUCE. 293T cells were either untransfected or transfected with FLAG-tagged Nrdp1 or its C-terminal half. BRUCE, c-IAP1, XIAP, actin, and Nrdp1 were detected by Western blotting. A nontagged Nrdp1, which could be detected by an antiserum against Nrdp1 in Western blot and was used to replace the FLAG-tagged Nrdp1 in some experiments, also reduced the levels of BRUCE. (**B**) The levels of BRUCE (red) were reduced in NIH/3T3 cells overexpressing the GFP–Nrdp1 fusion protein, as shown by immunostaining. More than 80% of GFP-positive cells showed almost complete disappearance of BRUCE. A representative cell that expresses GFP–Nrdp1 is indicated by an arrow. Staining with 4′-6-diamidino-2-phenylindole was used to visualize nuclei (blue). (**C**) Nrdp1 promotes BRUCE degradation by proteasomes. 293T cells were transfected with either control vectors or FLAG-tagged Nrdp1, labeled with ^{35}S-Met and ^{35}S-Cys for 12 h, and then chased for the indicated times with or without 10 μM MG132, 20 μM lactacystin, or 100 μM chloroquine. Numbers under the blot indicate relative amounts of BRUCE in the samples. The experiments were repeated at least three times. Each time, empty vectors were included as controls for Nrdp1. In some experiments, we also tried to use the C-terminal half of Nrdp1 as controls, and similar results were obtained.

Nrdp1 catalyzes ubiquitination of BRUCE in vitro and in vivo

Since Nrdp1 is a Ub ligase, we examined whether Nrdp1 can catalyze BRUCE ubiquitination. Immunopurified BRUCE was incubated with crude lysates of 293T cells that had been transfected with wild-type Nrdp1. This E3 promoted the formation of high-molecular-weight Ub conjugates, which were immunoprecipitated with an anti-BRUCE antibody and detected by Western blot with an anti-Ub antibody (Figure 3A). These conjugates could be eluted from the beads with SDS sample buffer and re-immunoprecipitated with the anti-BRUCE antibody. Thus, the Ub is covalently conjugated to BRUCE (Figure 3B). The BRUCE from the reaction containing the wild-type Nrdp1 ran slower on the gel than that from control extracts, presumably because of its ubiquitination (Figure 3A, middle).

When the RING finger mutant (D56V) of Nrdp1, which does not stimulate degradation of ErbB3 (Qiu and Goldberg, 2002), was transfected into cells, and a similar assay was performed in the lysates, BRUCE was not ubiquitinated (Figure 3A). The N-terminal half of Nrdp1 contains this RING finger domain, which is believed to function as a docking site for an E2 (Joazeiro and Weissman, 2000). Even though BRUCE contains an E2 motif, the N-terminal half of Nrdp1 did not associate with BRUCE in immunoprecipitation experiments (data not shown).

To investigate whether BRUCE may function as an E2 in its own ubiquitination, wild-type Nrdp1 was immunopurified from the lysates, eluted from the beads, and incubated directly with immunopurified BRUCE on the beads. No BRUCE ubiquitination was detected, despite the presence of E1, Ub, and ATP (Figure 3C). Although the bacterially expressed E2 motif of BRUCE can form a thioester bond with Ub (Hauser *et al*, 1998), when full-length BRUCE was incubated with ^{125}I-labeled Ub, E1, and ATP, thioester bond formation between BRUCE and Ub was not detected (data not shown). In contrast, UbcH7 or UbcH5c (a member of the UbcH5 family of E2 proteins) was found to form a clear ^{125}I–Ub thioester bond. Since UbcH5c supports Nrdp1-mediated ubiquitination of ErbB3 (Qiu and Goldberg, 2002), ubiquitination of BRUCE was assayed in the presence of UbcH5c. Addition of this E2 markedly stimulated the formation of BRUCE–Ub conjugates (Figure 3C). However, if Nrdp1 was replaced by the immunopurified RING finger mutant (D56V), no Ub conjugation of BRUCE was evident (Figure 3C). Thus, Nrdp1 catalyzes BRUCE ubiquitination, apparently by recruiting UbcH5 through its RING finger domain.

To test whether Nrdp1 also promotes ubiquitination of BRUCE *in vivo*, 293T cells were transfected with Nrdp1. BRUCE–Ub conjugates were detected in the cells transfected with Nrdp1 by immunoprecipitation of BRUCE and Western blot analysis using anti-Ub antibodies (Figure 3D). As we

