N-terminal Truncation of Antiapoptotic MCL1, but Not G2/M-induced Phosphorylation, Is Associated with Stabilization and Abundant Expression in Tumor Cells*

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The antiapoptotic BCL2 family member MCL1 is normally up- and down-modulated in response to environmental signals and conditions, but is constitutively expressed in cancer where it promotes cell survival and drug resistance. A post-translational modification identified here, truncation at the N terminus, was found to act along with previously described ERK- and GSK3-induced phosphorylation events to regulate the turnover of the MCL1 protein and thus its availability for antiapoptotic effects. Although both N-terminally truncated and full-length MCL1 contain sequences enriched in proline, glutamic acid, serine, and threonine and were susceptible to proteasomal degradation, the truncated form decayed less rapidly and was maintained for an extended period in the presence of ERK activation. This was associated with extended cell survival because the truncated form of MCL1 (unlike those of BCL2 and BCLX) retained antiapoptotic activity. N-terminal truncation slightly increased the electrophoretic mobility of MCL1 and differed from the phosphorylation/band shift to decreased mobility, which occurs in the G2/M phase and was not found to affect MCL1 turnover. The N-terminally truncated form of MCL1 was expressed to varying extents in normal lymphoid tissues and was the predominant form present in lymphomas from transgenic mice and human tumor lines of B-lymphoid origin. The degradation versus stabilized expression of antiapoptotic MCL1 is thus controlled by N-terminal truncation as well as by ERK- and GSK3 (but not G2/M)-induced phosphorylation. These modifications may contribute to dysregulated MCL1 expression in cancer and represent targets for promoting its degradation to enhance tumor cell death.

The antiapoptotic mediator MCL1 was discovered based on rapid, transient up-regulation in ML-1 human myeloblastic leukemia cells initiating differentiation in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA)7 (1). MCL1 has since been found to be induced in cells at various stages in differentiation, in response to specific growth, differentiation, and survival factors. Induction of MCL1 expression serves to promote viability in cells undergoing changes in proliferation or differentiation or responding to stress, infection, or other signals. Thus, when MCL1 is conditionally knocked out, hematopoietic stem cells do not survive, and early stage B- or T-lymphocytes die instead of continuing their differentiation (2, 3); mature lymphoid cells likewise exhibit impaired survival despite the presence of growth factors (4, 5). Just as induction of MCL1 expression serves to promote viability, its down-regulation is an early, pivotal event in apoptosis initiated by DNA damage and other death stimuli (6, 7). Overall, in response to changing signals, increases and decreases in MCL1 expression regulate cell viability, maintaining cells and lineages that are needed while eliminating those that are no longer needed or are damaged or overabundant (1).

Although MCL1 is normally expressed in particular cells in response to specific stimuli, it is expressed continuously and in abundance in various types of cancer, including leukemia/lymphoma and multiple myeloma. For example, whereas differentiating plasma cells express MCL1 at distinct stages and in response to survival signals, multiple myeloma cells frequently exhibit constitutive, growth factor-independent expression (8–11). The level of expression in these cells is in the range of, or greater than, that seen transiently upon TPA stimulation of ML-1 cells. Constitutive, abundant MCL1 expression in multiple myeloma and other cancers is associated with enhanced cell survival/drug resistance and poor prognosis (12–22). Mice expressing a human MCL1 transgene product also exhibit enhanced survival of hematopoietic and lymphoid cells and have a high probability of developing B-cell lymphoma upon long term observation (23, 24).

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7 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol 13-acetate; ANOVA, analysis of variance; BH, BCL2 homology; ERK, extracellular signal-regulated kinase; HPLC, high pressure liquid chromatography; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PEST, enriched in proline, glutamic acid, serine, and threonine; Z, N-benzoyloxycarbonyl; fmk, (O-methyl)fluoromethyl ketone; CMV, cytomegalovirus; CHO, Chinese hamster ovary.
MCL1 expression is regulated through multiple mechanisms that act both transcriptionally and post-transcriptionally. Thus, MCL1 up-regulation and stabilization by specific growth and differentiation factors promote viability, whereas MCL1 down-regulation and/or degradation occurs early in apoptosis induced by growth factor withdrawal, DNA damage, and other apoptosis-inducing stimuli (6, 7, 25, 26). The rapid transcriptional up-regulation seen in TPA-treated ML-1 cells is induced through an extracellular signal-regulated kinase (ERK)-mediated early response gene mechanism, whereas other signaling pathways operate in other cell types (AKT, STAT (27, 28)). Down-regulation can occur rapidly because the $t_\text{1/2}$ of the mRNA as well as the protein is generally in the range of several hours (29, 30). The MCL1 mRNA is also subject to alternative splicing to much smaller BH3-only variants (~34–35-kDa), which are pro- rather than antiapoptotic (4, 31). The multiple mechanisms that regulate MCL1 allow it to carry out its function as a rapid response viability mediator and aid its role in the maintenance of homeostatic tissue differentiation. These mechanisms also keep in check its potential for contributing to tumorigenesis.

The post-translational mechanisms that regulate MCL1 are beginning to be elucidated. Although the carboxyl half of the protein (residues 177–350) contains the BCL2 homology (BH) domains, the upstream half is replete with PEST and poor PEST sequences; these may contribute to the ability of the protein to undergo rapid turnover (32). Turnover has recently been found to be modulated by phosphorylation at a motif at the end of the PEST region (e.g. at Thr$^{163}$ and Ser$^{159}$). Phosphorylations at this motif determine whether the MCL1 protein undergoes rapid degradation to terminate its actions or, alternatively, is stabilized to promote/prolong its expression and antiapoptotic effects. An early clue that the antiapoptotic actions of MCL1 can be regulated by modulation of its turnover came from studies of granulocyte-macrophage colony-stimulating factor; this growth factor was found to promote MCL1 stabilization and neutrophil survival, acting through ERK and AKT (25). ERK activation (e.g. induced by TPA) results in phosphorylation at Thr$^{163}$ and stabilization of the MCL1 protein (33). Conversely, GSK3 activation (e.g. induced upon IL-3 deprivation and ensuing AKT inhibition) stimulates phosphorylation upstream at Ser$^{159}$; this targets MCL1 for proteosomal degradation and enhances cell death (26). When the latter phosphorylation is blocked, MCL1 turnover is slowed (~2-fold (34)), and cell viability is enhanced. Loss of GSK3-induced degradation has been shown recently to contribute to tumorigenesis in rodent systems and also appears to be important in human cancer (34, 35). Control of the effects of MCL1 through modulation of its degradation is reminiscent of certain cell cycle and DNA damage-response proteins, an example being cyclin E where phosphorylation-stimulated turnover is part of the normal cycle and is altered in tumors (36, 37). In addition to proteosomal degradation, MCL1 is subject to caspase cleavage to pro-apoptotic forms (at Asp$^{127}$ or Asp$^{157}$, yielding ~28- and 23-kDa bands, respectively (38)).

In previous studies, MCL1 was seen to undergo other post-translational modifications in addition to those above. On large format gels, MCL1 was found to consist of a closely spaced doublet (29, 39); this did not represent phosphorylation, because the doublet was not affected by treatment with phosphatase (33, 40). In addition, MCL1 was subject to phosphorylation through another pathway induced in $G_2$/M phase, which resulted in a band shift of the MCL1 protein to slightly reduced electrophoretic mobility (31, 38, 40).

In the present studies, MCL1 was found to be synthesized as a 42-kDa protein and to undergo a transition to a 40-kDa form, resulting in the formation of the doublet. As shown by mutational and mass spectrometry analysis, this is because of truncation at the N terminus, which occurs in a mildly hydrophobic segment (residues 9–27) that terminates in a stretch of small residues (e.g. Gly, Ala). The truncated form of MCL1 was found to turn over less rapidly than the full-length form and to be maintained for an extended period in the presence of ERK activation. This was associated with an extension of cell survival because the truncated form of MCL1 (unlike those of BCL2 and BCLX (41)) retained antiapoptotic activity. In contrast to the N-terminal truncation, the phosphorylation/band shift, which involved Ser$^{64}$ and Ser$^{121}$, did not affect MCL1 turnover, confirming other studies (42, 43). N-terminally truncated MCL1 was differentially expressed in normal lymphoid tissues and was the predominant form present in B-lymphoid tumor cells. Overall, rapid degradation versus extended expression of antiapoptotic MCL1 is controlled by N-terminal truncation as well as by ERK- and GSK3 (but not $G_2$/M)-induced phosphorylation. The identification of modifications that affect MCL1 turnover suggests approaches that could be exploited to enhance the degradation of this pro-survival protein and sensitize cancer cells to apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Cell lines were maintained as described, as were primary cells from MCL1 transgenic mice and age- and sex-matched nontransgenic controls (using an approved IACUC protocol) (9, 23, 29, 44, 45). Transfection was by electroporation for FDC-P1 cells and with Effectene (Qiagen, Valencia, CA) for CHO and COS-1 cells.

