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Mule/ARF-BP1, a BH3-Only E3 Ubiquitin Ligase, Catalyzes the Polyubiquitination of Mcl-1 and Regulates Apoptosis

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Summary

The elimination of Mcl-1, an anti-apoptotic Bcl-2 family member, is an early and required step for DNA damage-induced apoptosis. The degradation of Mcl-1 can be blocked by proteasome inhibitors, suggesting a role for the ubiquitin proteasome pathway in apoptosis. Here, we demonstrate that Mcl-1 is ubiquinated at five lysines. Biochemical fractionation of cell extracts allowed us to identify a 482 kDa HECT-domain-containing ubiquitin ligase named Mule (*M*cl-1 *u*biquitin *l*igase *E*3) that is both required and sufficient for the polyubiquitination of Mcl-1. Mule also contains a region similar to the Bcl-2 homology region 3 (BH3) domain that allows Mule to specifically interact with Mcl-1. Elimination of Mule expression by RNA interference stabilizes Mcl-1 protein, resulting in an attenuation of the apoptosis induced by DNA-damage agents. Thus, Mule is a unique BH3-containing E3 ubiquitin ligase apical to Bcl-2 family proteins during DNA damage-induced apoptosis.

Introduction

Apoptosis is a form of cell death orchestrated by chains of biochemical reactions. Cells undergoing apoptosis show characteristic morphological features such as condensation of cytoplasmic and nuclear contents, blebbing of plasma membranes, fragmentation of nuclei, and ultimately breakdown into membrane bound apoptotic bodies that are rapidly phagocytized (Kerr et al., 1972).

Mitochondria play an important role in regulating apoptosis induced by intracellular damaging signals such as DNA damage in mammalian cells (Danial and Korsmeyer, 2004). Apoptotic stimuli exert their effects on mitochondria to cause the release of proapoptotic factors like cytochrome c and Smac/Diablo. These factors either directly activate caspases, a group of intracellular cysteine proteases that execute apoptosis by cleaving their substrates or releasing caspase inhibition imposed by the inhibitor-of-apoptosis proteins (IAPs; Du et al., 2000; Liu et al., 1996; Verhagen et al., 2000).

Mitochondrial response to apoptotic stimuli is regulated by the pro- and antiapoptotic Bcl-2 family of proteins (Gross et al., 1999; Martinou and Green, 2001). Antiapoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1 protect mitochondrial integrity, while the proapoptotic members of the family promote the release of apoptogenic proteins from mitochondria. The fate of a cell under apoptotic stimulation is determined by the balance between the pro- and antiapoptotic members of the Bcl-2 family. Therefore, studying the regulation of such balance will be critically important for our understanding of apoptosis regulation.

Among the antiapoptotic members of the Bcl-2 family proteins, Mcl-1 is unique in that it is an early-response gene that can be rapidly induced and turned over (Kozopas et al., 1993; Yang et al., 1995; Yang et al., 1996). This property enables Mcl-1 to function at an early step in a signaling cascade, consisting of Bcl-2 family proteins, and provides an acute protective function against apoptosis induced by a variety of stimuli, including DNA damage, adenoviral infection, growth factors' withdrawal, and treatment of cytotoxic agents (Cuconati et al., 2003; Derouet et al., 2004; Huang et al., 2000; Le Gouill et al., 2004; Nijhawan et al., 2003; Piret et al., 2004; Zhang et al., 2002; Zhou et al., 1997). Consistently, disappearance of Mcl-1 is associated with the onset of apoptosis and is achieved by the combination of synthesis blockage and continuous degradation (Cuconati et al., 2003; Nijhawan et al., 2003).

The degradation of Mcl-1 in HeLa cells can be blocked by proteasome inhibitors, suggesting a role for the ubiquitin proteasome pathway in apoptosis upstream of Bcl-2 family of proteins (Derouet et al., 2004; Nencioni et al., 2004; Cuconati et al., 2003; Nijhawan et al., 2003). In the current report, we demonstrate that Mcl-1 is polyubiquitinated in vivo and in vitro. Using HeLa cell extracts as a source, we established a cell-free assay for Mcl-1 ubiquitination. Biochemical fractionation of HeLa cell extracts allowed us to identify and purify a 482 kDa E3 ubiquitin ligase that mediated the polyubiquitination of Mcl-1. This protein turned out to be a novel *h*omologous to *E*6-AP *c*arboxyl *t*erminus (HECT)-domain-containing ubiquitin ligase. We named this protein Mule, for *M*cl-1 *u*biquitin *l*igase *E*3.

Results

Ubiquitination of Mcl-1 In Vivo and In Vitro

Proteins targeted for proteasome degradation are usually modified by a polyubiquitin chain (Hershko and Ciechanover, 1998). To test whether Mcl-1 is ubiquitinated, we treated HeLa cells expressing Flag-Mcl-1 with a proteasome inhibitor MG132 to block the proteasome activity and performed immunoprecipitation analysis for Flag-Mcl-1 to test for the accumulation of higher-molecular-weight forms of Mcl-1 that could be ubiquitin modified. As shown in Figure 1A, higher-molecular-weight protein bands recognized by anti-Mcl-1 antibody were indeed accumulated upon MG132 treatment (Figure 1A, lanes 3 and 4). To rule out a possibility that these higher-molecular-weight bands were from Mcl-1-associated protein rather than Mcl-1 itself, the protein mixture was first heated in the presence of 1% SDS and 5 mM DTT to disassociate protein-protein

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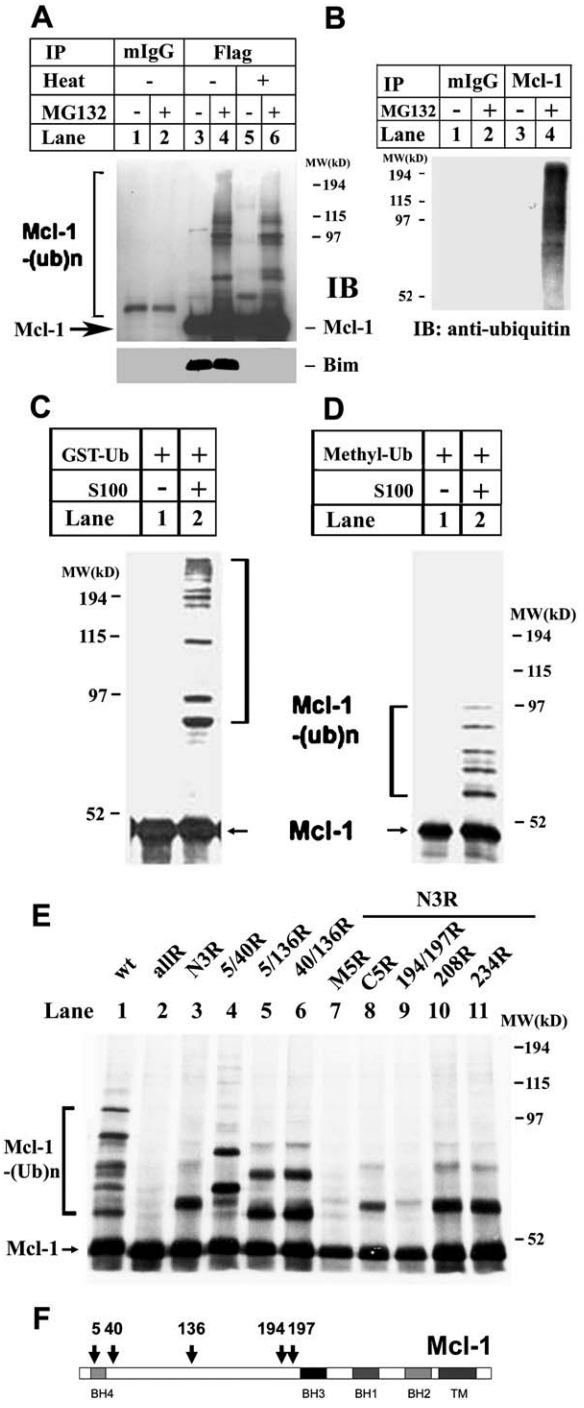