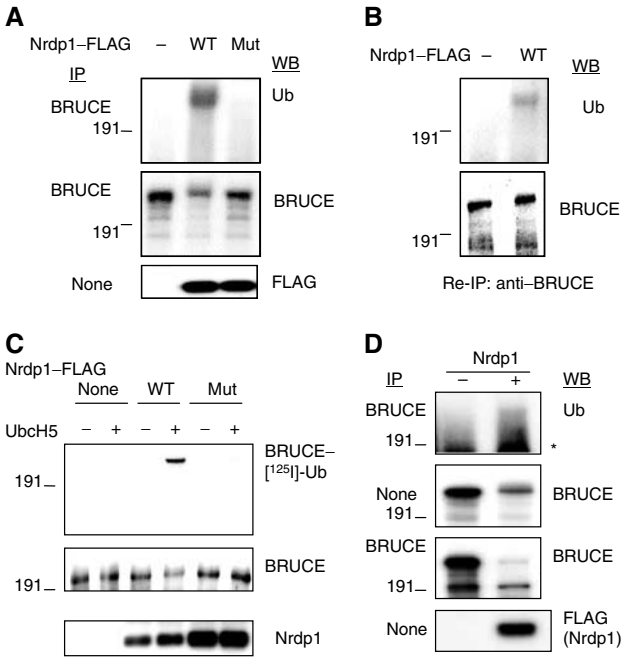


Figure 3 Nrdp1 catalyzes BRUCE ubiquitination both *in vitro* and *in vivo*. (**A**) Nrdp1 in crude cell lysates promotes the formation of Ub conjugates, which were precipitated by anti-BRUCE antibodies. BRUCE on beads immunopurified from the 293T cells was incubated with the lysates of 293T cells transfected with no, wild-type (WT), or D56V mutant (Mut) Nrdp1. Ubiquitination of BRUCE was detected by immunoblotting using anti-Ub antibodies. (**B**) Re-immunoprecipitation of the ubiquitinated BRUCE. Following ubiquitination assay as in (A), the proteins were eluted from the beads with SDS sample buffer and re-immunoprecipitated with anti-BRUCE antibodies. BRUCE ubiquitination was visualized as in (A). (**C**) *In vitro* ubiquitination of BRUCE by Nrdp1 requires an exogenous E2, UbcH5c. BRUCE on beads was incubated in the ubiquitination buffer supplemented with no Nrdp1 or the immunopurified wild-type (WT) or D56V mutant (Mut) Nrdp1, the E1, and ^{125}I-labeled Ub in the presence or absence of UbcH5. After incubation, BRUCE was re-immunoprecipitated as in (B) and analyzed by Western blot or by PhosphoImager. (**D**) Nrdp1 enhances ubiquitination of BRUCE *in vivo*. 293T cells were transfected with empty vectors or vectors encoding Nrdp1-FLAG. BRUCE was immunoprecipitated from the lysates and separated on SDS–PAGE. BRUCE and ubiquitinated BRUCE were detected by Western blot using antibodies against BRUCE or Ub. Asterisk denotes a contaminating band from the antibody preparation.

found after ubiquitination of BRUCE *in vitro* (Figure 3A), a large fraction of the BRUCE immunoprecipitated from the Nrdp1-transfected cells ran slightly slower than that from the untransfected cells (Figure 3D, second panel from top), apparently due to its conjugation to Ub. Thus, Nrdp1 promotes ubiquitination of BRUCE both *in vitro* and *in vivo*, and the E2 motif on BRUCE cannot support this process *in vitro*.

Apoptotic stimuli induce proteasomal degradation of BRUCE in various types of cells

Both c-IAP1 and XIAP in thymocytes are degraded by proteasomes in response to apoptotic stimuli, including glucocorticoids and etoposide (an inhibitor of topoisomerase II), and the resulting decrease in their levels may contribute to apoptotic death (Yang *et al*, 2000). To examine whether BRUCE levels also fall in response to apoptotic stimuli, 293T cells were treated with etoposide (5 μM). The levels of BRUCE decreased markedly by 24 h after treatment (Figure 4A). The highly specific proteasome inhibitor lactacystin greatly reduced the etoposide-induced loss of BRUCE (Figure 4B), suggesting that this decrease in BRUCE content is largely due to degradation by the 26S proteasome.

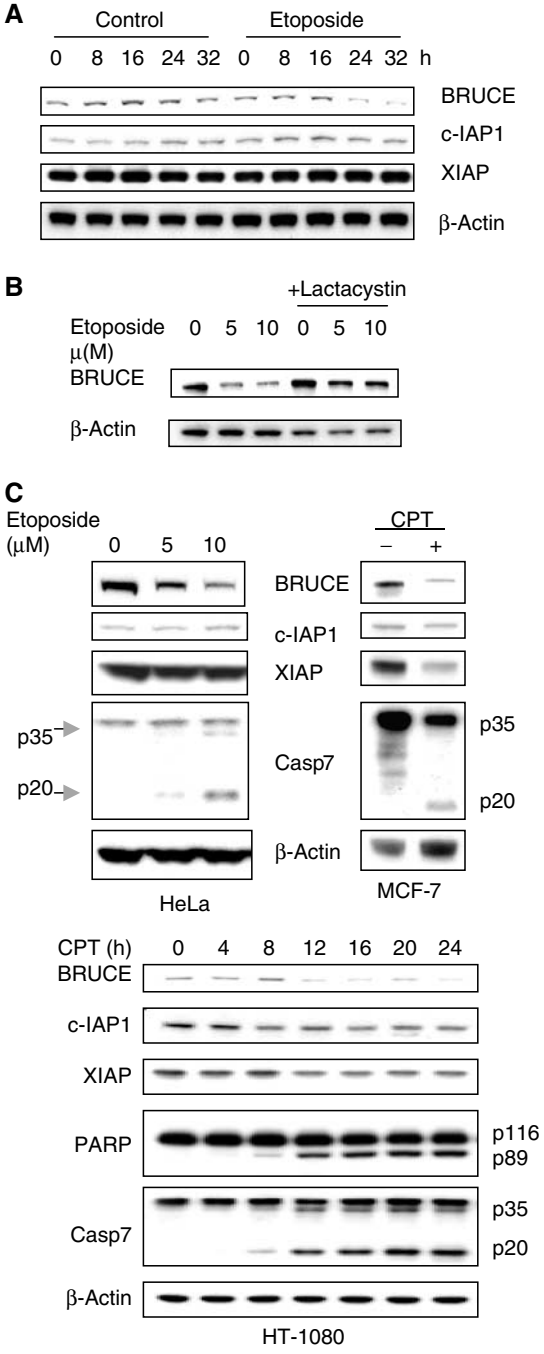


Figure 4 Apoptotic stimuli induce proteasomal degradation of BRUCE. (**A**) Immunoblot analysis of the time course of BRUCE loss in 293T cells following treatment with etoposide (5 μM). (**B**) Lactacystin inhibits etoposide-induced degradation of BRUCE in 293T cells as analyzed by immunoblot. Lactacystin (20 μM) was added at 16 h after addition of etoposide, and was incubated with the cells for an additional 16 h. (**C**) Degradation of BRUCE is associated with apoptosis induced by etoposide or camptothecin (CPT). The cellular levels of IAPs and other apoptosis-related proteins were analyzed by Western blot following treatment either with the indicated concentrations of etoposide (for HeLa cells) or with 5 μg ml^{-1} of camptothecin for 24 h or for indicated times (for MCF-7 and HT1080 cells).