Cell viability was assessed by hemocytometer using trypan blue dye exclusion (44) or by scoring apoptotic morphology (9). In some cases, the percentage of adherent cells was assessed by counting (Coulter Counter; Opa Locka, FL) nonadherent cells and adherent cells obtained by trypsinization.

**Pharmacologic Agents and Antibodies**—Carbobenzoxy-$\text{l}$-leucyl-$\text{l}$-leucyl-$\text{l}$-leucinal (MG132), clasto-lactacystin $\beta$-lactone (lactacystin), calpain inhibitors I and II, and calpeptin were from Calbiochem. N-Benzylloxycarbonyl-Val-Ala-Asp (O-methyl)fluoromethyl ketone (Z-VAD-fmk) was from Bio-Rad or Promega Corp. (Madison, WI). TPA was from Alexis Biochemicals (San Diego) or Sigma, and etoposide was from Sigma.

The antibodies used included a rabbit polyclonal (29) and mouse monoclonal antibody (24) directed against the human MCL1 protein, and a polyclonal antibody directed against a peptide representing human MCL1 (Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies detect human but not mouse MCL1 under standard conditions. Immunization of New Zealand White rabbits with a peptide representing the MCL1
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N-terminal 12 (MAP peptide) or 16 residues (keyhole limpet hemocyanin-conjugated) was carried out by, respectively, Hazelton Research Products, Inc. (Denver, PA; 2 rabbits), and Sigma Genosys (The Woodlands, TX; 2 rabbits).

In Vitro Transcription/Translation, Western Blotting, and Assay of \[^{35}S\]Met and \[^{32}P\] Incorporation—In vitro transcription/translation was carried out with the p3.2 construct containing the MCL1 cDNA (29), using the TNT T7 Quick Coupled Transcription/Translation System (Promega; control DNA provided).

Western blotting and densitometric scanning were carried out as described (9, 27). Assay of incorporation of \[^{35}S\]Met or \[^{32}P\] into MCL1 was as described (29), using Met- or phosphate-free RPMI 1640 medium and dialyzed serum. With \[^{32}P\]orthophosphate, cells were preincubated in this medium for 1.5 h. Pulse-chase experiments were carried out essentially as described previously (29), with 15 mg/ml nonradioactive methionine being present in the chase medium. MCL1 protein remaining was calculated relative to the initial (zero time) value, after normalization for tubulin expression.

Generation of Mutant Constructs—Mutations were prepared using QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA; primers shown in Table 1) and confirmed by sequencing. Mutants were cloned into the pcDNA3.1 (+) vector (Invitrogen) after digestion with BamHI and HindIII.

Mass Spectrometry—BL41-3 cells were lysed and incubated on ice for 15 min, using Nonidet P-40 lysis buffer (142.5 mM KCl, 5 mM MgCl2, 1 mM EGTA, 0.2% Nonidet P-40) containing Sigma protease and phosphatase inhibitor mixtures (1:100 each). After centrifugation (12,000 rpm for 5 min), MCL1 was immunoprecipitated from the lysate using an anti-MCL1 antibody (S-19; Santa Cruz Biotechnology) conjugated to Dynabeads (M-280; Dynal, Norway). Immunoprecipitation was carried out overnight at 4 °C. The Dynabeads were prepared as follows: (i) washing and suspension in 20 mM Tris buffer (pH 7.4) containing 142.5 mM KCl, 5 mM MgCl2, and 1 mM EGTA; (ii) conjugation to the anti-MCL1 antibody by incubation at 4 °C for 3 h with gentle rocking; and (iii) washing the conjugated Dynabeads with Nonidet P-40 lysis buffer. After centrifugation (13,000 rpm), the immunoprecipitates were suspended in SDS loading buffer, heated at 90 °C for 15 min with vortexing every 5 min, and subjected to SDS-PAGE. After staining using enhanced Coomassie Blue dye, the portion of the gel containing the MCL1 doublet was excised. This was submitted to the Harvard University Microchemistry and Proteomics Analysis Facility, Cambridge, MA. Liquid chromatography-tandem mass spectrometry was used because attempts to isolate sufficient MCL1 Edman degradation were unsuccessful.

For experiment 1, the MCL1 sample was reduced and alkylated using dithiothreitol and iodoacetamide, followed by digestion with trypsin and chymotrypsin together. Nano-capillary reversed phase HPLC was carried out using a 75-μm inner diameter glass capillary column with a 15-μm spray tip (New Objective Inc., Woburn, MA) packed with 10 cm of YMC C18 media. A Surveyor HPLC (Thermo Finnigan) was connected to a three-way tee where flow was split ~300:l, with ~350 nl/min flow directed to the analytical column. Sample loading was via a manual pressure bomb at 400 p.s.i. HPLC solvents were 0.29% acetic acid in water (A buffer) and acetonitrile (B buffer) with a linear gradient from 1% B to 51% B over 50 min, followed by a 95% B column wash and column re-equilibration. Mass spectrometry was performed on a Thermo Finnigan Deca XP+ ion trap operating in data-dependent mode (with the most intense four ions selected for MS/MS fragmentation from each full MS scan) with dynamic exclusion enabled (120-s exclusion duration for each MS/MS spectra acquired), as well as a selected ion monitoring mode targeting the N-terminal peptides of interest. Collected spectra were identified using the Sequest algorithm and manually verified for fidelity to MCL1.

For experiment 2, the sample was reduced and alkylated as described and digested with endoprotease Glu-C. The nanocapillary reversed phase HPLC was performed using a similar arrangement as above, but using a Hewlett-Packard 1100 HPLC system equipped with a FAMOS (LC Packings) autosampler. Data analysis was also as described previously; however, no further selected ion monitoring experiments were performed.

Experiment 3 was conducted in a similar fashion, with the sample reduced and alkylated, followed by separate digestions of endoprotease Glu-C and chymotrypsin. The HPLC system used was a Waters Nano Acquity UPLC, which enabled the use of a trapping column and direct flow chromatography at 300 nl/min flow rates using 0.1% formic acid buffers (both A and B) and a similar gradient. In this case, 100-μm inner diameter IntegraFrit columns (New Objective Inc.) packed with 2 cm of Magic C18-AQ (Microm Biosources Inc.) 5-μm beads were used for the trapping column (trapping a 5-μl injection for 5 min at 5 μl/min in 100% A buffer), with 15 cm of Magic C18-AQ 3-μm beads used in the 75-μm inner diameter analytical column. The sample was analyzed using an LTQ-Orbitrap hybrid linear ion trap/Orbitrap instrument (Thermo Finnigan), which acquired full MS scans in the Orbitrap at high resolution (60,000 resolution (full peak width at half-maximum height)) and low parts per million mass accuracy, whereas the linear ion trap concurrently acquired MS/MS spectra of the four most abundant ions per full MS scan, with dynamic exclusion duration of 30 s per ion as described previously. The use of this instrument led to a dramatic increase in protein coverage and overall confidence of results, with the obtained spectra identified using Sequest with manual verification.

Sequence and Statistical Evaluations—Alignment was with ClustalW. Sequence of low amino acid complexity was identified by using the software SEG and compositional bias was confirmed with CAST. PEST sequences were identified (46) and potential hydrophobic/membrane-associated segments were assessed using Membrane Protein Explorer2 in interfaced mode (47). Statistical analyses were with SYSTAT5 for the MAC.

RESULTS

MCL1 Is Synthesized as a 42-kDa Protein and Converted to a 40-kDa Form—Our studies of MCL1 have made extensive use of ML-1 as well as BL41 Burkitt lymphoma cells and the MCL1-

\(^a\) The software for the Membrane Protein Explorer was designed in 2006 by S. Jaysinghe, K. Hristova, W. Willey, C. Snider, and S. H. White.
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amplified BL41-3 subline. These endogenously expressing lines have complementary uses as ML-1 cells exhibit basal MCL1 transcription that can be induced by TPA (29), whereas BL41-3 cells exhibit constitutive MCL1 overexpression (39). With large format gels, the 42/40-kDa MCL1 doublet is seen in both systems. It was originally seen in ML-1 cells in pulse-chase experiments demonstrating rapid MCL1 up-regulation and turnover (29). In the latter study, the 42-kDa upper band was also noted to label more rapidly during the pulse (within 1 h, when labeling of the 40-kDa band was faint). The relative intensity of the upper band then decreased after the chase, whereas that of the lower band increased. This suggested that the upper band was synthesized first and underwent conversion to the lower band. In recent studies of phosphorylation, carried out in BL41-3 cells because of abundant expression, both bands exhibited ERK-induced 32P incorporation but did not collapse to a single band upon phosphatase treatment (40). This suggested that differential phosphorylation did not underlie the difference between the bands. These observations have now been confirmed in reciprocal experiments, which monitored the formation and decay of the doublet bands in BL41-3 cells and their phosphorylation in ML-1 cells. Large format gels were used in these and other studies below, although the doublet bands separated to somewhat differing extents in different experiments.