Figure 1. In Vivo and In Vitro Ubiquitination of Mcl-1

(A) HeLa cells were mocked treated (lanes 1, 3, and 5) or treated with 10 μM proteasome inhibitor MG132 (lanes 2, 4, and 6) for 8 hr, then lysed in Flag lysis buffer. The cell extracts were immunoprecipitated by anti-Flag mAb or control mouse IgG as described in the Experimental Procedures. Flag-Mcl-1-immune complex from the M2 beads was eluted by 3× Flag peptide (0.5 mg/ml) at 4°C overnight. Resulting eluants were either boiled in the presence of 1% SDS and 5 mM DTT or without treatment. The boiled eluant was diluted ten times then subjected to second immunoprecipitation with anti-Flag M2 agarose beads. The resulting eluants were subjected to Western blotting with rabbit anti-Mcl-1 or Bim antibodies. Mcl-1(Ub)n denotes ubiquitin conjugates of Mcl-1.

interactions before being subjected to immunoprecipitation. The disassociation was measured by the absence of Bim, a known Mcl-1-associated protein in the immunoprecipitates (Figure 1A, lanes 3–6, lower panel). The heat treatment removed Bim from Mcl-1 immunoprecipitate, but the higher shift of Mcl-1 bands remained (Figure 1A, lanes 5 and 6). This result suggested that polyubiquitination of Mcl-1 might occur. The immunoprecipitates were also probed by an anti-ubiquitin antibody to verify that these higher-molecular-weight bands were indeed caused by polyubiquitin conjugation of Mcl-1. As shown in Figure 1B, the higher-molecular bands that were immunoprecipitated with anti-flag antibody were also recognized by the anti-ubiquitin antibody, indicating that the shift of Mcl-1 protein bands were caused by polyubiquitin conjugation (Figure 1B, lane 4).

We then established an in vitro ubiquitination assay for Mcl-1. The system consists of an ATP regenerating system, GST-ubiquitin or methylated ubiquitin (ubiquitin was reductively methylated to prevent polyubiquitination chain formation as described by Carrano et al. [1999]), ubiquitin aldehyde to block the deubiquitin enzymes, radio-labeled recombinant human Mcl-1 protein generated by in vitro translation, and an S100 fraction from HeLa cells. As shown in Figures 1C and 1D, both GST-ubiquitin and methylated ubiquitin were readily conjugated to the radio-labeled Mcl-1. Furthermore, each component was indispensable for this ubiquitin ligating activity (data not shown).

Mapping Mcl-1 Ubiquitination Sites

This in vitro ubiquitination assay with methylated ubiquitin provided us a tool to map the Mcl-1 ubiquitination

(B) Mock- (lanes 1 and 3) or MG132-treated (lanes 2 and 4) HeLa cells were lysed in Flag lysis buffer, and the cell extracts were immunoprecipitated by anti-Flag mAb or control mIgG. The immune complexes were eluted by 3× Flag peptide (0.5 mg/ml) at 4°C overnight. Resulting eluants were subjected to Western blotting with an anti-ubiquitin antibody (P4D1 from Santa Cruz).

(C) In vitro ubiquitination (Ub) assays were performed with in vitro translated ^{35}S-labeled Mcl-1. ^{35}S-labeled Mcl-1 was incubated without (lane 1) or with (lane 2) 3 μl (20 μg) HeLa cytosolic extracts (S100), as well as 0.8 μl (0.3 μg) of ubiquitin aldehyde, ATP-regenerating system, and 1.5 μl (15 μg) of GST-ubiquitin at 37°C for 1 hr in a final volume of 15 μl adjusted with buffer A. The samples were then subjected to 10% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a Phosphorimaging plate for 16 hr at room temperature.

(D) The same reaction was performed except that 1.5 μl (15 μg) methyl-ubiquitin was used instead of GST-ubiquitin.

(E) In vitro ubiquitination assays were performed with ^{35}S-labeled wild-type Mcl-1 or mutants as described in (D). WT denotes wild-type Mcl-1 protein (lane 1); all R denotes Mcl-1 with all lysines mutated to arginines (R) (lane 2); N3R denotes Mcl-1 with N-terminal three lysines mutated to arginines (R) (lane 3); 5/40R (lane 4), 40/136R (lane 5), and 5/136R (lane 6) denote Mcl-1 with lysines 5/40, 40/136, and 5/136 mutated to arginines (R), respectively. N3R was also mutated in combination with middle five lysines (m5R) (lane 7) or C-terminal five lysines (c5R) (lane 8); or in combination with individual lysine residues 194/197R (lane 9), or 208R (lane 10), or 234R (lane 11), respectively.

(F) Diagram of lysine residues for ubiquitination sites of Mcl-1. BH denotes Bcl-2 homology region, and TM denotes transmembrane domain.

sites. In principle, methylated ubiquitin cannot be polymerized; therefore, each shifted band with the addition of 7 kDa molecular mass is caused by ubiquitination on a single site. If ubiquitin is present, excess amounts of methylated ubiquitin can block polyubiquitin chain formation, resulting in the formation of short polyubiquitin chains. Since Mcl-1 was synthesized in vitro in reticulocyte lysates and an S100 was utilized as a source for ubiquitin ligase activity, polyubiquitin chains derived from ubiquitin present in the reticulocyte lysates or the S100 were terminated by methylated ubiquitin, producing short polyubiquitin chains (mono-, di-, and triubiquitin; Figure 1D).

We used this assay in combination with site-specific mutagenesis to examine which lysine residue(s) is required for Mcl-1 ubiquitination, and the data are shown in Figure 1E. Mcl-1 contains total of 13 lysine residues and could be ubiquitinated to multiple major upshifted bands with an ~7 kDa interval with methylated ubiquitin in the HeLa S100 (Figure 1E, lane 1). None of these ubiquitinated bands was formed when all the lysine residues in Mcl-1 were mutated to arginines (Figure 1E, lane 2).

When the first three lysine residues (5, 40, and 136) were mutated, the majority of Mcl-1 ubiquitination was eliminated, and only one major band remained (Figure 1E, lane 3). Among these three lysines, if lysine 136 was intact, a strong short polyubiquitin chain was formed, indicating that lysine 136 was one major ubiquitin target site (Figure 1E, lane 4). The short polyubiquitin chain ligated to lysine 136 disappeared when lysine 136 was mutated (Figure 1E, lanes 5 and 6), and another short polyubiquitin chain appeared (Figure 1E, lanes 5 and 6). This chain could be ligated to either lysine 5 or 40, since ablation of both sites blocked its formation (Figure 1E, lane 3), but not when either one of them was mutated (Figure 1E, lanes 5 and 6).

To search for additional sites beyond first three lysines, we combined mutations at the first three lysines with five lysine resides in the middle of Mcl-1, and this change completely abolished Mcl-1 ubiquitination (Figure 1E, lane 7). In contrast, combining the aforementioned mutations with mutations in the last five lysine residues showed the same ubiquitination pattern as the first three lysine mutant alone, indicating that they did not contribute to Mcl-1 ubiquitination (Figure 1E, lane 8). Among the five lysine residues in the middle of Mcl-1, mutations at lysine 194 and 197 in combination with the first three lysine mutants abolished ubiquitination, while the mutations at the lysine residues 208 and 234 did not have much effect (Figure 1E, lanes 9–11). Therefore, five lysine residues (5, 40, 136, 194, and 197) in Mcl-1 protein are involved in ubiquitin conjugation (Figure 1F). Consequently, mutations in these five lysine residues lead to prolongation of Mcl-1 half-life (see Figure S1 in the Supplemental Data available with this article online). Among these five lysine residues, lysines 136, 194, and 197 are conserved among human, mouse, and rat (data not shown).

Identification of Mcl-1 Ubiquitination Activity

To isolate the enzyme that carries out Mcl-1 ubiquitination, we fractionated HeLa cell S100 extracts and reconstituted the Mcl-1 ubiquitination reaction using different column fractions. We found that the Mcl-1 generated by in vitro translation gave high background in this assay. Therefore, we switched to the purified bacterially expressed recombinant Flag-tagged Mcl-1 protein as a substrate. This modification dramatically increased the specificity and sensitivity of the assay since the Mcl-1 ubiquitin conjugates now could be detected by anti-Flag antibody without interference from reticulocyte lysates. The recombinant Mcl-1 could be efficiently ubiquitinated by the ubiquitin ligase present in the HeLa cell extracts (Figure 2A, lane 3).