Although etoposide induces degradation of c-IAP1 and XIAP in thymocytes (Yang *et al*, 2000), this agent did not significantly alter their levels in 293T cells (Figure 4A). In HeLa cells, the levels of BRUCE, but not of c-IAP1 or XIAP, also decreased after exposure to etoposide for 24 h (Figure 4C). We then examined whether net degradation of BRUCE is associated with apoptosis in other cell types. In response to etoposide or camptothecin (another topoisomerase inhibitor), the levels of BRUCE also dramatically decreased in NIH/3T3, MCF-7, and HT1080 cells (Figure 4C and Supplementary Figure 2-s). This degradation of BRUCE was associated with apoptosis, since a marked activation of caspase 7 and cleavage of PARP were detected following these treatments (Figure 4C). While the levels of c-IAP1 did not fall in these cell lines, the levels of XIAP dramatically decreased in MCF-7 cells following treatment with camptothecin (Figure 4C). Thus, these apoptotic stimuli induce degradation of BRUCE in all five cell lines tested, while the degradation of other IAPs occurs only in relatively few cell types.

siRNA for Nrdp1 reduces etoposide-induced loss of BRUCE

To investigate whether Nrdp1 may mediate BRUCE degradation induced by apoptotic stimuli, 293T cells transfected with Nrdp1 or an empty vector were incubated with or without etoposide. This agent potentiated the Nrdp1-dependent decrease in BRUCE levels, but did not affect the levels of c-IAP1 (Figure 5A). These observations suggest that Nrdp1 mediates the etoposide-induced loss of BRUCE.

If Nrdp1 plays a key role in BRUCE degradation *in vivo*, decreasing the levels of endogenous Nrdp1 by RNAi should reduce BRUCE ubiquitination and degradation. To suppress the expression of Nrdp1, we utilized the DNA vector (BS/U6)-based RNAi method of Sui *et al* (2002) to construct a DNA vector (BS/U6/Nrdp1) that expresses 21-nt small interference RNAs (siRNAs) for the coding region of Nrdp1. In the 293T cells transfected with FLAG-tagged Nrdp1, cotransfection of BS/U6/Nrdp1 prevented expression of transfected Nrdp1 as shown by Western blot (Supplementary Figure 3-s). Moreover, real-time reverse transcriptase–PCR analysis indicated that siRNA for Nrdp1 reduced the mRNA levels of endogenous Nrdp1 by 40–70% (Figure 5B). In the absence of etoposide, the siRNA for Nrdp1 increased BRUCE levels slightly, presumably because it reduced the slow degradation of this quite stable protein. However, after treatment of the cells with etoposide, the levels of BRUCE decreased markedly (Figures 4A and 5B), and the expression of Nrdp1 siRNAs greatly reduced this etoposide-induced loss of BRUCE (Figure 5B). The levels of BRUCE in the cells expressing GFP siRNA were slightly higher than those for the empty vector, but were dramatically lower than those for Nrdp1 siRNA. In contrast, Nrdp1 siRNAs did not affect the levels of XIAP. Thus, endogenous Nrdp1 mediates specifically the degradation of BRUCE induced by etoposide.

siRNA for BRUCE promotes apoptosis

Since apoptotic stimuli trigger Nrdp1-mediated degradation of BRUCE, we tested the possibility that this loss of BRUCE may by itself cause apoptosis. Expression of siRNAs for BRUCE markedly reduced the levels of this protein in HeLa cells as demonstrated by immunostaining (Figure 6A).

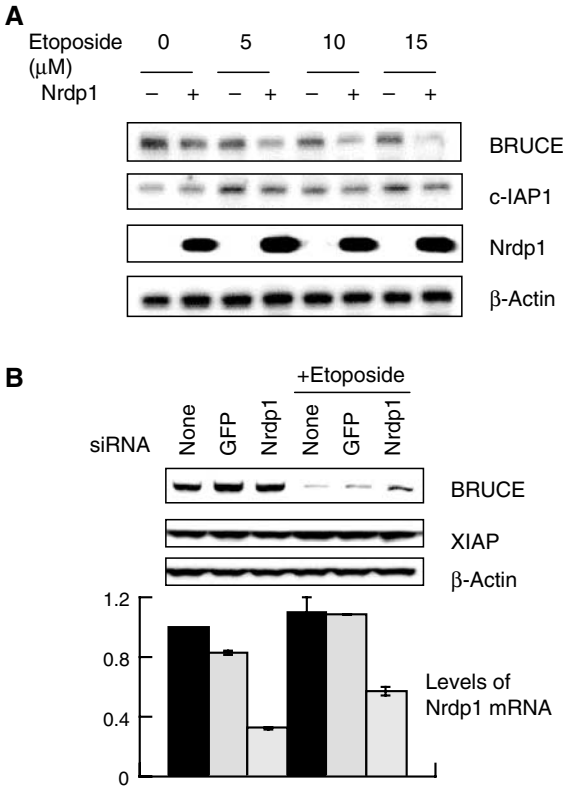


Figure 5 Nrdp1 mediates the etoposide-induced loss of BRUCE. (**A**) Etoposide potentiates the Nrdp1-mediated BRUCE degradation. 293T cells were transfected with empty vectors or vectors encoding Nrdp1, incubated for 24 h, and then treated with the different concentrations of etoposide for 15 h. The levels of BRUCE, Nrdp1, c-IAP1, XIAP, and β-actin were detected by immunoblotting of cell lysates. (**B**) Nrdp1 siRNA reduces the decrease in BRUCE content induced by etoposide. 293T cells were transfected with BS/U6, BS/U6/GFP, or BS/U6/Nrdp1, and were split at 1 to 5 at 3 days after transfection. After 24 h, the cells were treated with or without etoposide (10 μM), and incubated for another 24 h. The levels of BRUCE, XIAP, and β-actin were determined by Western blot. The relative levels of Nrdp1 mRNA (normalized to those of GAPDH) were quantitated by real-time reverse transcriptase–PCR.

Following transfection of HeLa cells with control vectors, 4.53 ± 0.03% of the cells were apoptotic, as shown by condensed chromatin. However, following transfection of BRUCE siRNAs, the fraction of apoptotic cells increased to 16.7 ± 1.9%, even though the transfection efficiency was only about 27% (Figure 6B). Meanwhile, expression of siRNA for BRUCE led to a clear increase in the levels of active caspase 3, especially the 19-kDa band (Figure 6C, left panels). This increase was even larger if the cells were also treated with tumor necrosis factor α (TNFα) (Figure 6C). In 293T cells, which are known to be quite resistant to apoptotic stimuli, the expression of siRNA-BRUCE had no effect on the levels of active caspase 3 (the 17-kDa band) by itself, but caused a marked increase in active caspase 3 in the presence of TNFα (Figure 6D). These results indicate that decreasing the cellular content of BRUCE promotes apoptosis or enhances sensitivity to other apoptotic stimuli.