The decay of the doublet bands in BL41-3 cells was first monitored by Western blotting after exposure to cycloheximide. In the absence of this agent, the lower band was more prominent than the upper band (Fig. 1A, top photograph). Upon addition of cycloheximide, both bands decayed rapidly as expected, and loss of the upper band occurred even more rapidly than that of the lower band (t1/2 of ~1 h versus ~3 h, similar to previous estimates in ML-1 cells (29)). Upon [35S]Met pulse-chase labeling, the upper MCL1 band labeled more intensely during the pulse and declined relative to the lower band as both decayed during the chase (Fig. 1A, bottom photograph). Thus, in BL41-3 as in ML-1 cells (29), the MCL1 upper band was synthesized first and decayed in tandem with a transition to the lower band, the latter exhibiting more abundant steady state expression. The upper band also co-migrated with the single band obtained upon in vitro translation (Fig. 1B). Initial synthesis of the upper band and conversion to the lower band were reflected in findings below showing that stimulation of MCL1 expression markedly increased the upper band whereas proteasome inhibition caused prominent accumulation of the lower band.

Both MCL1 bands exhibited 32P incorporation and did not collapse to a single band upon phosphatase treatment in ML-1 as in BL41-3 cells (Fig. 1C and data not shown (40)). Thus, findings from both lines, in addition to demonstrating the transition between the bands, showed that this does not reflect differential phosphorylation.

Proteasome Inhibitors Do Not Prevent the Formation of the 42/40-kDa MCL1 Doublet but Can Inhibit the Degradation of Either Band—To assess whether the formation of the 42/40-kDa doublet related to proteasomal degradation, we monitored expression of the MCL1 bands in the presence of proteasome inhibitors. Our rationale was that if the transition from the upper to the lower band involved the proteasome, this transition would be blocked by such inhibitors, leading to accumula-

FIGURE 1. MCL1 is synthesized as a 42-kDa band and undergoes a transition to a 40-kDa form. A, MCL1 doublet bands were monitored in BL41-3 cells exposed to cycloheximide (CHX, 2.5 μg/ml, Western blots, top photograph) or to [35S]Met for 2 h followed by a chase (immunoprecipitation/autoradiography, bottom photograph). For the Western blots, a lighter exposure and a darker exposure are shown for better visualization of the bands. In the case of the pulse-chase experiment, a nonspecific band on the autoradiograph did not show any consistent change with time; here, the 0.5-h sample was loaded at 50% of the others. The 42-kDa upper and 40-kDa lower MCL1 bands are indicated with longer and shorter lines, respectively. The mean (± S.E.) of three independent experiments is shown below the Western blot, and the pulse-chase experiment shown is representative of two independent experiments. B, in vitro transcription and translation were carried out with a full-length MCL1 cDNA using rabbit reticulocyte lysate (left), or wheat germ extract (right) where the product obtained was compared with the endogenous MCL1 doublet present in BL41-3 cells. The experiment shown is representative of two independent experiments. C, MCL1 doublet bands were assayed for incorporation of [32P]orthophosphate or [35S]Met in ML-1 cells exposed to TPA (1.7 μM for 3 h). Preimmune serum was used in lanes 1 and 3, and antiserum to MCL1 in lanes 2 and 4.
allowing continued transition to the lower band (29, 30). In view of this, we reasoned that TPA could be used to enhance our ability to assess whether the transition of the upper to the lower band occurred in the presence of proteasome inhibitors. TPA was therefore used to induce MCL1 expression in the presence or absence of such inhibitors. Time points of 3.5 and 10 h were examined because, upon application of TPA by itself, the upper band was predominant at 3.5 h (Fig. 2A, right (upper photograph)), and the lower band became more prominent at 10 h (Fig. 2A, right (lower photograph)) (29, 30). Upon application of TPA in the presence of proteasome inhibitors, the effects of both types of agent were in evidence. At 3.5 h, both the upper and lower bands were prominent (Fig. 2A, middle (upper photograph)), consistent with the fact that the former was prominent with TPA alone and the latter with proteasome inhibitors alone. At 10 h, expression of the lower band was dramatically increased relative to that of the upper band, with the lower band becoming so abundant that it nearly obscured the small amount of remaining upper band (Fig. 2A, middle (lower photograph)). In sum, from 3.5 to 10 h, the relative abundance of the lower versus the upper band increased in cells exposed to TPA, either by itself or along with proteasome inhibitors. The transition of the MCL1 upper to the lower band thus continued in the presence of inhibition of proteasomal degradation.

Similar studies were carried out using murine FDC-P1 cells stably transfected with an inducible MCL1 cDNA (44). Under basal conditions, the transfected MCL1 gene product was nearly undetectable in these cells (trace amounts were faintly seen upon prolonged exposure). Upon exposure to the inducer alone (dexamethasone), MCL1 was expressed predominantly as the upper band (i.e. the initial translation product; Fig. 2B). Upon exposure to MG132 alone, both bands were detectable at 2 h. This was presumably due to stabilization of the trace amounts of MCL1 present in untreated cells and suggested that MG132 was capable of affecting both bands (see below). The lower band then exhibited increased predominance at 5 h, suggesting that MG132 did not prevent the transition to the lower band. Upon exposure to the inducer and MG132 in combination, both bands were present in abundance (4-fold fewer cells were loaded). Here, the upper band was more prominent at 2 h, whereas the lower band became increasingly prominent at 5 and 24 h. In sum, although the upper band was the primary product observed when MCL1 expression was induced, it underwent progressive accumulation in the form the lower band in the presence of MG132. The progressive increase in the relative abundance of the lower versus the upper band in FDC-P1 cells mirrored and underscored the above results in ML-1 cells. This confirmed that the transition from the upper to the lower band can occur in the presence the proteasome inhibition.

In addition to the above inducible transfectants, we have FDC-P1 transfectants in which MCL1 is constitutively expressed under the control of the CMV promoter. Large format gels yielded results similar to those obtained with the inducible transfectants in that basal MCL1 expression consisted of primarily the MCL1 upper band along with lesser amounts of the lower band, and MG132 resulted in a marked transition to the lower band continued, whereas degradation was slowed, consistent with the possibility that the proteasome was not critical for the transition. The MCL1 band pattern seen with MG132 differed from that seen with TPA; the latter stimulates MCL1 transcription and thus expression of the upper band (i.e. the initial translation product; Fig. 1B) while also

FIGURE 2. Proteasome inhibition does not block the transition of MCL1 from the 42- to the 40-kDa band but can inhibit the degradation of both forms. A, MCL1 doublet bands were monitored in ML-1 cells exposed to MG132 or TPA (0.5 μM or 0.5 nM, respectively; left panels) or to proteasome inhibitors (20 μM lactacystin or 10 μM MG132 for 1 h) followed by the addition of TPA (0.5 nM; middle and right panels). Each lane represents 4 (left) or 5 × 10^5 (right) cell equivalents. The experiments shown are representative of 2–3 independent experiments. B, introduced MCL1 gene product was assayed in 7.5-MAMmcl1 and vector-transfected 8.5-MAMneo cells exposed to MG132 (1 μM for 1 h) followed by the addition of dexamethasone (0.1 μM) for the indicated times. Each lane represents 2 × 10^5 cell equivalents except that 5 × 10^6 cell equivalents were loaded in the case of 7.5-MAMMcl1 cells exposed to MG132 plus dexamethasone. ML-1 cells exposed to TPA (0.5 nM for 3.5 h; 5 × 10^5 cells) and BL41 cells (2.5 × 10^5 cells) were included on the blot. C, upper and middle panels, the introduced MCL1 gene product was assayed in S8-CMVMcl1, S3-CMVMcl1, and vector-transfected V9-CMVneo and V6-CMVneo cells exposed to MG132 (1.5 μM) for 1 h followed by the addition of TPA (5 nM) for 3.5 h. Each lane represents 5 × 10^5 cell equivalents. The time point used was chosen based on preliminary experiments where the increase in MCL1 expression in the presence of TPA was found to occur with a time course similar to that seen in ML-1 cells in that it was prominent at ~2–6 h (not shown). Lower panel, cells were exposed to either MG132 or TPA as above. Each lane contains 5 × 10^5 cell equivalents except that 10^5 cell equivalents were loaded in the case of untreated controls.
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In addition to assessing the effect of proteasome inhibitors on the transition to the lower band, it was of interest to know whether these agents were capable of inhibiting the degradation of both bands. This seemed likely in the above experiments, but it was difficult to discern in the case of the upper band because of continued conversion to the lower band. In addition, conditions appropriate for monitoring the abundant MCL1 expression seen in the presence of inhibitors plus inducers were not ideal for cells treated with either type of agent alone. We therefore repeated the above experiments focusing on cells treated with single agents and with MG132 in particular. Expression of the upper as well as the lower MCL1 band was found to be increased in the presence of this inhibitor (note that twice as many untreated cells were loaded; Fig. 2C, lowest photograph). In sum, the degradation of both bands, but not the transition between the two, was blocked by proteasome inhibition.