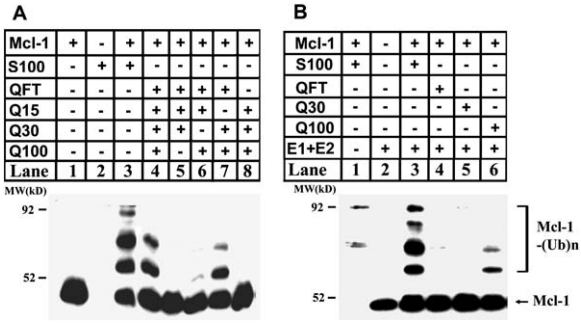

Figure 2. Fractionation and Reconstitution of Mcl-1 Ubiquitin Conjugating Activity

HeLa cell S100 was prepared and fractionated by Q-Sepharose into four fractions (QFT [flow-through], Q15, Q30, and Q100 represent eluant with 150 mM, 300 mM, and 1000 mM NaCl) as described in the Experimental Procedures.

(A) QFT and bound fractions (Q15, Q30, and Q100) were collected and dialyzed against buffer A. Aliquots of all four fractions (2 μl each; lane 4) or without QFT (lane 8), Q30 (lane 6), or Q100 (lane 5) were incubated with an aliquot of methyl-ubiquitin (15 μg in 1.5 μl) in the presence of Flag tagged Mcl-1 (200 ng in 2 μl) at 37°C for 1 hr in a final volume of 15 μl of buffer A. Mcl-1 alone (lane 1) and the reaction mixture incubated with S100 (lane 2) are served as negative and positive controls, respectively. The samples were then subjected to Western blotting with a Flag antibody (M2).

(B) Recombinant E1 (15 ng in 0.25 μl) and E2 (150 ng of Ubch7 in 0.3 μl) were incubated alone (lane 2) or in combination with QFT (lane 4), Q30 (lane 5), and Q100 (lane 6) in the presence of methyl-ubiquitin (15 μg in 1.5 μl) and recombinant Mcl-1 (200 ng in 2 μl) at 37°C for 1 hr in a final volume of 15 μl. Reactions with HeLa S100 alone (lane 1) or with S100 plus all other ubiquitination assay components as in (A) (lane 3) served as negative and positive controls, respectively. The samples were then subjected to Western blotting with an anti-Flag M2 antibody.

Ubiquitin-protein ligation requires the sequential action of three enzymes. The C-terminal Gly residue of ubiquitin is activated in an ATP-requiring step by a ubiquitin-activating enzyme, E1. Activated ubiquitin is next transferred to an active-site Cys residue of a ubiquitin-carrier protein, E2. Finally, the ubiquitin is linked to the substrate by a ubiquitin-protein ligase, or E3 enzyme (Hershko and Ciechanover, 1998). Specificity of ubiquitin-protein ligation and subsequent degradation of target proteins appears to be determined through selective interaction between specific E3s and protein substrates (Varshavsky, 2000).

To study how many factors are involved in Mcl-1 ubiquitination, we first fractionated HeLa S100 through an ion exchange Q column into four fractions: Flow-

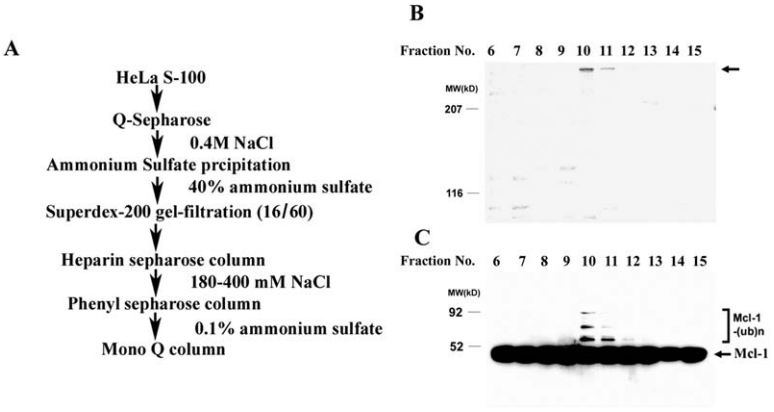

Figure 3. Purification of Mcl-1 Ubiquitin Ligase E3 (Mule)

(A) Diagram of the purification scheme for Mule. See the Experimental Procedures for details.

(B) Aliquots (30 μl) of the indicated fractions from the Mono Q column were subjected to 6% SDS-PAGE followed by Colloidal blue staining using a Colloidal blue staining kit from Invitrogen.

(C) Aliquots of 4 μl of the indicated Mono Q column fractions were incubated with 1.5 μl (15 μg) of Methyl-Ub, 0.25 μl (15 ng) of human E1, 0.3 μl (150 ng) of Ubch7, 0.8 μl (0.3 μg) of ubiquitin aldehyde, and ATP-regenerating system at 37°C for 1hr in a final volume of 15 μl adjusted with buffer A. After 1 hr at 37°C, the samples were subjected to Western blotting using a rabbit anti-Mcl-1 antibody.

through (QFT), proteins bound to the column and eluted at 150 mM NaCl (Q15), 300 mM NaCl (Q30), and 1 M NaCl (Q100), respectively. Three fractions, QFT, Q30, and Q100 were necessary and sufficient to catalyze the Mcl-1 ubiquitination in this reaction (Figure 2A, lanes 4–8). Using a similar ion exchange column, it was reported that majority of E2 activity is present in the QFT, while the E1 resides in the Q30 fraction (Hershko et al., 1983). We therefore substituted Q30 activity with the purified human E1 enzyme and the QFT fraction with several purified recombinant human E2s. We found that the recombinant human E1 could indeed substitute for the Q30 fraction, and one of the E2 enzymes, UbcH7, could substitute the QFT fraction (data not shown). However, E1 and UbcH7 were not sufficient for the Mcl-1 ubiquitin ligase activity (Figure 2B, lane 2). Addition of the Q100 fraction, rather than QFT or Q30, to the reaction containing E1 and UbcH7, restored Mcl-1 ubiquitin ligase activity, indicating that the Q100 fraction contained an E3 enzyme for Mcl-1 ubiquitination (Figure 2B, lanes 4–6). We referred to this Mcl-1 ubiquitin E3 ligase activity present in the Q100 fraction as Mule for *M*cl-1 *u*biquitin *l*igase *E*3.

Biochemical Purification of Mule

Using Mcl-1 ubiquitination as an assay, we purified the Mule activity from the Q100 to apparent homogeneity through a six step procedure (Figure 3A; see the Experimental Procedures for details). The last step of purification, a Mono Q column chromatographic step, is shown in Figures 3B and 3C. Mule activity was eluted from the Mono Q column at roughly 400 mM NaCl (Figure 3C, fractions 10 and 11). The same protein fractions were subjected to SDS-PAGE followed by Colloidal blue staining. A protein band with a molecular mass much larger than the highest molecular weight standard used (207 kDa) correlated with the Mcl-1 ligase activity (Figure 3B).

Molecular Cloning of Mule

To obtain the sequence identity of this Mcl-1 ubiquitin ligase activity, the protein was excised from the gel and subjected to tryptic digestion followed by mass spectral analysis. The mass fingerprinting of the digested peptides was initially mapped to a previously reported protein Ureb1, a 310 amino acid HECT-domain-containing protein (Gu et al., 1994). But it was quite apparent that Ureb1 did not represent Mule in its entity because of the size difference. We subsequently performed several rounds of BLAST searching using the NCBI genebank and TIGR human gene index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=human) in combination with a Genome Scan using the human genomic sequence (http://genes.mit.edu/genomescan.html) to assemble the full-length Mule sequence (see the Experimental Procedures). The finally assembled Mule sequence based on these resources is 4374 amino acid residues long (Figures 4A and S2). Furthermore, the peptides we identified with mass spectrometry technology using the purified endogenous Mule were matched throughout the newly assembled full-length Mule, including the new N terminus (Figure S2).