Activation of caspases follows mitochondrial damage and release of caspase activators, such as cytochrome *c*. This process can be inhibited by the antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-x_L (Merry and Korsmeyer, 1997;

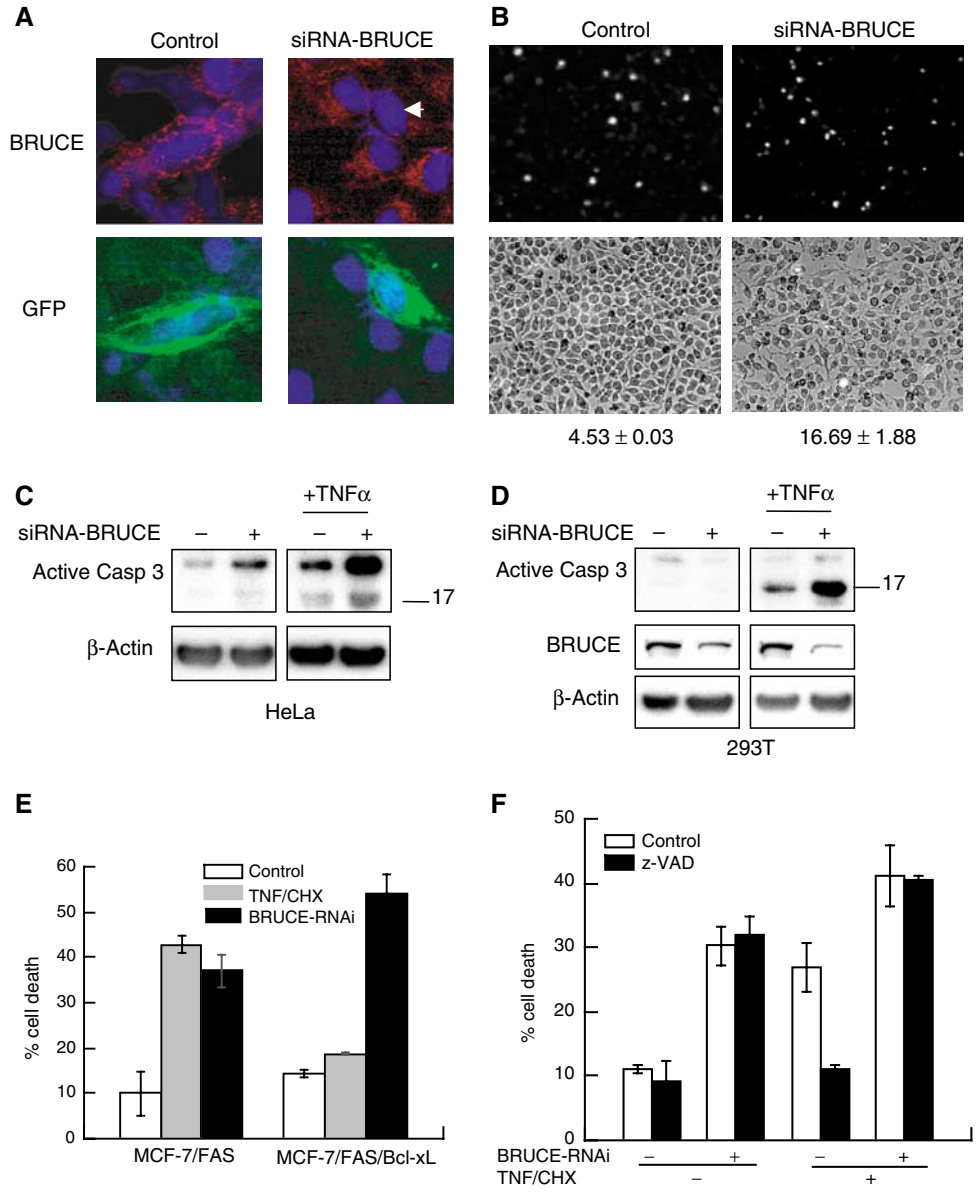


Figure 6 Expression of siRNAs for BRUCE promotes apoptosis or enhances sensitivity to TNFα-induced apoptosis in diverse cell types. (**A**) BRUCE siRNA reduces the cellular levels of BRUCE in HeLa cells. HeLa cells were cotransfected with BS/U6 (control) or BS/U6/BRUCE (siRNA-BRUCE) and the vectors encoding GFP (pEGFP, Clontech), and were split at 1 to 4 at 3 days after transfection. After 16 h, the cells were fixed with methanol and immunostained with mouse anti-BRUCE antibodies/anti-mouse IgG–Cy3 conjugates to visualize BRUCE (red) under a confocal microscope. Visualization of nuclei (blue) was achieved by co-staining the cells with 4′-6-diamidino-2-phenylindole. The siRNA vectors and GFP vectors were cotransfected at a ratio of 10:1 to ensure that the GFP-positive cells also received the siRNA vectors. More than 90% GFP-expressing cells showed reduced levels of BRUCE. A representative GFP-positive cell with reduced levels of BRUCE is indicated by an arrow following transfection with BS/U6/BRUCE. (**B**) Expression of siRNA for BRUCE causes apoptosis in HeLa cells. HeLa cells were transfected and processed as in (A), but were finally stained with 2 μM Hoechst 33342 (without fixing with methanol) to visualize their nuclei. In some experiments, the control BS/U6 was replaced by BS/U6/GFP, but similar results were obtained. Apoptotic cells with condensed chromatin (upper panels) were counted under a microscope using UV lights. All the cells (lower panels) were photographed under visible lights. The levels of apoptosis were expressed as a percentile of the number of apoptotic cells, relative to total cell number. Transfection efficiency with both treatments was about 27%. (**C**) Expression of siRNA-BRUCE promotes caspase 3 activation in HeLa cells. HeLa cells were transfected and split as in (A). At 10 h after splitting, the cells were either untreated or treated with 20 ng ml^{-1} TNFα for 6 h. The levels of β-actin and active caspase 3 were analyzed by immunoblotting. (**D**) Expression of siRNA-BRUCE potentiates caspase 3 activation induced by TNFα in 293T cells. 293T cells were transfected and split as in (A). At 16 h after splitting, the cells were either untreated or treated with 20 ng ml^{-1} TNFα for 6 h. The levels of BRUCE, β-actin, and active caspase 3 were analyzed by immunoblotting. (**E**) The cell death induced by BRUCE RNAi is resistant to overexpression of Bcl-x_L. MCF-7/Fas and MCF-7/Fas/Bcl-x_L cells were transfected and processed as in (A). Percentage of cell death (round) was determined for the transfected cells, which express GFP. To monitor whether the transfected Bcl-x_L functions properly, both cell lines were treated with 20 ng ml^{-1} TNFα in the presence of 1 μM cycloheximide (CHX) for 5 h. (**F**) The cell death induced by BRUCE RNAi is independent of caspase activation in MCF-7/Fas cells. MCF-7/Fas cells were transfected, split, treated with TNFα/CHX, and analyzed as in (E). In addition, the cells were untreated or treated with 20 μM of z-VAD-fmk at 2 days after transfection, and 24 h later, fresh z-VAD-fmk was added following splitting.