The TPA/ERK-induced Slowing of MCL1 Degradation Is Manifested as Extended Expression of the 40-kDa Lower Band and Is Accompanied by Enhanced Cell Survival—Our previous studies had shown that MCL1 protein turnover is slowed when BL41 and BL41-3 cells are exposed to TPA, applied at a concentration that induces ERK activation and stimulates MCL1 phosphorylation at Thr163 (33). We thus wondered whether one or both bands would manifest the TPA/ERK-induced stabilization. As in the earlier studies, we examined this in BL41 cells, where effects on expression of the MCL1 protein can be followed in the absence of the wide fluctuations in transcription that occur in ML-1 cells. We thus exposed BL41 cells to cycloheximide to inhibit new protein synthesis, and we monitored the decay of the doublet bands in the absence or presence of TPA. Although both bands decayed in the presence of cycloheximide (the full-length more rapidly than the truncated form, as expected), the truncated form was maintained for an extended period in the presence of ERK activation (Fig. 3). Extended expression of this form was associated with enhanced cell survival, as cells exposed to cycloheximide by itself exhibited more apoptosis than cells exposed to cycloheximide plus TPA (>50% versus ~20%; Fig. 3A, legend). ERK activation thus resulted in stabilization of the MCL1 protein without preventing the transition to the lower band, where extended expression of the latter was associated with enhanced survival.

The MCL1 Doublet Does Not Arise through Calpain-/Caspase-mediated Cleavage or Other Modifications Seen in the BCL2 Family in Association with Cell Death—Various BCL2 family members undergo post-translational modifications at the N terminus (41, 48–51), suggesting this as a possibility in the case of MCL1. Although both bands of the doublet were membrane-associated, previous work showed that deletion of the MCL1 C terminus resulted in a loss of this association (29). Formation of the doublet thus did not appear to involve C-terminal truncation. The difference between the doublet bands corresponded to ~1–2 kDa, which could represent ~10–25 amino acid residues. MCL1 residues 14–15 are Leu-Tyr, sequences frequently present in calpain substrates at the P2-P1 sites (52). Because other PEST proteins are affected by calpains (46), the formation of the MCL1 doublet was monitored in the presence of calpain inhibitors. The appearance of the doublet

increase in the relative expression of the latter (Fig. 2C). Because expression from the CMV promoter can be stimulated by TPA, we reasoned that this agent might induce increased expression in these clones, much as dexamethasone did in the inducible clones. Indeed, TPA stimulated MCL1 expression, largely in the form of the upper band. Interestingly, along with the 42-kDa upper and 40-kDa lower bands, the 38-kDa “still lower” band was detected in some cases (Fig. 2C, middle photograph). The latter is present at low levels in ML-1 cells exposed to TPA for ~3 days (29). The addition of MG132 along with TPA did not interfere with, but rather enhanced, the expression of all these bands. We note that the formation of these bands in transfected cells confirmed that alternate splicing was not involved, because the transfected cells express an MCL1 cDNA construct lacking introns. We also note that the lower band accumulated in the presence of MG132 in BL41 cells (Fig. 3A, lower photograph) and in the CHO cells transfected with MCL1 that were used in experiments below (not shown). Thus, in both endogenously expressing cells and inducible or constitutive transfected cells, the 42-kDa MCL1 band produced initially is converted to lower molecular weight forms, prominently the 40-kDa band, through a process that is not blocked by proteasome inhibition.
with the appearance of the lower band (Fig. 4C). We also assayed for MCL1 deamidation but observed no change under alkaline conditions using BCLX as a positive control (48). Overall, the MCL1 doublet is not formed through a variety of modifications that occur in other BCL2 family members in association with cell death. This is perhaps not surprising as the MCL1 doublet is present in viable cells (29, 39).

MCL1 Undergoes N-terminal Truncation in a Mildly Hydrophobic Segment Containing a Gly/Ala-rich Stretch—In trials in four separate rabbits, the N-terminal residues of MCL1 were not found to be strongly antigenic, although early but not later bleeds from one immunized animal detected the upper band very weakly on Western blotting. When this preparation was used in immunoprecipitation, examination of the pellet was not informative because of background staining. However, examination of the supernatant remaining after serial immunoprecipitation demonstrated preferential immunodepletion of the MCL1 upper band (Fig. 5A, left panel). In another trial approach, we used a construct containing an N-terminal Myc tag (p9E10-MCL1D157A (38)). We ultimately did not pursue this approach because only a minute proportion of the tagged MCL1 protein appeared to undergo conversion to lower molecular weight forms even upon extensive overexpression, and it was difficult to be sure that these were generated in the normal fashion (Fig. 5A, right panel). Nonetheless, the Myc tag was only detected on the major high molecular weight product obtained, even upon extensive exposure of the autoradiograph. Because these trial approaches were consistent with the possibility of N-terminal truncation, we prepared a series of block mutations across residues 14–29, the region likely to be involved. This region of human MCL1 contains a moderately hydrophobic segment (residues 9–27, Fig. 10A), contiguous with a stretch of low complexity rich in Gly and Ala (residues 17–33, Fig. 10A). Because of the abundance of Gly and Ala, we did not introduce these amino acids but used larger and charged residues (Table 1). Four constructs were prepared, each altered at four residues (pMCL1Δ14–17, pMCL1Δ18–21, pMCL1Δ22–25, and pMCL1Δ26–29; Fig. 5E); a construct altered at residues 2–5 was made for assay with the others (pMCL1Δ2–5). Our purpose was to test for effects on the MCL1 lower band. Standard (nontagged) constructs were used, because of the above observations with a tagged construct (Fig. 5A, right panel). Upon transfection into FDC-P1 cells, expression of MCL1 was low in the G418-resistant populations obtained. To screen rapidly without waiting for the outgrowth of clones with robust expression (44), we monitored the MCL1 bands in the presence of MG132 or calpain inhibitor I to stabilize expression. An initial trial with the former agent (1 μM for 4.5 h, not shown) and a further experiment with the latter (Fig. 5B, left) revealed a change with pMCL1Δ22–25 in that the lower MCL1 band exhibited reduced electrophoretic mobility. This was seen upon comparison to pMCL1Δ26–29, which contains the same introduced amino acids. It was also apparent with the other constructs, although, for reasons that remain to be explored, expression was low in the presence of mutations further upstream (even upon addition of TPA to stimulate MCL1 expression; Fig. 5B, right). Overall, the lower component of the

![Figure 4: The MCL1 doublet does not arise through calpain- or caspase-mediated cleavage.](Image)

A, MCL1 doublet bands, along with BAX and tubulin, were monitored in BL41-3 cells exposed to calpeptin (30 μM, found in preliminary experiments to be a maximal concentration that did not produce substantial cytotoxicity while slowing cell growth). No change in the band pattern was seen in cells treated with vehicle alone (Me2SO; originally loaded after the zero time point and not shown to save space). The experiment shown is representative of two independent experiments with calpeptin. B, MCL1 doublet bands were monitored in ML-1 cells exposed for 1 h to calpain inhibitor II or I (50 or 1 μM, respectively), selected as above, followed by the addition of TPA (0.5 nM) for the indicated times. Each lane represents 5 × 10^6 cell equivalents. Samples not exposed to calpain inhibitor were originally on the right side of the blot and are shown on the left for ease of interpretation. The experiment shown is representative of two independent experiments with calpain inhibitors I and II. C, MCL1 doublet bands were monitored in ML-1 cells exposed to Z-VAD-fmk (10 μg/ml) for 1 h followed by the addition of TPA (0.5 nM) for the indicated times.

was not affected by several such inhibitors, including calpeptin (Fig. 4A) and calpain inhibitors I and II (Fig. 4B). The latter two were examined in ML-1 cells, which were used because TPA can be applied to increase MCL1 transcription and expression of the upper band; this allows one to monitor for inhibitor effects on the appearance of the lower band, as was done above with proteasome inhibitors (Fig. 2A). Upon application of TPA alone (Fig. 4B), the upper band was more prominent at early times and the lower band at later times, as expected (Fig. 2A) (29). This was also seen upon application of calpain inhibitor I or II with TPA, where it occurred in the presence of an overall elevation of MCL1 expression. In other words, the inhibitors caused an increase in both bands but did not block the transition between the two; the overall increase may be due to effects on calpains or the proteasome, which can also be affected by these inhibitors. Congruently, studies below did not detect cleavage C-terminal to Tyr<sup>15</sup> (Fig. 5E). This is consistent with the fact that the residues surrounding MCL1 Leu<sup>14</sup>-Tyr<sup>15</sup> differ from other calpain sites (52).