The 4374 amino acid Mule contains multiple recognizable domains (Figure 4A). The COOH-terminal 350 amino acid residues were well conserved among the HECT ubiquitin ligase domains (Figure 4B). Mule also contains a ubiquitin-associated (UBA) domain and a WWE domain in the middle of the protein (Aravind, 2001; Hofmann and Bucher, 1996). In between the WWE and the HECT domains, there is a well-conserved BH3 domain that is shared by all the Bcl-2 family of proteins (Figure 4C), and none of the sequence matching with BH1, BH2, and BH4 domains was found in Mule. At the N terminus of Mule, there are two unclassified domains: DUF, for domain of unknown function. From the structure analysis, these two domains resemble ARM (Armadillo)-like repeats; therefore we named them as ARLD1 (*A*RM *r*epeat-*l*ike *d*omain *1*) and ARLD2 (Figure 4A). Mule is highly conserved in mammals. The amino acid sequence among human and mouse share more than 90% identity throughout the entire protein, and all the domains are well conserved (Figure S3).

Recombinant Activity of Mule

After cloning of the putative full-length Mule, we generated and purified recombinant Mule protein using a baculovirus-based expression system (Figure 5A). In the presence of E1, Ubch7 (E2), and an ATP-regenerating

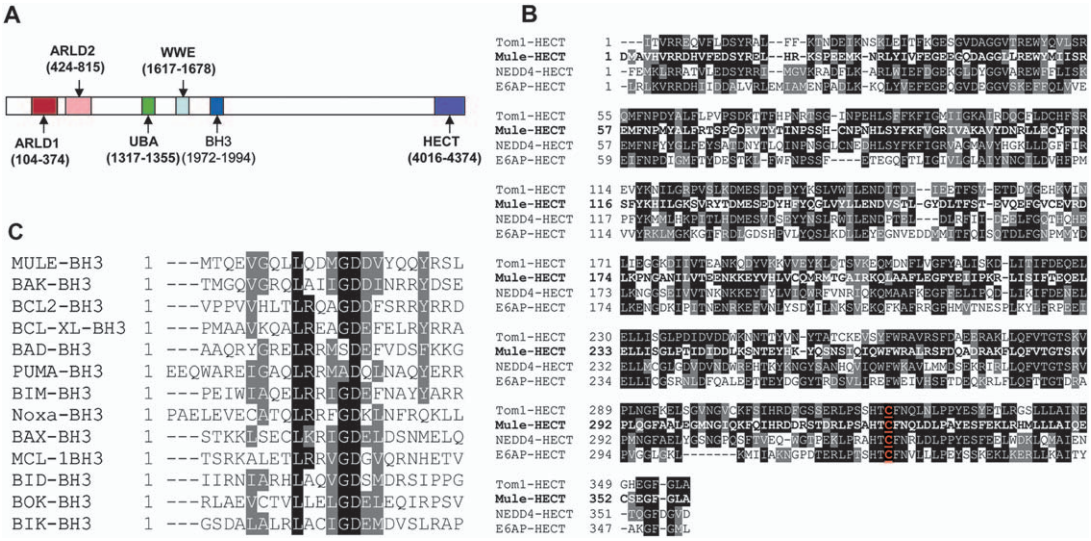

Figure 4. Domain Structure of Mule

(A) A schematic representation of Mule is shown by the horizontal bar with the numbers corresponding to the amino acid residues. The predicted Mule is 4374 amino acids long. The *A*rm *r*epeat-*l*ike *d*omains (ARLD) 1 and 2, the WWE domain, the UBA domain, the BH3 domain, and the HECT domain are indicated.

(B) The amino acid sequence of the HECT domain of Mule was aligned with HECT domains of human E6-AP, budding yeast TOM1, and human NEDD4 by the T-coffee program (Notredame et al., 2000). Identical amino acids and conserved amino acids are marked by black and gray boxes, respectively. The cystine residues in the active site for HECT domains are marked in red.

(C) The amino acid sequence of the BH3 domain of Mule was aligned with the BH3 domains of human Bcl-2 family proteins by the T-coffee program. Identical amino acids and conserved amino acids are marked by black and gray boxes, respectively.

system, the purified recombinant Mule was able to stimulate Mcl-1 ubiquitination with methylated or GST-tagged ubiquitin, but not with a ubiquitin-like protein SUMO (Figure 5B). We used methylated ubiquitin for further biochemical analysis because it gave clearer signal. Mule ubiquitinated Mcl-1 in a dose-dependent manner, and the ubiquitination activity relied on the presence of E1 and E2 (Figure 5C).

To further confirm that the HECT domain was required for E3 activity, the active-site Cys residue that was deduced from conservation among various HECT domain E3s (Figure 4B) was mutated to an Ala by site-specific mutagenesis. The mutant Mule was purified through the same procedure as wild-type Mule. In contrast to wild-type Mule, the point mutant lost E3 activity for Mcl-1 (Figure 5D, lanes 1–3). Furthermore, the Cys mutant Mule showed no dominant-negative effect over wild-type Mule on Mcl-1 ubiquitin ligase activity (Figure 5D, lanes 4–9).

The members of the Bcl-2 family of proteins containing only the BH3 domain use this domain to interact with other family members. If Mule also uses its BH3 domain to interact with Mcl-1, a peptide containing this domain should compete for Mule-Mcl-1 interaction, therefore blocking Mcl-1 ubiquitination in the reconstituted system. To test this hypothesis, we synthesized a 26 amino acid Mule BH3-containing peptide and a mutant peptide in which four hydrophobic residues required for protein-protein interaction were switched with glutamates (4E). As shown in Figure 5E, the Mule BH3 peptide can efficiently block Mcl-1 ubiquitination at 4 μM (Figure 5E, lane 4), while the Mule BH3-4E mutant has no obvious effect even at 20 μM (Figure 5E, lane 9).

To further confirm that Mule can promote Mcl-1 polyubiquitination, we used wild-type ubiquitin to perform an Mcl-1 in vitro ubiquitination assay. Mule stimulates Mcl-1 polyubiquitination in the presence of E1 and E2 (Figure 5F, lanes 3–7 and 9). Mutation of the active cystine site abrogates ubiquitin ligase activity (Figure 5F, lane 8). Addition of BH3 peptide rather that 4E mutant blocked Mcl-1 polyubiquitination (Figure 5F, lanes 10–13).

Characterization of Interaction between Mule and Mcl-1

We generated a monoclonal antibody against the bacterially expressed Mule (amino acids 2219–2396) and performed Western blotting and immunoprecipitation experiments with this antibody. This antibody specifically recognizes the 482 kDa band in human cell extracts (data not shown). The specificity of this antibody was confirmed by either antigen competition or small RNA interference (data not shown and Figure 7). Mule was unstable upon high-temperature treatment displaying significant breakdown products when boiled in SDS-PAGE loading buffer (data not shown).

Having an antibody that recognizes the endogenous protein allowed us to specifically deplete Mule from the HeLa extracts. As shown in Figures 6A and 6B, depletion of Mule with this anti-Mule monoclonal antibody dramatically compromised Mcl-1 ubiquitination in the extracts, indicating that Mule is the major E3 for Mcl-1 in HeLa cell extracts (Figures 6A and 6B, lane 2). In