Kroemer and Reed, 2000). To examine whether the apoptosis induced by BRUCE-siRNA is resistant to Bcl-2/Bcl-x_L, an MCF-7/Fas cell line that overexpresses Bcl-x_L was employed. Although Bcl-x_L suppressed the cell death induced by TNFα and cycloheximide (an inhibitor of protein synthesis) as shown previously (Li *et al*, 1998), the cell death induced by BRUCE RNAi was not inhibited by Bcl-x_L (Figure 6E). To test whether caspase activation mediates the effect of BRUCE-siRNA, we treated MCF-7/Fas cells with the general inhibitor of caspases, z-VAD, for 2 days. Although z-VAD suppressed TNFα-induced cell death, it had no effect on the cell death induced by BRUCE-siRNA at 4 days after transfection (Figure 6F). In other experiments (not shown), when fresh z-VAD was added daily from the beginning of transfection, there was still no inhibition of the cell death induced by BRUCE-siRNA (although there was some toxicity induced by z-VAD itself). Thus, while BRUCE-siRNA leads to caspase activation, it also appears to promote apoptosis by a mechanism not sensitive to Bcl-2 or inhibition of caspases.

Overexpression of Nrdp1 promotes caspase activation and apoptosis

These findings strongly suggest that degradation of BRUCE by Nrdp1 should promote apoptosis. To test this idea, we first transfected NIH/3T3 cells (which do not express ErbB3) with Nrdp1-FLAG and selected Nrdp1-FLAG-positive cells by incubating them with geneticin for 11 days. The growth of the cells transfected with Nrdp1 was much less than that of cells transfected with an empty vector (Figure 7A). This effect was due to the Ub ligase activity of Nrdp1, since transfection of the Nrdp1 N-terminal half or the inactive RING finger mutant, Nrdp1D56V, did not reduce cell growth. Thus, the ability of Nrdp1 to suppress the growth of NIH/3T3 cells requires both the RING finger domain and the substrate-binding region. In similar experiments, overexpression of Nrdp1 also suppressed the growth of HCT116 human colon carcinoma cells (data not shown).

To determine whether Nrdp1 promotes apoptosis, HeLa cells were transiently transfected with Nrdp1 and stained with Hoechst 33342 to visualize condensed chromatin. While transfection with Nrdp1 by itself had no significant effect on the frequency of cell death within 24 h, it increased by over 100% the frequency of apoptosis induced by TNFα in the presence of cycloheximide (Figure 7B, left panel). In addition, even in 293T cells, which are normally quite resistant to apoptosis induced by TNFα, Nrdp1 transfection also led to a three-fold increase in the low level of cell death upon TNFα treatment (Figure 7B, right panel). Following treatment with TNFα, the 293T cells overexpressing Nrdp1 also showed a marked activation of caspase 3, as demonstrated by enzymatic assays (Figure 7C, upper panel) and immunoblotting of the active fragments (Figure 7C, lower panels). Thus, TNFα appears to enhance sensitivity of the cells to overexpression of Nrdp1. However, the TNFα treatment also increased the levels of transfected Nrdp1 (Figure 7C), perhaps by increasing promoter activity of the plasmid, since the