Caspase cleavage of MCL1 yields much shorter bands (38), and MCL1 does not contain a potential caspase site that could account for the doublet. Nonetheless, to rule out indirect as well as direct caspase involvement, we used Z-VAD-fmk. As above, the inhibitor affected both bands without interfering

![Figure 5: Block mutations in MCL1.](Image)
Tumor Abundance/Stabilization of N-terminally Truncated MCL1

**FIGURE 5.** MCL1 undergoes truncation in a moderately hydrophobic segment near the N terminus. **A**, left panel, S3-CMV[mcl1] cells were exposed to 50 nM TPA for 3.5 h to stimulate MCL1 expression, and the cell lysate was serially immunodepleted with antiserum (IgG purified) from a rabbit immunized using a MAP peptide representing MCL1 amino acids 1-12. The presence of the doublet bands in the initial lysate (0 cycles of immunodepletion) and the supernatant remaining after 1-3 cycles of immunodepletion were monitored by Western blotting using an antibody that recognizes both bands. The ratio of the upper to the lower band was estimated by densitometric scanning to decrease from 1.6 to 0.4. A similar result was obtained using ML-1 cells exposed to TPA (1.7 nM). In a control experiment, serial immunoprecipitation was carried out with an antibody that recognizes both bands; no selectivity was seen for either band. **Right panel**, CHO cells were transiently transfected with either pcDNA3.1MCL1 (pMCL1) or p9E10-MCL1D157A (pmycMCL1), or pMCL1Δ22-25 (Multi). Each lane represents 5 x 10⁵ cell equivalents. Two complementary approaches were used to examine the above possibility. In a molecular approach, we prepared a pair of constructs as follows: pMCL1ΔN term, which lacked residues 2–23, and pMCL1ΔMulti, which contained mutations at residues 17–20, 22, and 24 (Table 1). Our purpose was 2-fold. Given the above observations with pMCL1Δ22–25, we wished to determine how the mobility of the pMCL1ΔN term-encoded protein, lacking 22 residues, compared with that of the endogenous lower band. In addition, we wished to determine whether the broader span of mutations present in pMCL1ΔMulti would further interfere with the generation of this band. In a second approach, discussed further below, a sufficient amount of the MCL1 protein was purified for analysis of fragments representing the N terminus by mass spectrometry.

Upon transfection into a CHO cell derivative (33), the protein encoded by pMCL1ΔN term exhibited an electrophoretic mobility similar to that of the endogenous MCL1 lower band (Fig. 5C), whereas that encoded by pMCL1ΔMulti did not exhibit conversion to the lower form. These findings were in accord with the hypothesis that truncation occurred in the vicinity of residues 17–24, with multiple mutations in this region interfering with the generation of the lower band. A caveat was that expression with pMCL1ΔMulti was reproducibly low compared with that obtained with other constructs. To better evaluate pMCL1ΔMulti for conversion to the lower band, we utilized COS-1 cells, which yield abundant, transient expression (29). The 40-kDa lower band was not seen with pMCL1ΔMulti (Fig. 5D), although the 38-kDa band was detected and was also seen with pMCL1ΔN term. The mutations present in pMCL1ΔMulti thus specifically interfered with the generation of the 40-kDa band. The basis for formation of the 38-kDa band remains to be determined but could relate to further cleavage of the 42- and 40-kDa bands (e.g. possibly at another stretch of low complexity at residues 47–64; see Fig. 5C).

We reasoned that N-terminal truncation of MCL1 might occur at more than one position, accounting for the above observations. In other words, mutation of residues 22–25 could have prevented cleavage in a localized area, but cleavage could have occurred in an adjacent area, yielding a slightly larger protein. This stretch in human MCL1 contains a repetitive sequence, 17GGAGLGA(S/G)28 (consensus X(Gly/Ala)(Gly/Ala)(Gly/Ala)), further prompting us to consider this possibility.

For the introduced MCL1-encoded protein. Thirty percent more cell lysate was loaded for MCL1ΔMulti. D, following transient transfection of the indicated constructs into COS-1 cells, the MCL1 encoded proteins were assayed as above. The samples in the first 4 lanes represent a different experiment from those in the last 2 lanes. E, MCL1 from BL41-3 cells was subjected to in-gel digestion with the indicated enzymes, and the fragments obtained were identified by mass spectrometry. The graph depicts fragments from the N terminus in which the N-terminal residue did not conform to the consensus expected based on the enzyme used for digestion. Coverage of the protein was 37, 65, and 94% in experiments 1, 2, and 3, respectively. Nonconsensus ends occurred nonrandomly at the N terminus as the majority of fragments representing downstream portions of MCL1 contained cleavage sites expected based on the enzymes used for digestion or, in a small number of cases, contained an end that fell 1 residue from such a site or represented a site of downstream cleavage by trypsin. Nonconsensus N-terminal residues representing the fragments obtained most frequently are indicated with arrows. The block mutants used in B are diagrammed.

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from Gly17 to Arg30. Although the Arg30–Pro31 bond represents a potential trypsin cleavage site, the Cys16–Gly17 bond does not. All of the other fragments representing downstream portions of MCL1 contained precise trypsin or chymotrypsin cleavage sites at both ends, suggesting that nonspecific cleavage had not occurred and was unlikely to account for the presence of Gly17 at the N terminus. Sequences from further upstream (i.e. representing the full-length protein) were not detected in this initial trial, probably because of the predominance of the lower band. We attempted to identify such sequences by slicing the gel between the doublet and processing the two halves separately. However, because of the close proximity of the bands, particularly in preparative quantities, complete separation could not be obtained, and the N-terminal fragment initiating at Gly17 was detected in both halves of the gel slice.

We next scaled up the isolation, used additional enzymes for in gel digestion, and we characterized a large number of fragments. Of >350 fragments analyzed altogether, the majority exhibited cleavage as expected given the enzyme used for digestion. However, we consistently observed N-terminal fragments in which the N-terminal end was unexpected given the enzymes used for digestion. The fragment with Gly17 at the N terminus was seen with trypsin, chymotrypsin, or GluC, although a consensus cleavage site is not present for any of these enzymes. This fragment thus was not simply a product of anomalous cleavage by one of them. Some fragments initiating at other sites were observed. This was clear in experiment 3, where careful attention was paid to meticulous MCL1 isolation, and upgraded instrumentation allowed for greater precision in the characterization of the fragments. For example, a fragment initiating at Leu21 was seen, where Gly17 and Leu21 (underlined) lie in the 17GGAGLGAG repetitive sequence noted above. In experiment 3, we also achieved 94% coverage of the full-length (non-truncated) MCL1 protein. This included coverage of both the N terminus (eight fragments, including chymotryptic fragments initiating after residue 2; see overlining in Fig. 10A) and the C terminus (eight fragments extending downstream to residue 347). Thus, results obtained using this biochemical approach were in keeping with findings from mutant analysis and showed that, in addition to the full-length form, MCL1 is present in forms that are N-terminally truncated in the mildly hydrophobic segment containing a Gly/Ala-rich stretch.

**N-terminal Truncation Promotes MCL1 Stabilization and Extends Cell Survival**—Because endogenous MCL1 is not detected, an advantage of the CHO cell system used above is that transfected MCL1 gene products can be readily monitored (33). We therefore selected stable CHO cell transfectants expressing WT-MCL1, pMCL1 Multi, and pMCL1 ΔN term. Western blotting showed that WT-MCL1 was expressed primarily as the upper band, with truncated products being present at low abundance. The predominance of the upper band in CHO as well as FDC-P1 transfectants may relate to the fact that synthesis of this band is driven by the strong CMV promoter. pMCL1 Multi was also detected as the upper band, but it was expressed at very low levels hindering studies with this construct (see below). WT-MCL1- and pMCL1 ΔN term-transfected cells were therefore used to study the full-length and truncated forms of MCL1, respectively.