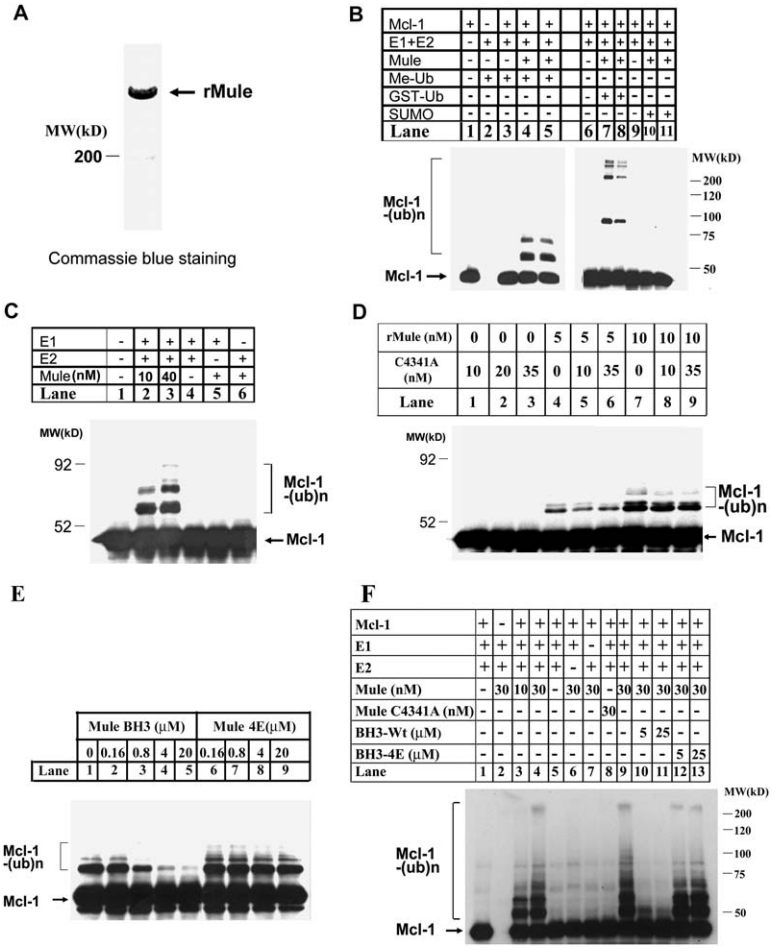

Figure 5. In Vitro Reconstitution of Mcl-1 Ubiquitination Using Recombinant Mule

(A) N-terminal His_{12}-tagged Mule was expressed using a baculovirus expression system and purified as described in the Experimental Procedures. 0.8 μg of recombinant Mule was subjected to 6% SDS-PAGE followed by Colloidal blue staining using a Colloidal blue staining kit from Invitrogen.

(B) Recombinant Mule was incubated with ATP regenerating buffer, E1, E2, recombinant Mcl-1, and ubiquitin in a 10 μl reaction volume at 37°C for 1 hr. The reactions were performed as duplicates in lanes 4 and 5, 7 and 8, and 10 and 11. Methyl-Ub (125 μM) was used in lanes 2–5, GST-Ub (125 μM) in lanes 6–8, and GST-SUMO (125 μM) in lanes 9–11.

(C) Mcl-1 ubiquitination reactions were performed in the presence of methyl-Ub using two different doses of Mule (lane 2, 1×—15 nM and lane 3, 4×—60 nM) as above. As shown in lanes 4–6, the in vitro ubiquitination reactions were performed in the absence of Mule (lane 4), E2 (lane 5), or E1 (lane 6) and analyzed as described in the Experimental Procedures.

(D) Recombinant Mule-C4341A mutant protein was added to the Mcl-1 ubiquitination reaction at different doses (0 nM—lanes 4 and 7; 10 nM—lanes 1, 5, and 8; 20 nM—lane 2; 35 nM—lanes 3, 6, and 9) without wild-type recombinant Mule (rMule, lanes 1–3), or with 5 nM rMule (lanes 4–6), or with 10 nM rMule (lanes 7–9) and analyzed as described in the Experimental Procedures.

(E) A Mule BH3 peptide or a Mule BH3-4E mutant peptide was added to the Mcl-1 ubiquitination reaction at different doses (0 nM—lane 1, 0.16 μM—lanes 2 and 6, 0.8 μM—lanes 3 and 7, 4 μM—lanes 4 and 8, 20 μM—lanes 5 and 9) with wild-type recombinant Mule (rMule, 10 nM).

(F) Mcl-1 ubiquitination reactions were performed in the presence of ubiquitin instead of methyl-Ub to visualize polyubiquitin chain formation. The reaction mixture is subjected to immunoprecipitation by anti-Flag M2 beads followed by Western blotting with rabbit anti-Mcl-1 antibody. Mcl-(Ub)n denotes ubiquitin conjugates of Mcl-1.

contrast, the same depletion experiment performed with another monoclonal antibody against β-tubulin did not deplete Mcl-1 ubiquitin ligase activity (Figures 6A and 6B, lane 1).

We next tested whether Mule interacts with Mcl-1 in vivo. Cell extracts from Flag-Mcl-1-expressing cells were immunoprecipitated with an anti-Flag monoclonal antibody. The immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with antibodies against Flag or Mule. As shown in Figure 6C, the antibody against Flag precipitated Flag-Mcl-1 in Flag-Mcl-1-expressing cells, but not in the control cells (Figure 6C, lower panel, lanes 1 and 2). The Flag antibody also precipitated Mule, and the amount of coprecipitated Mcl-1 and Mule increased upon MG132 treatment (Figure 6C, upper panel, lanes 2 and 3). These results indicate that Mcl-1 interacts with Mule in vivo.

To examine whether the BH3 domain of Mule is sufficient for interaction with Mcl-1, we used a biotin-labeled version of the 26 amino acid Mule BH3 peptide and the 4E mutant to pull down proteins from cell extracts. As shown in Figure 6D, Mule BH3 domain specifically pulled down Mcl-1, but not Bcl-xL, Bcl-2, and Bax in HeLa S100 (Figure 6D, lane 3), while no interaction between Mule BH3-4E mutant with Mcl-1 was detected (Figure 6D, lane 4). In contrast, the BH3 peptide from another proapoptotic BH3-only containing protein Bim was able to pull down all four proteins as reported before (Figure 6D, lane 5; Harada et al., 2004; Chen et al., 2005).

Effects of Mule on Mcl-1 Degradation and Apoptosis

To confirm that Mule was responsible for Mcl-1 degradation in vivo, we eliminated Mule expression by an RNA interference approach. Transient expression of each of four different siRNAs against Mule was able to eliminate the majority of Mule; however, two or three days after transfection, residual Mule still could be detected, likely due to its long half-life or limitations of transfection efficiency (data not shown). Long-lived proteins are always difficult to be manipulated by a transient siRNA strategy. We therefore established a stable cell line that only expressed small hairpin RNA (shRNA) against Mule upon addition of tetracycline. Cell growth was not dramatically affected by tetracycline addition (data not

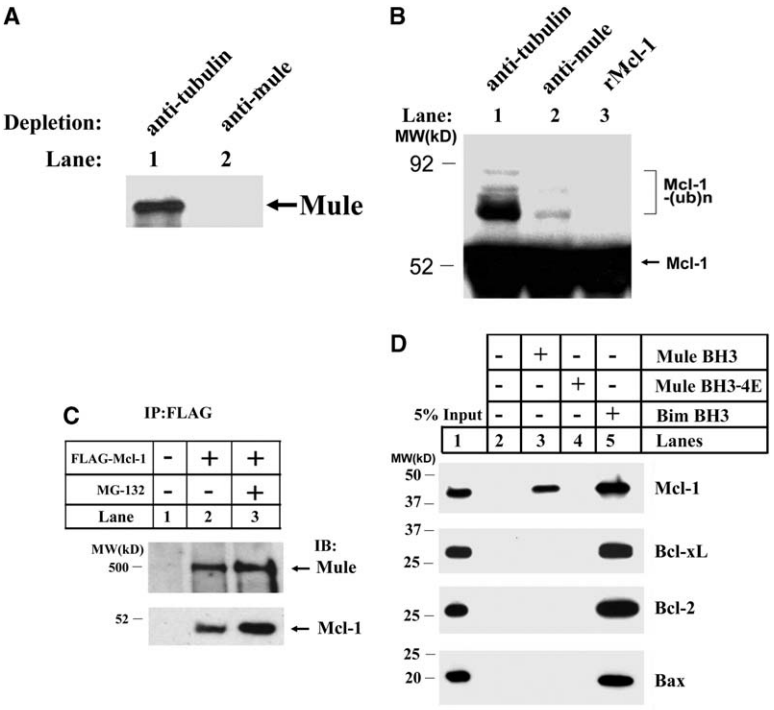

Figure 6. Mule Is the Major E3 for Mcl-1 Ubiquitin Ligase Activity and Interacts with Mcl-1 through the BH3 Domain

(A) Immunodepletions were performed in HeLa S100 with the use of the control anti-β-tubulin monoclonal antibody (lane 1) or anti-Mule monoclonal antibody (lane 2). Mule protein was detected in the supernatants by Western blotting with the use of the corresponding antibody.

(B) Aliquots of 10 μg HeLa S100 immunodepleted with the β-tubulin (lane 1) or anti-Mule monoclonal antibodies (lane 2) were used for Mcl-1 in vitro ubiquitination assay. A probe alone served as a control (lane 3). The reaction mixtures were subjected to Western blotting with anti-Flag M2 antibody.