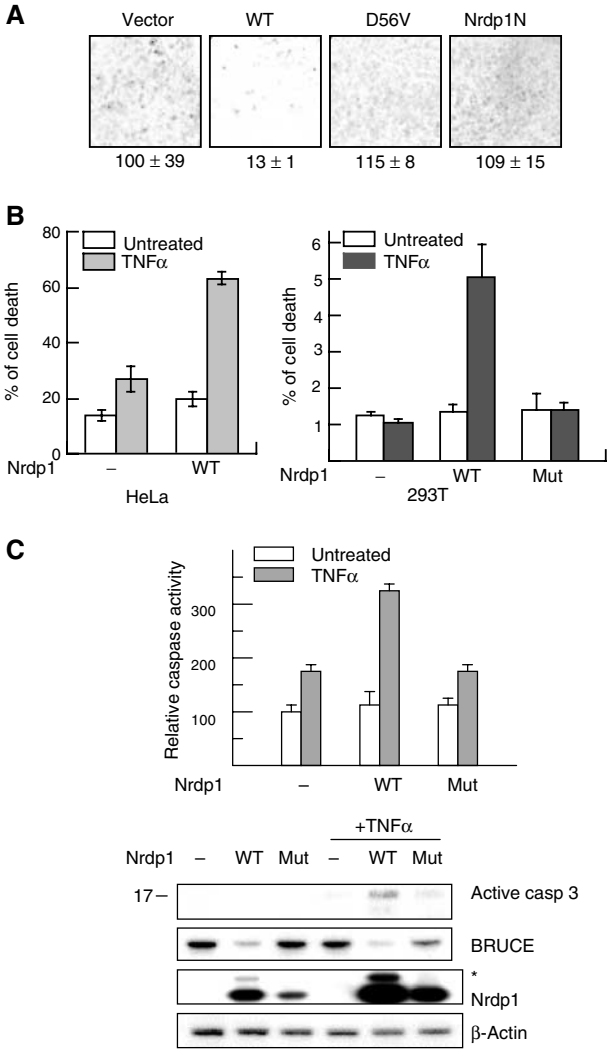


Figure 7 Overexpression of Nrdp1 sensitizes cells to caspase 3 activation and apoptosis. (**A**) Overexpression of Nrdp1 inhibits growth of NIH/3T3 cells. NIH/3T3 cells were transfected with empty pcDNA3.1(+) vector (Invitrogen) or with the vector encoding wild-type (WT), mutant, or truncated Nrdp1. At 3 days after transfection, the cells were subcultured, and the same number of cells for each transfection was seeded. The transfection efficiency was about 15% in all the cases, as monitored in parallel using a GFP-expressing vector. Then, the cells were selected for 8 days by 800 μg ml^{-1} geneticin (Gibco) and were photographed. The percentage of colonies relative to cells transfected with the empty vector (768 ± 295 colonies plate^{-1}) is presented under each photograph. (**B**) Overexpression of Nrdp1 sensitizes cells to apoptosis induced by TNFα. (Left panel) HeLa cells were cotransfected with GFP and either empty vectors or the vectors encoding Nrdp1 for 24 h, and then were treated with 10 ng ml^{-1} TNFα and 1 μM of cycloheximide for 3 h. The cells were finally stained with 2 μM Hoechst 33342 to visualize their nuclei. Apoptotic cells with condensed chromatin in GFP-positive cells were counted under a microscope using UV lights. (Right panel) 293T cells were transfected with either empty vectors or the vectors encoding wild-type or mutant (D56V) Nrdp1 for 16 h, and then were treated with 20 ng ml^{-1} TNFα for 6 h. The cells were finally stained with 2 μM Hoechst 33342 to visualize their nuclei. Apoptotic cells with condensed chromatin were counted under a microscope using UV lights. (**C**) Nrdp1 overexpression promotes caspase 3 activation in response to TNFα. 293T cells were transfected as in (B), and then were treated with 20 ng ml^{-1} TNFα for 6 h. (Upper panel) Caspase activity was assayed by using DEVD-7-amino-4-methylcoumarin as a substrate. The caspase activity in untreated cells (23.9 pmol min^{-1} mg^{-1} protein) was assigned as 100. (Lower panels) Levels of active caspase 3, BRUCE, and Nrdp1 were detected by immunoblotting. β-Actin is a control for loading. The band marked with an asterisk is probably ubiquitinated Nrdp1.

levels of transfected GFP were also elevated by this treatment (data not shown). In any case, overexpression of Nrdp1, like the suppression of BRUCE content by RNA interference or by apoptotic stimuli, promotes apoptosis or enhances sensitivity to apoptotic stimuli.

Discussion

BRUCE inhibits apoptosis

The present studies have demonstrated that the Ub ligase Nrdp1 catalyzes ubiquitination and proteasomal degradation of BRUCE, and that the net loss of BRUCE can play an important role in inducing apoptosis. Antisense oligonucleotides against BRUCE enhance the sensitivity of the SNB-78 glioma cells to apoptosis induced by DNA-damaging agents (Chen *et al*, 1999). In certain cell types (HeLa and MCF-7 cells), BRUCE is essential to prevent apoptosis, since decreasing BRUCE levels by RNAi could by itself induce apoptosis. In other cells (e.g., 293T cells), knockdown of BRUCE enhanced sensitivity to TNFα-induced cell death. In contrast to BRUCE, no other mammalian IAPs have been shown to be essential for viability, even though overexpression of virtually any IAP suppresses apoptosis. For example, mice lacking XIAP showed no sign of increased sensitivity to apoptotic stimuli (Harlin *et al*, 2001).

An important question raised by these findings is how BRUCE inhibits apoptosis. BRUCE appears to act downstream of Bcl-2/Bcl-x_L (Figure 6E), which inhibits the release of caspase activators from the mitochondria (Merry and Korsmeyer, 1997; Kroemer and Reed, 2000). Hence, one possibility is that BRUCE, like other IAPs, may inhibit caspases. On the other hand, IAPs, such as XIAP, have anti-apoptotic activities unrelated to their ability to inhibit caspases (Silke *et al*, 2001, 2002). Since *Drosophila* BRUCE seems unable to block the *Drosophila* caspase (Dronc) activity (Vernooy *et al*, 2002), the mammalian BRUCE may also not inhibit caspases directly. Several IAPs can bind the mammalian RHG motif-containing proteins, such as Smac/DIABLO and HtrA2/Omi, which promote apoptosis (Du *et al*, 2000; Suzuki *et al*, 2001a). Interestingly, *Drosophila* BRUCE suppresses cell death induced by the *Drosophila* RHG proteins, Rpr and Grim (Vernooy *et al*, 2002). In related studies, we have found that BRUCE interacts with both Smac/DIABLO and HtrA2/Omi (Qiu *et al*, in preparation). Thus, one mechanism by which mammalian BRUCE inhibits apoptosis is probably through downregulation of Smac/DIABLO and related proapoptotic proteins.