Decay of the MCL1 protein was monitored using the two approaches above, pulse-chase labeling and exposure to cycloheximide. In the first of these approaches, both the [35S]Met-labeled upper band present with WT-MCL1 and the pMCL1 ΔN term-encoded band declined over a relatively short time frame, decay of the upper band occurring particularly rapidly (Fig. 6A; half-life of ∼1 h for the upper and 2 h for the lower band as determined by PhosphorImager analysis). In the second approach involving exposure to cycloheximide, the decay of the WT-MCL1-encoded upper band was likewise rapid (Fig. 6B, left side of blot). Expression of the 40- and 38-kDa bands was detectable by Western blotting but at low levels. The pMCL1 ΔN-term-encoded MCL1 band declined more slowly than the WT-MCL1-encoded upper band and was persistent at ≈2 h (Fig. 6B, right side of blot). During the course of these studies, we noted some unexpected features of the CHO cell transfectant system. For example, we were unable to meaningfully assess the effect of TPA-induced ERK activation in the [35S]Met incorporation assay. This was because the presence of TPA during the chase markedly stimulated MCL1 synthesis from the CMV promoter, as above (Fig. 2C). By mass action, this led to artifactual incorporation of label into MCL1, likely from [35S]Met that remained or was released by the turnover of other proteins. Another observation that was surprising at first was the extended persistence of the pMCL1 ΔN term-encoded band seen upon assay by Western blotting after application of cycloheximide (Fig. 6B). Stabilized expression was consistently observed from 2 to 6 h, recalling the stabilization seen in BL41-3 cells in the presence of TPA-induced ERK activation (Fig. 3). Furthermore, the addition of TPA along with cycloheximide

### Table 1

| Construct | Downstream primer | Amino acid residues affected | Sequence in construct |
|-----------|-------------------|-----------------------------|-----------------------|
| pMCL1 Δ2–5 | CCAGCCCTGCGATATTTTTTCGCTATGAGAAACCGCGGAATCC | 2–5 | FGKL |
| pMCL1 Δ14–17 | GGTACCGTTCTGACGGAATCCGCGATATTTCGCTATGAGAAACCGCGGAATCC | 14–17 | LYG |
| pMCL1 Δ18–21 | GACGCGTTCTGACGGAATCCGCGATATTTCGCTATGAGAAACCGCGGAATCC | 18–21 | GAGL |
| pMCL1 Δ22–25 | GATGCGTTCTGACGGAATCCGCGATATTTCGCTATGAGAAACCGCGGAATCC | 22–25 | GAGS |
| pMCL1 Δ26–29 | CGTTGAGCTCAGGCCGCTATGAGAAACCGCGGAATCC | 26–29 | GGAT |
| pMCL1 Multi | CGTCTGACGGAATCCGCGATATTTCGCTATGAGAAACCGCGGAATCC | 5, 17, 18, 19, 20, 22, 24 | GAGL |
| pMCL1 ΔNerm | CCGGCTGCGATATTTTTTCGCTATGAGAAACCGCGGAATCC | 2–23 deleted | MGSGG |
resulted in little if any further MCL1 stabilization beyond seen with pMCL1/H9004 Nterm itself (slight if any effect in four independent experiments, data not shown). We wondered whether ERK activation might play a role in these observations, a possibility we initially conceived of because we had previously detected basal expression of activated ERK in CHO (but not BL41-3).

FIGURE 6. N-terminally truncated MCL1 exhibits enhanced stabilization and extends cell survival. A, CHO cells stably transfected with MCL1ΔNterm were assayed along with cells that had been transfected with WT-MCL1, where the latter expressed primarily the MCL1 upper band. Decay of the MCL1 bands was assessed using [35S]Met pulse-chase as in Fig. 1. The mean of two independent experiments is shown to the right of the autoradiograph. B, cell lines in A were exposed to cycloheximide (CHX) (25 μg/ml), and the decay of the MCL1 bands was assessed by Western blotting. The mean of three independent experiments is shown to the right of the autoradiograph. C, CHO cells were exposed to cycloheximide in the absence or presence of TPA and assayed for activated ERK (phosphorylated ERK; pERK) at the indicated times. D, CHO cells stably transfected with MCL1ΔMulti or WT-MCL1 were exposed to cycloheximide (25 μg/ml) to assess decay of the MCL1 bands by Western blotting. E, CHO cells stably transfected with WT-MCL1 or MCL1ΔNterm were incubated in the absence or presence of cycloheximide as in B. The plates were stained with crystal violet at the indicated times.
cells (33). Upon monitoring ERK, we found that cycloheximide resulted in ERK activation in the CHO cell system such that little if any further activation occurred in upon addition of TPA (Fig. 6C). The fact that cycloheximide increases ERK activation in addition to inhibiting protein synthesis in this system was consistent with the observed persistence of the truncated band. Because the truncated band did not decay as rapidly as the full-length band and persisted in the presence of cycloheximide-induced ERK activation, these results in transfected CHO cells provided a parallel to those in endogenously expressing BL41/BL41-3 cells. As in the latter, the persistence of the truncated band in CHO cells was associated with extended cell survival (Fig. 6E).

We also examined pMCL1ΔMulti-transfected cells, where very low levels of expression were obtained in multiple independent transfectants. It seemed likely that this related to the rapid turnover of the upper band. Indeed, the pMCL1ΔMulti-encoded protein exhibited rapid turnover, although this could not be rigorously quantified due to the low levels of expression (Fig. 6D).

Full-length and N-terminally Truncated MCL1 Promote Cell Survival upon Enforced Expression—Previous studies of FDC-P1 transfectants showed that expression of the introduced MCL1 gene product extended cell survival by about a day in the presence of apoptotic stimuli such as etoposide (44). However, it was not clear which MCL1 band(s) produced this effect, because standard gels were used. The present studies using large format gels showed that MCL1 was present in these cells primarily as the 42-kDa initial translation product (Fig. 2, B, lane 3, and C and Fig. 5C). Expression of this band was confirmed to result in viability protection upon exposure to etoposide (cell death reduced from ~80–90% to ~40–70%, with some variability between independent clones as seen previously (44); Fig. 7A). Thus, despite its lability, the 42-kDa upper band was capable of enhancing cell viability when its rapid turnover was circumvented by enforced expression. This did not require supraphysiologic levels of MCL1, because expression in the FDC-P1 transfectants is in the range attainable endogenously (~30% of the expression seen in ML-1 cells stimulated with TPA (44)).

When the inducible FDC-P1 transfectants were exposed to MG132 along with dexamethasone, expression of the 42-kDa MCL1 upper band was accompanied by increasing accumulation of the 40-kDa lower band (Fig. 2B, 4th, 10th, and 11th lanes). Prolonged exposure to MG132 (for ≥1 day) resulted in the death of cells not exposed to dexamethasone, but this was reduced in cells concurrently exposed to dexamethasone (from ≥80% to about 25%; Fig. 7B, left set of bars). Increased expression of the lower band and decreased cell death were likewise seen in stable transfectants exposed to MG132 (Fig. 2C and Fig. 7B, right set of bars). Thus, the lower band was expressed in association with enhanced viability as above (Fig. 3A, legend, and Fig. 6E). Indeed, enhanced viability was seen when the above MCL1−ΔNterm- and WT-MCL1-transfected CHO cells were exposed to etoposide (Fig. 7C), where these lines express approximately equivalent levels of the 40-kDa and primarily the 42-kDa band, respectively (Fig. 5, lanes 1 and 2); this observation extended previous findings with WT-MCL1- and parental or vector-transfected CHO cells (45). In both the FDC-P1 and CHO cell systems, viability protection with MCL1 is short lived. For example, MCL1 expression was maintained in FDC-P1 transfecteds exposed to etoposide, but cell death on day 2 approached that seen on day 1 in control cultures not expressing MCL1 (44). Similar results were seen in the presence of MG132 (Fig. 7D) and in CHO cells (Fig. 7C, legend). Moderate viability protection in cells in culture appears to be a consistent characteristic of MCL1, and the present experiments in FDC-P1, CHO, and MCL1 transgenic cells (Fig. 7F, below) suggested that this occurred in the presence of the upper and/or the lower band. N-terminal truncation thus slows the turnover of MCL1 but does not have an adverse effect on its antiapoptotic activity, allowing for enhanced survival upon extended or enforced expression of this form (Figs. 3, 6, and 7). This contrasts with the loss of antiapoptotic activity seen upon N-terminal truncation of BCL2 and BCLX (41).