(C) Interaction of Mule and Mcl-1 in vivo. Control HeLa cells (lane 1) or HeLa cell stable expression Flag-tagged Mcl-1 (lanes 2 and 3) and were treated without (lane 2) or with MG132 (lane 3). Cells were lysed in Flag lysis buffer. Cell extracts were immunoprecipitated by anti-Flag mAb. Aliquots of 20 μl of immunoprecipitates were subjected to Western blotting with anti-Mule or anti-Mcl-1 antibody as marked.

(D) BH3 domain of Mule selectively interacts with Mcl-1. Ten micromolar biotin-labeling Mule BH3, Mule BH3-4E, and Bim BH3 peptides were incubated with HeLa cell extracts at 4°C for 2 hr and then precipitated with streptavidin agarose beads (Novagen) overnight in Flag lysis buffer. Aliquots of 20 μl of immunoprecipitates were subjected to Western blotting with antibody against Mcl-1, Bcl-xL, Bcl-2, and Bax.

shown). This inducible shRNA approach provides superior knockdown efficiency and broadened the time window to evaluate the cellular effect on Mule depletion. To increase knockdown efficiency, we also increased the copy number of shRNA driven by H1 promoter from one to seven (Figure 7A).

Mule expression was eliminated by tetracycline in a time-dependent fashion. After induction of the tetracycline for three days, Mule levels became completely undetectable (Figures 7A and 7B). At the same time, Mcl-1 gradually accumulated, and its level of increase correlated well with diminishing Mule (Figure 7A, lower panel). In contrast to Mcl-1, the levels of the other Bcl-2 family of proteins including Bcl-xL, Bcl-2, Bax, Bak, Bim, Bid, and a control protein actin did not change upon elimination of Mule, a result that was consistent with the specificity of the Mule BH3 domain for Mcl-1 (Figure 7C). Accordingly, half-life of Mcl-1 was prolonged in Mule knockdown cells with or without apoptotic stimuli (Figure S4). We then treated cells with their Mule level knocked down with several DNA-damaging agents including UV irradiation and two commonly used chemotherapeutic drugs etoposide and cisplatin. As shown in Figure 7C, cell death induced by all three agents was reduced when Mule was knocked down. The cell death was correlated well with the caspase-3-like (DEVD) activity (Figure 7D). Among these DNA-damaging agents, cisplatin showed the least ability to induce apoptosis when Mule was knocked down. We therefore performed a detailed biochemical analysis in cells treated with this drug with or without Mule knocked down. In cells in which Mule was there, we started to observe caspase-3 activation as measured by its cleavage into active form 4 hr after cisplatin treatment, and it continued to increase up to 8 hr (Figure 7E, lanes 1–5), while in cells that Mule was knocked down, we did not see any caspase-3 activation even 6 hr after cisplatin treatment. Only after 8 hr did we start to see weak caspase-3 activation (Figure 7E, lanes 6–10). Consistently, the cleavage of endogenous caspase-3 substrate poly(ADP-ribose) polymerase, PARP, showed the same pattern. However, Mule protein level did not change significantly upon cisplatin treatment.

To test whether the delayed apoptosis when Mule was knocked down has a long-lasting effect, cells were switched to fresh medium after 6 hr of cisplatin treatment and continued in culture for 8 days. As shown in Figure 7F, there were few, if any, colonies grown up after cells containing Mule were treated with cisplatin. In contrast, there were more than 9-fold more colonies that appeared after the same treatment after Mule was knocked down.

Discussion

Mule Is the E3 Ubiquitin Ligase for Mcl-1

Although Mule is an E3 ubiquitin ligase that is identified solely based on its in vitro biochemical activity, we believe that it is the enzyme that regulates Mcl-1 half-life in vivo. First, it seems to contribute to the major Mcl-1 ubiquitination activity in HeLa cell extracts since immunodepletion of this protein with a specific monoclonal antibody also depleted most of the activity. Second, four different siRNA oligonucleotides that target different regions of Mule mRNA specifically knocked down Mule protein with a corresponding increase in

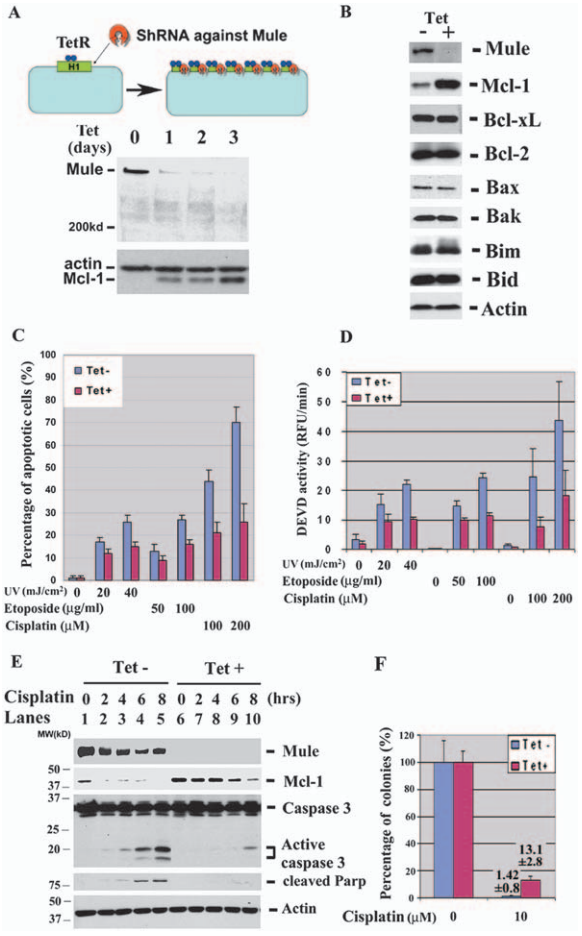

Figure 7. Inducible Expression of Mule shRNA Knocked Down Mule

(A) Scheme of tetracycline-inducible Mule shRNA construct (see the Experimental Procedures for detail). Mule shRNA was induced by adding 2 μg/ml tetracycline to the culture medium of a U_2OS cell line stably transfected with pcDNA6/TR and pSuperior/Mule-shRNA. Treated cells were collected in lysis buffer at indicated time, and cell extracts were subjected to SDS-PAGE and subsequent Western blotting for Mule, Mcl-1, and actin.

(B) The same samples in (A) were subjected to 15% SDS-PAGE and subsequent Western blotting for Mcl-1, Bcl-xL, Bcl-2, Bim, Bax, Bak, and Actin.

(C) U_2OS cells with pcDNA6/TR and pSuperior/Mule-shRNA stably transfected were treated with or without tetracycline for 3 days followed by treatment of UV irradiation at 0, 20, and 40 mJ/cm^2, etoposide at 0, 50, 100 μg/ml for 20 hr, or cisplatin at 0, 100, 200 μM for 20 hr. Cell death (% of total population) was quantified by Trypan blue staining. Values and error bars represent the average and standard derivation of three independent experiments.

(D) U_2OS cells with pcDNA6/TR and pSuperior/Mule-shRNA stably transfected were treated with or without tetracycline for 3 days followed by treatment of UV irradiation at 0, 20, and 40 mJ/cm^2, etoposide at 0, 50, 100 μg/ml for 20 hr, or cisplatin at 0, 100, 200 μM for 20 hr. Cell extracts were collected in lysis buffer and applied to the fluorogenic assay for capase-3 activity as described in the Experimental Procedures. The caspase-3 activity was expressed as RFU (relative fluorescence units)/min/mg protein. Values and error bars represent the average and standard derivation of three independent experiments.

(E) U_2OS cells with pcDNA6/TR and pSuperior/Mule-shRNA stably transfected were treated with or without tetracycline for 3 days followed by treatment of cisplatin at 200 μM for 0, 2, 4, 6, and 8 hr. Cell extracts were collected and subjected to Western blotting for Mule, Mcl-1, caspase-3, PARP, and actin.

Mcl-1 protein levels (data not shown). And finally, an inducible shRNA construct stably transfected into cells confirmed increased Mcl-1 protein levels that perfectly correlated with the induction of shRNA for Mule (Figure 7).