Nrdp1 promotes ubiquitination and proteasomal degradation of BRUCE in response to apoptotic stimuli

The *Drosophila* IAP, DIAP1, binds and inhibits *Drosophila* caspases, and genetic studies indicate that the cellular content of DIAP1 determines the threshold for apoptosis (Goyal, 2001). Perhaps in an analogous way, the level of BRUCE may also determine the sensitivity of certain mammalian cells to apoptotic stimuli. In fact, reducing BRUCE by overexpression of Nrdp1 also caused caspase 3 activation and promoted apoptosis. Furthermore, accelerated degradation of BRUCE appears to be a key event in apoptosis. During the etoposide-induced apoptosis, the levels of c-IAP1 and XIAP fall markedly in thymocytes (Yang *et al*, 2000), but did not change in HeLa and 293T cells (Figure 4). In MCF-7 cells, the levels of both BRUCE and XIAP, but not of c-IAP1, decreased sharply in response to another apoptotic stimulus, camptothecin. The levels of BRUCE also fell dramatically in HeLa, 293T, HT1080, and NIH/3T3 cells upon treatment with either etoposide or camptothecin. Thus, degradation of BRUCE appears to be a general event during initiation of apoptosis in many cell types, while degradation of other IAPs may or may not occur, depending on the nature of the cell and, perhaps, the apoptotic stimulus. Since the loss of BRUCE could be inhibited by RNAi for Nrdp1 and by lactacystin, these results indicate that Nrdp1 catalyzes proteasomal degradation of BRUCE in response to these apoptotic stimuli.

The present findings also raise the possibility that Nrdp1 or BRUCE may have to be modified to cause ubiquitination of BRUCE. Transient transfection with Nrdp1 itself for up to 3 days did not cause cell death in any cell type tested. However, cell death was triggered following either prolonged incubation (11 days) in the presence of geneticin (Figure 7A) or 1-day incubation in the presence of TNFα (Figure 7B) after transfection with Nrdp1. One possible explanation is that most Nrdp1 may be normally present in an inactive form or that BRUCE may normally exist in an Nrdp1-resistant form, and apoptotic stimuli (such as TNFα) activate BRUCE degradation by causing enzyme or substrate modification (e.g., by phosphorylation). Alternatively, the levels of BRUCE may need to be quite low in order to trigger apoptosis, and the prolonged expression of Nrdp1 or treatment with TNFα may trigger apoptosis, because they lower the levels of BRUCE appreciably. The latter notion is also supported by the results in Figure 6, where siRNA-BRUCE decreased BRUCE levels sufficiently to trigger apoptosis in HeLa and MCF-7 cells, but needed TNFα to do so in 293T cells. Although all the five lines of cells tested showed some cell death in response to the treatments that reduced BRUCE, 293T cells were relatively resistant to apoptosis. For example, while a combination of Nrdp1 transfection and TNFα treatment led to a three-fold increase in the rate of cell death in 293T cells, the total cell death was only about 5% (in contrast to 60% in HeLa cells).

In addition to BRUCE, Nrdp1 might promote ubiquitination and degradation of other protein(s) that may also be involved in the regulation of apoptosis. The present findings were obtained in cells lacking ErbB3. In cells expressing this receptor, the Nrdp1-catalyzed degradation of ErbB3 perhaps also contributes to apoptosis, since EGF, which activates both the EGF receptor and ErbB3, suppresses apoptosis (Zhou *et al*, 2000; Gilmore *et al*, 2002).

Nrdp1 seems to be proapoptotic by promoting degradation of BRUCE and perhaps ErbB3. However, in related experiments, we found that knockdown of Nrdp1 by RNAi enhanced the sensitivity of 293T cells to TNFα, as evidenced by the elevated levels of active caspase 3 in 293T cells transfected with siRNA for Nrdp1 (Supplementary Figure 4-s). The simplest explanation for these findings is that although Nrdp1's effect on BRUCE can induce apoptosis, Nrdp1 can also inhibit apoptosis by promoting the degradation of additional substrate(s), which are proapoptotic. There are several instances where the same E3 seems to have very different, even opposite, physiological effects in degrading different substrates. For example, c-IAP1 can catalyze ubiquitination and degradation of TRAF2, which is antiapoptotic, but c-IAP1 is best known as an inhibitor of caspases and thus should retard apoptosis (Deveraux *et al*, 1998; Li *et al*, 2002). In such

cases, the susceptibility of the different substrates to E3 is regulated by distinct mechanisms, and the effects of knockdown of the E3 must depend on the physiological conditions and the relative roles of the substrates. Understanding the effects of Nrdp1 knockdown must await the identification of the additional Nrdp1 substrates and elucidation of their roles in apoptosis.

BRUCE is a very unusual IAP

Unlike most human IAPs, which are E3s containing a RING finger domain and undergo autoubiquitination (Yang *et al*, 2000; Wilson *et al*, 2002), BRUCE bears an E2 motif in its C-terminal region. Despite this E2 motif, we were unable to obtain any evidence that BRUCE may function as an E2 in its own ubiquitination. The Nrdp1-mediated ubiquitination of BRUCE *in vitro* required an exogenous E2, UbcH5. Also, immunopurified BRUCE could not serve as an E2 for autoubiquitination of Nrdp1 (data not shown). Nevertheless, it remains possible that BRUCE may function as an E2 for ubiquitination of other proteins under other conditions.

Another important feature of BRUCE is its association with membranes including the Golgi apparatus (Hauser *et al*, 1998). Interestingly, Nrdp1 also appears to be membrane associated (Qiu and Goldberg, 2002). Transfected Nrdp1 was localized on the cytoplasmic membrane as well as in the cytosol. Although most membrane proteins are degraded by lysosomes, BRUCE, like ErbB3, appears to be degraded by proteasomes, since MG132 and lactacystin reduced the Nrdp1-mediated degradation of BRUCE (Figure 2C). In contrast, the lysosomal inhibitor chloroquine did not reduce BRUCE degradation (Figure 2C) under conditions where it can inhibit the Cbl-mediated degradation of the EGF receptor, a lysosomal substrate (Levkowitz *et al*, 1998). Since BRUCE is one of the largest proteins known (530 kDa), it is intriguing that proteasomes can digest such a long polypeptide chain.

In certain mammalian cells, apoptotic stimuli induce autoubiquitination and degradation of c-IAP1 and XIAP, but no proteins are known to regulate degradation of mammalian IAPs. In contrast, Rpr, Grim, and Hid in *Drosophila* have been found to reduce the levels of DIAP1 by promoting its ubiquitination and degradation (Palaga and Osborne, 2002). Also, a *Drosophila* E2, UBCD1, and an F box protein (a subunit of some E3s), Morgue, can synergize with Rpr in promoting the degradation of DIAP1 (Hays *et al*, 2002; Ryoo *et al*, 2002; Wing *et al*, 2002). Therefore, the Nrdp1-mediated degradation of BRUCE is the first example where degradation of a mammalian IAP is regulated by another protein and can contribute to apoptosis.