The Truncated Form of MCL1 Is Present to Varying Extents in Normal Lymphoid Tissues and Is Prominent in Tumor Cells of B-lymphoid Origin—In previous studies of transgenic mice expressing human MCL1 (under the control of its endogenous promoter (23)), MCL1 expression was assessed using standard gels. The MCL1 transgene product was found to be readily detectable and expressed at similar levels in hematolymphoid tissues (lymph nodes, bone marrow, thymus, and spleen), with lower levels being present in other tissues such as the liver. Large format gels were used in the present studies, and these demonstrated tissue-specific differences in the proportions of the doublet bands in different lymphoid tissues. Both bands were present in lymph nodes and bone marrow; both were also present in the thymus and spleen, the upper band was more prominent in the thymus whereas the lower band was more prominent in the spleen (Fig. 7E). This provided a further opportunity to examine cell survival in the presence of the two forms of MCL1. Cells explanted from the thymus of transgenic animals exhibited enhanced survival in tissue culture (Fig. 7F) paralleling previous observations in the spleen (23). Thus, in both transfected and transgenic cells, the capacity to enhance cell viability was seen when either the full-length or the truncated form of MCL1 was predominant.

MCL1 transgenic mice demonstrate splenic enlargement and have a high probability of developing B-cell lymphoma upon long term observation (24). In the lymphomas, transgene expression (assessed using standard gels) is generally at least as high as that seen in the enlarged spleen of transgenic, nontumor-bearing animals. Expression at these levels was seen in an earlier examination of tumors from five animals, where some variability between animals was noted (24). Examination of an additional five tumor-bearing animals showed that four of these exhibited expression at least as high as, or higher than, that seen in enlarged, nontumorigenic spleen (Fig. 7G, upper panel). Large format gels revealed that the tumors expressed the lower MCL1 band more abundantly than the upper band. This was true whether tumor tissue was harvested from the thymus, spleen, or lymph nodes (Fig. 7G, lower panel). The lymphomas that develop in transgenic mice thus express MCL1 abundantly in the form of the lower band and, in this respect, demonstrate parallels to human cancer cells (see below).

Human multiple myeloma cells and cell lines also frequently exhibit abundant MCL1 expression, generally at levels in the range of, or greater than, those seen in ML-1 cells at the peak of
stimulation with TPA (8, 9). These levels are above the threshold for effects on cell viability (44), and depletion of MCL1 expression results in enhanced multiple myeloma cell death (53–55). Examination of a series of multiple myeloma cell lines showed that MCL1 underwent conversion to the truncated form in the majority of lines (Fig. 8A). This was seen in lines exhibiting highly elevated MCL1 overexpression (KMS-12-BM, KMS-12-PE, KMS-18-PE, and KMS-21-PE), where a short autoradiographic exposure is shown because of the abundance of MCL1 in these lines. Conversion to the lower band likewise occurred in lines exhibiting MCL1 expression in the range of that seen in ML-1 cells stimulated with TPA (MM1.R, MM1.S, and RPMI8226). Out of eight cell lines examined, only one (U266) did not exhibit extensive conversion of MCL1 to the lower band.

Burkitt lymphoma cell lines and malignant B-cells also express abundant MCL1 (38). MCL1 is primarily present as the 40-kDa lower band in BL41 cells and the BL41-3 subline. Interestingly, although MCL1 expression is higher in BL41-3 than in the BL41 cells, the reverse is true of BCL2, which is more abundant in BL41 cells (39). A similar reciprocal relationship between MCL1 and BCL2 was noted upon examination of a series of B-lymphoid lines. Thus, Raji, Daudi, Ramos, and two Burkitt lymphoma lines of group I (biopsy-like (56)) phenotype expressed MCL1 but not BCL2 in abundance (Fig. 8B, upper panel). Conversely, Epstein-Barr virus-immortalized lymphocytes (LCL) and a Burkitt lymphoma group III line expressed abundant BCL2 but scant MCL1. In cell lines that expressed MCL1, the 40-kDa lower band was predominant (Fig. 8B, lower panel). A human lymphoma sample exhibiting elevated expression of MCL1 (Fig. 8C, left panel) likewise expressed primarily the MCL1 lower band (Fig. 8C, right panel).

GSK3 Inhibition Stabilizes Both Bands of the MCL1 Doublet, whereas Induction of the G2/M Phase Phosphorylation/Band Shift Does Not Affect Turnover—The realization that MCL1 undergoes N-terminal truncation brought up the question of how this relates to other modifications that regulate MCL1. Both MCL1 bands undergo ERK-induced phosphorylation, as described above (Fig. 1C) (33, 40). Because this is thought to prime MCL1 for GSK3-induced phosphorylation and targeting for degradation (26), it seemed likely that both bands would also be susceptible to the latter pathway. To examine this, we incubated ML-1 cells in the absence or pres-
ence of TPA (to increase MCL1 expression) and LiCl (an inhibitor of GSK3). The expression of both MCL1 bands was elevated in cells exposed to these two agents, as was expression of β-catenin, which is also targeted for degradation by GSK3 (Fig. 9A). Little increase occurred in the presence of either TPA or LiCl alone, suggesting that more complete GSK3 inhibition was achieved upon exposure to both agents (57). An extended exposure time (18 h) was used in this experiment, both to better detect stabilization and because prolonged exposure to TPA produces some ML-1 cell death (58). Not surprisingly (26), death was reduced in the presence of the increased expression of both MCL1 bands seen in the presence of the GSK3 inhibitor (Fig. 9A, legend).

MCL1 is also subject to phosphorylation through another pathway, which acts in cells in G2/M phase and causes MCL1 to shift to a slightly reduced electrophoretic mobility (33, 40). This phosphorylation/shift is prominent in cells exposed to microtubule-directed agents. Exposure of BL41-3 cells to taxol causes the 42-kDa MCL1 band to shift to an apparent molecular mass of ~43–44 kDa. This occurs gradually (over ~12 h or more; Fig. 9B, upper photograph) in tandem with accumulation in G2/M phase (33, 40). This phosphorylation/shift did not markedly affect the turnover of MCL1. This was seen in cells in which essentially all of the 42-kDa band was induced to shift to the phosphorylated ~44-kDa form by exposure to taxol. The 44-kDa shifted band present in taxol-treated cells turned over rapidly, as did the 42-kDa band present in untreated cells (t1/2 ~ 1 h in both cases, where the taxol-treated cells in Fig. 9B, lower photograph were assayed alongside the untreated cells in Fig. 1A). The phosphorylation shifted band present in taxol-treated cells exhibited a considerable decline at 1 h, when the unshifted 40-kDa band was just beginning to decay. In overall summary, although MCL1 turnover is modulated by N-terminal truncation and ERK or GSK3 activation, it is not markedly affected by the G2/M-associated phosphorylation/band shift.

Okadaic acid also induces the MCL1 phosphorylation/band shift and acts over a much shorter time frame than taxol (~2 h with 1 μM okadaic acid). This agent induces all of the MCL1 protein present in BL41-3 cells to undergo the phosphorylation/shift and produces the same phosphopeptides as taxol (33). Because this synchronous and complete MCL1 phosphorylation/shift would aid in the identification of the sites involved, mass spectrometry was used to identify these in okadaic acid-treated BL41-3 cells. Phosphorylation in these endogenously expressing cells was identified at MCL1 Ser121 and Ser122 (supplemental Fig. 1), sites previously identified in MCL1-transfected cells (42, 43). The finding that turnover of the endogenous phosphorylation/shifted band was not markedly altered (Fig. 9B) agreed with fact that no effect on turnover was seen in cells transfected with MCL1-containing mutations at these sites (42, 43).

**MCL1 N-terminal Truncation Is Not Sensitive to a Variety of Protease Inhibitors**—In addition to using inhibitors of calpain and the proteasome (Figs. 2 and 4), we used a variety of other protease inhibitors to assess their effects on the appearance of the truncated band. Various concentrations were initially applied to identify those that had an effect (cell growth inhibition) without causing substantial cytotoxicity. These concentrations were then used in experiments similar to those above (Figs. 2 and 4), using either ML-1 or BL41-3 cells. We did not observe notable inhibition of the transition from the upper to the lower band in the presence of any of the inhibitors tested. These included 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (up to 50 μg/ml), tosyl phenylalanyl chloromethyl ketone (0.5 μg/ml), leupeptin (50 μg/ml), bestatin (40–120 μM), and Ala-Ala-Phe-chloromethyl ketone (10–20 μM). The latter two agents inhibit certain aminopeptidases; the fact that they did not inhibit the formation of the lower MCL1 band suggests that enzymes sensitive to these inhibitors may not be involved. Overall, a variety of common exo- and endoprotease inhibitors did not have a substantial effect on the truncation of MCL1. In any case, future identification of the protease involved will depend on isolation of the N-terminal truncation activity and the development of an in vitro means of assaying for MCL1 truncation.