Mule belongs to the HECT-domain-containing group of E3 ligases. The HECT domain was first identified in the enzyme that binds to the papilloma virus E6 protein responsible for degradation of the p53 tumor suppressor protein (Huibregtse et al., 1995). The active site Cys within the HECT domain receives ubiquitin from the corresponding E2 and transfers it to the protein substrate (Scheffner et al., 1995). Mutation of the conserved Cys in the HECT domain of Mule abolishes its Mcl-1 ubiquitination activity (Figure 5D). Mule also contains recognizable UBA and WWE domains. Both domains are commonly found in other E3 enzymes. The UBA domain is known to bind mono- or polyubiquitin, and the WWE domain is a conserved globular domain that is predicted to mediate specific protein-protein interactions in ubiquitin and ADP-ribose conjugation systems (Aravind, 2001; Hofmann and Bucher, 1996; Wilkinson et al., 2001).

But the precise roles of these three domains, as well as the two ARM repeat-like domains (ARLD1 and ARLD2) at the Mule N terminus, remain to be defined by further structure-function studies. In addition to these recognizable domains, the other regions in this enormous protein may serve other unknown functions as well.

Mule Is a New Member of BH3-Only Proapoptotic Proteins

Interestingly, Mule also contains a well-conserved BH3 domain through which the Bcl-2 family members interact with each other (Figure 4C; Kelekar and Thompson, 1998). The BH3 domain of Mule seems to function as its substrate binding surface specific for Mcl-1. Similar mutations in the BH3 domain that abolish the function of other BH3-ohly proteins (4E) also eliminate the ability of Mule BH3-containing peptide to bind Mcl-1 and inhibit Mcl-1 ubiquitination catalyzed by Mule (Figure 5E; Chen et al., 2005). In addition, elimination of Mule attenuates apoptosis upon DNA damage. All this evidence qualifies Mule as a novel member of BH3-only, proapoptotic family proteins.

Among the Bcl-2 family members, the BH3 domain of Mule mostly resembles to the BH3 domain of Bak (Figure 4C). Not surprisingly, Bak has been found to be associated with Mcl-1 (Cuconati et al., 2003). It is conceivable that Mule not only binds to free Mcl-1 through its BH3 domain but also competes with Bak to break up the Mcl-1/Bak complex and promotes Mcl-1 degra-

(F) Long-term survival of Mule knockdown cells upon DNA damage. Two thousand U_2OS cells (in 150 mm plates) with pcDNA6/TR and pSuperior/Mule-shRNA stably transfected were treated with or without tetracycline for 3 days followed by treatment of cisplatin at 10 μM for 6 hr. The number of colonies formed after 8 days was scored and present as relative percentage to colonies formed in untreated cells. The data represent the average number of colonies formed from at least two experiments.

dation in the proteasome. The recognition of Mcl-1 by Mule seems to be specific, as the case of other BH3-only containing proteins (Figure 6D; Chen et al., 2005), and we did not observe any interaction between the BH3 domain of Mule with other anti- or proapoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL, and Bax. The level of these proteins also did not change when Mule was knocked down.

Regulation of Mcl-1 Level by Proteolysis

As an E3 ligase for Mcl-1, Mule is the first such enzyme specifically identified that regulates the Bcl-2 family of proteins. Since Mcl-1 functions at an early step in apoptosis induced by various death stimuli and serves as a major survival factor, the activity that regulates Mcl-1 levels could be critically important in many physiological and pathological situations. The importance of Mcl-1 during development and lymphocytes homeostasis has been demonstrated by gene knockout studies. Deletion of the Mcl-1 gene from the mouse genome leads to peri-implantation embryonic lethality (Rinkenberger et al., 2000). Conditional knockout of Mcl-1 in mouse thymus and spleen results in profound reduction of both B and T cells (Opferman et al., 2003). Mcl-1 is also obligate for the survival of hematopoietic stem cells (Opferman et al., 2005). In mice, Mcl-1 overexpression apparently sets the stage for the development of lymphomas, very much like overexpression of Bcl-2 does (Reed, 1997). At the cellular level, Mcl-1 is a target of a viral oncogene (E1A) and for several survival factors including MCSF, VEGF, stem cell factor and interleukin-5, and HIF (Cuconati et al., 2003; Derouet et al., 2004; Huang et al., 2000; Le Gouill et al., 2004; Nijhawan et al., 2003; Piret et al., 2004; Zhang et al., 2002; Zhou et al., 1997). Overexpression of Mcl-1 has been found in a variety of human malignancies and often correlates with adverse clinical outcome (Zhang et al., 2002). Dysfunction of Mule is a potential reason for accumulation of Mcl-1. The function of Mule in tumor suppression will be addressed after the establishment of a murine knockout model and large-scale screening of human cancer patients for Mule mutations.

As important as Mcl-1 is, there seems to be other activities in addition to Mule that proteolyses Mcl-1. For example, it has been reported that Mcl-1 is a caspase substrate; a property that may contribute to the feed-forward amplification of apoptotic signals once caspases are activated (Clohessy et al., 2004; Herrant et al., 2004; Weng et al., 2005). In addition, we have observed that in several cell lines upon apoptosis induction, Mcl-1 still decreased even in the presence of proteasome or caspase inhibitors or when Mule level was knocked down to an undetectable level (Figure 7 and data not shown). So there might be another DNA-damaging inducible protease that cleaves Mcl-1 and also contributes to the onset of apoptosis in addition to Mule and caspases. Such a protease may contribute to the difference in half-life when cells were treated with cisplatin in the presence and absence of Mule (Figure S4). Nevertheless, the Mule knockdown experiment provides strong evidence that regulation of Mcl-1 by Mule through proteasomal degradation is a major contributor for the prosurvival role of Mcl-1 upon DNA damage, and this notion will be further consolidated and extended as soon as an animal model is established.

Biological Functions of Mule

Mule is the first ubiquitin E3 ligase that has been identified solely based on its biochemical activity to carry out the ubiquitination on Mcl-1. It is therefore possible that there are other Mule substrates in cells. More importantly, if there are other substrates for Mule, whether the degradation of these proteins is coordinated biologically with the degradation of Mcl-1 will be an interesting question for future studies.

With the inducible RNAi knockdown cells at hand and an anticipated animal model with Mule knocked out, we can start to address this interesting question.

Experimental Procedures

Reagents

The following antibodies were used for Western blots: monoclonal anti-Mcl-1 (Pharmingen), polyclonal anti-Mcl-1 (Santa Cruz), polyclonal anti-Bcl-2 (Ancell), polyclonal anti-Bax (Santa Cruz), polyclonal anti-Bak (Santa Cruz), polyclonal anti-Bim (Pharmingen), polyclonal anti-Bcl-xL (Pharmingen), polyclonal anti-caspase-3 (Cell signal), polyclonal anti-actin (Sigma), and anti-ubiquitin P4D1 monoclonal antibody (Santa Cruz). Anti-Mule monoclonal antibody was generated against the bacterially expressed His-tagged Mule (amino acids 2219–2396) from University of Texas Southwestern antibody core facility. Immunoprecipitations for Flag-Mcl-1 were performed with monoclonal antibody to Mcl-1 or Flag (Sigma). MG132, caspase-3 fluorogenic II substrate, phosphatase inhibitor cocktail II, and protease inhibitors were all obtained from Calbiochem. siRNA against Mule (si5635 TGCCGCAATCCAGACATAT, si9931 CATACCGAGAAGCATGCAA, si1301 GAGTGGTTGACCTTAT CAC, si1826 GTGCACTCTGTTTGAATGC, si2206 CAGGCCATGCA GAGCTTTA) were ordered from Dharmacon and University of Texas Southwestern RNA oligosynthesis core facility and used following the manufacturer's protocols. N-terminal biotin labeled Mule BH3 peptide (PGVMTQEVGQLLQDMGDDVYQQYRSL) and Mule BH3-4E peptide (PGVMTQEEGQLEQDEGDDEYQQYRSL) were ordered from New England Peptide. Biotin-labeled Bim BH3 peptide (DMRPEI WIAQELRRIGDEFNAYYARR) is ordered from University of Texas Southwestern peptide synthesis core facility.