Materials and methods

Cells

293T adenovirus-transformed human embryo kidney cells, HT1080 human fibrosarcoma cells, HeLa human epitheloid carcinoma cells, and HCT116 human colon carcinoma cells were maintained in DMEM (Gibco), supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2, while the DMEM was supplemented with 10% calf serum for NIH/3T3 mouse embryonic cells. All the above cell lines were obtained from the American Type Culture Collection. MCF-7/Fas and MCF-7/Fas/Bcl-xL human breast cancer cells were obtained and cultured as described (Li *et al*, 1998). Transfection was carried out using the SuperFect transfection kit (Qiagen).

Identification of BRUCE by mass spectrometry

A total of 10 100 mm plates of 293T cells were transfected with FLAG-tagged Nrdp1C, and incubated for 2 days. The cells were then treated with 10 μM of MG132 for 3 h and lysed in buffer containing 20 mM Tris–HCl, pH 8.0, 100 mM KCl, 0.2% Nonidet P-40, 10% glycerol, 1 mM $ZnCl_2$, 10 mM β-glycerophosphate, 5 mM tetrasodium pyrophosphate, 1 mM NaF, 1 mM $NaVO_3$, and a protease inhibitor cocktail (Roche). The lysates (about 15 mg of protein) were immunoprecipitated with anti-FLAG resin (Sigma), eluted with 100 mM Gly, pH 3.5, resolved by 4–12% gradient SDS–PAGE, and visualized by Coomassie staining. The band at about 500 kDa was excised for analysis by mass spectrometry (Department of Cell Biology, Harvard Medical School).

Expression vectors

To express GFP–Nrdp1 fusion protein in mammalian cells, the cDNA encoding mouse full-length Nrdp1, obtained from Research Genetics (GenBank accession number AA692083), was amplified by PCR and subcloned into pEGFP-C1 (Clontech) at *Eco*RI/*Bam*HI sites. The construction of BS/U6/Nrdp1 and BS/U6/BRUCE was carried out as described (Sui *et al*, 2002). The parent plasmid BS/U6 for siRNA expression and BS/U6/GFP for GFP RNAi were kindly provided by Drs G Sui and Y Shi. Four DNA oligos, which form an inverted repeat, were synthesized based on the 21-nt sequence in the coding region of the Nrdp1 cDNA (5′-GGGATGATCTCGACTCCT GAT-3′) or the BRUCE cDNA (5′-GGGCCAGTATGGCT-TGCTAGTA-3′) that bears no significant homology with other genes.

Analysis of Nrdp1 mRNA by real-time reverse transcriptase–PCR

Total RNA was isolated from 293T cells using TRIZOL reagent (Invitrogen). cDNA synthesis was carried out from 0.5 μg of total RNA using ProSTAR ultra-HF RT–PCR system (Stratagene). The cDNA from each sample was processed for relative quantification by real-time PCR. The primers for amplifying a 321-bp fragment of Nrdp1 cDNA are: 5′-TGCATTAAGCAC-CTGCGC-3′ and 5′-AGCAG GACAGCCACTCTCC-3′. The primers for amplifying a 450-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA are: 5′-CTCAGACACCATGGGGAAGGT-3′ and 5′-ATGATCTT GAGGCTGTTGTCATA. Templates and primers were mixed with components from the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Applied Science). Reactions in triplicate were carried out in the LightCycler real-time PCR machine (Roche Applied Science) under the following conditions: initial denaturation at 95°C for 10 min, and then 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 25 s. Melting curves were obtained to examine the purity of amplified products. In addition, the products were separated on 1.2% agarose gels in the presence of ethidium bromide to ensure the right sizes of the amplified products. The relative amount of Nrdp1 mRNA in each sample was normalized to that of GAPDH mRNA.

Western blot analysis

Cells were lysed as described above for analysis of all the proteins. BRUCE, c-IAP1, XIAP, PARP, caspase 7, active caspase 3, actin, Ub, and Nrdp1 were detected by Western blot with horseradish peroxidase-conjugated secondary antibodies using standard procedures. Anti-Nrdp1 serum was generated by immunizing rabbits with a C-terminal peptide of Nrdp1 (GDDMVQEPGLVMIFAHGVEE). Other antibodies were purchased from BD Biosciences (IAPs), Sigma (caspase 7, actin, and FLAG), Cell Signaling Inc. (active caspase 3), Santa Cruz (PARP), and Zymed (Ub).

Measurement of protein degradation

293T cells were incubated in serum-free medium for 45 min, metabolically labeled with ^{35}S-Met and ^{35}S-Cys (Amersham), and then chased in nonradioactive medium. Cell extracts were immunoprecipitated with anti-BRUCE antibodies, separated on 4–12% gradient SDS–PAGE, and analyzed with a PhosphoImager (Bio-Rad).

Ubiquitination assay

The reaction was carried out in the ubiquitination buffer containing 40 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.5 mM DTT, and 2 mM ATP. Unless indicated otherwise, this buffer (20 μl in total volume) was supplemented with 20 ng of the purified human E1 expressed in *Escherichia coli* (CalBiochem), 0 or 0.1 μg of the purified UbcH5c,

0.2 μg of [^{125}I]-Ub, Nrdp1-FLAG, and BRUCE. The immunopurified BRUCE on beads was incubated with the reaction mix at 30°C for 90 min, washed twice in the same buffer, resolved by SDS–PAGE, and analyzed by immunoblotting or PhosphoImager.

Spectrofluorometric assay of caspase 3

Caspase 3 activity was assayed similarly as described (Du *et al*, 2000). Aliquots of cell lysates were analyzed in 200 μl of a reaction mixture containing 0.1 mM HEPES (pH 7.4), 2 mM DTT, 0.1% (w/v) CHAPS, 1% (w/v) sucrose, and 20 μM of Z-DEVD-7-amino-4-methylcoumarin at 37°C for 30 min. The release of 7-amino-4-methylcoumarin from the substrates was monitored continuously at wavelength pair 380/460 nm (excitation/emission).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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