In Vitro Ubiquitination Assay

In a 15 μl reaction, 200 ng of recombinant Flag-Mcl-1 was incubated with an ATP regenerating system (50 mM Tris [pH 7.6], 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase), 10 μg of methyl-ubiquitin, 10 ng human E1, 100 ng Ubch7, 2 μM of ubiquitin aldehyde, and 10 μg of S100 or designated fractions at 37°C for one hour. After terminating the reactions with SDS sample buffer, reaction products were fractionated by SDS-PAGE (10%) and analyzed by Western blotting with anti-Flag M2 antibody during purification and anti-Mcl-1 when purified components were used.

Generating Inducible Mule shRNA Cell Line

Mule shRNA sequence si5635 (TGCCGCAATCCAGACATAT) was annealed following the protocol from Oligoengine and cloned into BglII and HindIII sites of pSuperior.puro vector (Oligoengine). The histone H1 promoter and shRNA was released from pSuperior.puro. shRNA construct and subcloned into pBluescript (modified) at sites of EcoRI and HindIII. The H1 promoter and shRNA were released from the pBluescript Mule shRNA constructs at the sites of BamHI and BglII and reinserted into the pBluescript Mule shRNA vector linearized with BglII to generate multiple copies of H1 + shRNA cassette. This cloning strategy was repeated several rounds until seven tandem cassettes were inserted into the same vector. These seven cassettes were then cloned into the BamHI site of the pSuperior.puro vector (modified without H1 promoter).

The Mule shRNA construct was cotransfected with tetracycline repressor expression construct into 5 × 10^5 attached U_2OS cells grown in DMEM with 10% FBS using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's protocol. One day later, the cells were selected against 1 μg/ml blasticidine and 2 μg/ml puromycin. After two weeks, individual clones were lifted and tested for expression of tetracycline repressor. Positive clones were further tested for Mule shRNA expression. Clones were treated with 2 μg/ml tetracycline for 3 days. Cell extracts were collected and subjected to 6% SDS-PAGE and Western blotting with anti-Mule monoclonal antibody. Clones with the best inducible knockdown of Mule were chosen for further experiments.

Supplemental Data

Supplemental Data include four figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/121/7/1085/DC1/.

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References

Aravind, L. (2001). The WWE domain: a common interaction module in protein ubiquitination and ADP ribosylation. Trends Biochem. Sci. *26*, 273–275.

Carrano, A.C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. *1*, 193–199.

Chen, L., Willis, S.N., Wei, A., Smith, B.J., Fletcher, J.I., Hinds, M.G., Colman, P.M., Day, C.L., Adams, J.M., and Huang, D.C. (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol. Cell *17*, 393–403.

Clohessy, J.G., Zhuang, J., and Brady, H.J. (2004). Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells. Br. J. Haematol. *125*, 655–665.

Cuconati, A., Mukherjee, C., Perez, D., and White, E. (2003). DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev. *17*, 2922–2932.

Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: Critical control points. Cell *116*, 205–219.

Derouet, M., Thomas, L., Cross, A., Moots, R.J., and Edwards, S.W. (2004). Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1. J. Biol. Chem. *279*, 26915–26921.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell *102*, 33–42.

Gross, A., McDonnell, J.M., and Korsmeyer, S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev. *13*, 1899–1911.

Gu, J., Ren, K., Dubner, R., and Iadarola, M.J. (1994). Cloning of a DNA binding protein that is a tyrosine kinase substrate and recognizes an upstream initiator-like sequence in the promoter of the preprodynorphin gene. Brain Res. Mol. Brain Res. *24*, 77–88.

Harada, H., Quearry, B., Ruiz-Vela, A., and Korsmeyer, S.J. (2004). Survival factor-induced extracellylar signal-regulated kinase phosphorylate Bim, inhibiting its association with Bax and proapoptotic activity. Proc. Natl. Acad. Sci. USA *101*, 15313–15317.

Herrant, M., Jacquel, A., Marchetti, S., Belhacene, N., Colosetti, P., Luciano, F., and Auberger, P. (2004). Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. Oncogene *23*, 7863–7873.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. *258*, 8206–8214.

Hofmann, K., and Bucher, P. (1996). The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. Trends Biochem. Sci. *21*, 172–173.

Huang, H.M., Huang, C.J., and Yen, J.J. (2000). Mcl-1 is a common target of stem cell factor and interleukin-5 for apoptosis prevention activity via MEK/MAPK and PI-3K/Akt pathways. Blood *96*, 1764–1771.

Huibregtse, J.M., Scheffner, M., Beaudenon, S., and Howley, P.M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. USA *92*, 2563–2567.

Kelekar, A., and Thompson, C.B. (1998). Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol. *8*, 324–330.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239–257.

Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P., and Craig, R.W. (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc. Natl. Acad. Sci. USA *90*, 3516–3520.

Le Gouill, S., Podar, K., Amiot, M., Hideshima, T., Chauhan, D., Ishitsuka, K., Kumar, S., Raje, N., Richardson, P.G., Harousseau, J.L., and Anderson, K.C. (2004). VEGF induces Mcl-1 up-regulation and protects multiple myeloma cells against apoptosis. Blood *104*, 2886–2892.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. Cell *86*, 147–157.

Martinou, J.C., and Green, D.R. (2001). Breaking the mitochondrial barrier. Nat. Rev. Mol. Cell Biol. *2*, 63–67.

Nencioni, A., Hua, F., Dillon, C.P., Yokoo, R., Scheiermann, C., Barbieri, E., Rocco, I., Garuti, A., Wesselborg, S., Belka, C., et al. (2004). Evidence for a protective role of Mcl-1 in proteasome inhibitor-induced apoptosis. Blood *105*, 3255–3262. Published online December 21, 2004. 10.1182/blood-2004-10-3984.

Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., and Wang, X. (2003). Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev. *17*, 1475–1486.

Notredame, C., Higgins, D.G., and Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. *302*, 205–217.

Opferman, J.T., Letai, A., Beard, C., Sorcinelli, M.D., Ong, C.C., and Korsmeyer, S.J. (2003). Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature *426*, 671–676.

Opferman, J.T., Iwasaki, H., Ong, C.C., Suh, H., Mizuno, S., Akashi, K., and Korsmeyer, S.J. (2005). Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. Science *307*, 1101–1104.

Piret, J.P., Minet, E., Cosse, J.P., Ninane, N., Debacq, C., Raes, M., and Michiels, C. (2004). Hypoxia-inducible factor-1-dependent overexpression of myeloid cell factor-1 protects hypoxic cells against tert-butyl hydroperoxide-induced apoptosis. J. Biol. Chem. *280*, 9336–9344. Published online December 17, 2004. 10.1074/jbc.M411858200.

Reed, J.C. (1997). Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin. Hematol. *34*, 9–19.

Rinkenberger, J.L., Horning, S., Klocke, B., Roth, K., and Korsmeyer, S.J. (2000). Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev. *14*, 23–27.

Scheffner, M., Nuber, U., and Huibregtse, J.M. (1995). Protein ubiquitination involving an E1–E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81–83.

Varshavsky, A. (2000). Recent studies of the ubiquitin system and the N-end rule pathway. Harvey Lect. *96*, 93–116.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J., and Vaux, D.L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell *102*, 43–53.

Weng, C., Li, Y., Xu, D., Shi, Y., and Tang, H. (2005). Specific cleavage of MCL-1 by caspase-3 in trail-induced apoptosis in Jurkat leukemia T cells. J. Biol. Chem. *280*, 10491–10500.

Wilkinson, C.R., Seeger, M., Hartmann-Petersen, R., Stone, M., Wallace, M., Semple, C., and Gordon, C. (2001). Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. Nat. Cell Biol. *3*, 939–943.

Yang, T., Kozopas, K.M., and Craig, R.W. (1995). The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. J. Cell Biol. *128*, 1173–1184.

Yang, T., Buchan, H.L., Townsend, K.J., and Craig, R.W. (1996). MCL-1, a member of the BLC-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. J. Cell. Physiol. *166*, 523–536.

Zhang, B., Gojo, I., and Fenton, R.G. (2002). Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood *99*, 1885–1893.

Zhou, P., Qian, L., Kozopas, K.M., and Craig, R.W. (1997). Mcl-1, a Bcl-2 family member, delays the death of hematopoietic under a variety of apoptosis-inducing conditions. Blood *89*, 630–643.

Accession Numbers

The GenBank accession number for the human Mule sequence is DQ097177 and for the mouse Mule sequence is DQ097265.