**FIGURE 7.** The 40- and 42-kDa forms of MCL1 enhance cell viability and are differentially expressed in normal lymphoid tissues, but the 40-kDa form is predominant in B-cell lymphomas from transgenic mice. A, indicated FDC-P1 MCL1 transfected lines were assayed for cell death after exposure to 20 μg/ml etoposide for 1 day. Bars represent the S.E. of two (inducible transfectants) or three independent (constitutive transfectants) experiments. Untreated cells and inducible transfectants treated with dexamethasone (Dex) exhibited <1% death. *p < 0.05 upon comparison to control samples (ANOVA with post hoc testing using Fisher’s least significant difference test). B, FDC-P1 MCL1 transfected lines were assayed for cell death 1 day after exposure to MG132 (1 μM with inducible and 1.5 μM with constitutive transfectants). Bars represent the S.E. of three experiments. *p < 0.05 (determined as in A). C, indicated CHO cell lines were assayed for cell death 2 days after exposure to 50 μM etoposide for 6 h. The bars represent the S.E. of three experiments with pMCL1 Nterm-transfected and untransfected CHO cells; in one of these experiments, WT-MCL1 transfecteds were assayed in parallel, and loss of cell adherence was also monitored. Essentially all cells were dead on day 3 regardless of the presence or absence of MCL1. *p < 0.05 (two-tailed paired Student’s t test) upon comparison of pMCL1 Nterm-transfected and control cells. D, indicated FDC-P1 MCL1 transfecteds were assayed for cell death 1–2 days after exposure to MG132 (0.5 μM). Bars represent the S.E. of three independent experiments. *p < 0.05 (ANOVA/Fisher’s least significant difference test) upon comparison with control samples. E, tissues from two healthy MCL1 transgenic mice were assayed for expression of the introduced MCL1 gene product. Each lane represents 5 × 105 (left) or 106 (right) cell equivalents. Lanes representing TPA-treated ML-1 cells contain 5 × 105 cell equivalents. F, cells from the thymus and spleen of MCL1 transgenic (TR) and nontransgenic mice were assayed for cell viability after explantation into tissue culture. The squares represent the S.E. of cells from the thymus of eight transgenic and five nontransgenic mice, where the values for spleen (circles) are from the same mice. The latter were previously reported within a larger series (shown with permission [44]); the coefficient of variation for the spleen cells shown averaged 36%. The arrows indicate the time at which ~50% loss of viability occurred in nontransgenic and transgenic cultures. *p < 0.05 upon comparison of nontransgenic versus transgenic cells (from both thymus and spleen; ANOVA/Fisher’s least significant difference test). The difference between transgenic and nontransgenic cells was of borderline significance on day 1 (p < 0.08). G, tumor-infiltrated tissues from transgenic mice with lymphoma or spleens from nontumor-bearing transgenic animals were assayed for expression of the introduced MCL1 gene product or BAX. The upper panel represents a standard gel and the lower panel a large format gel. In the upper panel, the samples in the 1st, 3rd, and 4th lanes are from mice with non disseminated lymphomas, and those in 2nd and 5th lanes are from mice with disseminated lymphoma; each lane represents 5 × 105 cell equivalents. In the lower panel, disseminated tumors were present in all cases except that shown in lane 8; samples from different mice are indicated with separate lines. Here, the lanes represent 2 × 10⁶ cells (1st to 7th lanes), 10⁶ cells (8th and 10th lanes), or 5 × 10⁵ ML-1 cells treated with TPA.
DISCUSSION

We have proposed the following two-part model of the role of MCL1 in normal tissues and in cancer (1). (i) MCL1 regulates the viability of normal cells at particular stages in differentiation and in response to specific growth, differentiation, survival, and stress signals. For example, rapid MCL1 up- or down-regulation, promoting or inhibiting viability, is a determinant of whether cells remain viable and differentiate along various pathways within the differentiation continuum. MCL1 also functions in situations requiring the ability to readily switch from the maintenance of viability to cell death. This is seen in the presence of microtubule disruption (9); it is also seen during infection where initial MCL1 up-regulation prevents macrophage toxicity to allow bacterial engulfment, whereas subsequent down-regulation promotes macrophage death and thus bacterial clearance (4). (ii) Alterations that affect the normal pattern of MCL1 expression can contribute to the development of cancer. Cell viability is prolonged when MCL1 expression is sustained unnecessarily rather than being induced in a regulated or transient fashion. When dysregulated expression occurs in a susceptible cell type in a conducive, growth factor-rich environment, viability is maintained for an indefinite period (23). This predisposes cells to transformation because, with unlimited survival, they can acquire additional changes that contribute to tumorigenesis.

Because of its important role, a host of mechanisms regulate MCL1 at the mRNA as well as the protein level. Transcription is
induced by different signaling pathways under different conditions (9). Emerging findings suggest that the antiapoptotic actions of the MCL1 protein are regulated by different post-translational modifications that affect either (i) its degradation versus stabilization or (ii) its antiapoptotic activity (Fig. 10B). (i) ERK- and GSK3-induced phosphorylations in the Ser159/Thr163-containing motif at the end of the PEST region regulate the degradation versus stabilization of MCL1. This affects its presence in or elimination from the cell and the level and duration of its expression. ERK-induced phosphorylation promotes MCL1 stabilization, which prolongs cell viability (25, 33). This may then prime for GSK3-induced phosphorylation, which promotes degradation to terminate antiapoptotic effects (26, 34). Similar mechanisms regulate c-Myc. Here they are thought to serve to stabilize expression for a self-limited period of time, preventing elevated expression from being sustained when it is no longer needed (59). (ii) Other phosphorylation events do not affect the turnover of MCL1 but influence its intrinsic antiapoptotic activity. This was observed previously with Ser121 (42) and recently with Ser64 (43), where these sites are phosphorylated as part of the MCL1 band shift pathway. Phosphorylation at Ser64 and Ser121 is mediated by cyclin-dependent kinases or
The present findings show that, along with phosphorylation at the end of the PEST region, the turnover of MCL1 is regulated by N-terminal truncation. Initial work suggested the involvement of this modification and ruled out a variety of others. Mass spectrometry then demonstrated truncation near the N terminus, but not elsewhere, in cells endogenously expressing MCL1. This occurred in a region of MCL1 consistent with the size of the 40-kDa band, and indeed, a construct truncated in this region migrated alongside the authentic 40-kDa band. In addition, the introduction of multiple mutations into this region specifically inhibited the generation of the 40-kDa band. Truncation occurs in a moderately hydrophobic segment that contains a Gly/Ala-rich, repetitive stretch. Upon analysis of a large number of fragments by mass spectrometry, truncation products were observed with N termini lying primarily within the region 15YCGGAGL21. Truncation was altered rather than blocked with scanning mutations of four amino acid residues and was inhibited in the presence of multiple mutations spanning this region. MCL1 may thus be similar to other proteins demonstrating the potential for cleavage at several nearby sites, such as the amyloid precursor protein (61). Because mass spectrometry is nonquantitative, it remains to be determined whether particular site(s) are preferred in the case of MCL1.

Upon induction of MCL1 expression, the initial translation product observed is the full-length form, which has activity but is highly labile. This may serve as a rapidly inducible means for short term enhancement of viability. This labile form can undergo either rapid decay for a transient effect or stabilization by N-terminal truncation/ERK activation for extended cell survival. These effects can be terminated by GSK3 activation, which targets both bands for degradation. These various mechanisms aid in the fine temporal control of the MCL1 protein, providing means for maintaining expression as needed while allowing rapid turnover when no longer needed.

The N-terminal half of MCL1 is replete with regulatory sequences (Fig. 10A), whereas the C-terminal half is important for antiapoptotic BCL2 family function (62) and is highly conserved (~75% for the species shown in Fig. 10A). The extreme N terminus is also highly conserved in mammals (85% for the first 20 residues), whereas the remainder of the N-terminal half is not as well conserved (~50%) although it uniformly contains PEST sequences. The N terminus proper is not conserved in Zebrafish and is not as well conserved in chicken as in mammals. Its involvement in MCL1 regulation may therefore represent a recent evolutionary development.

Modifications affecting the N terminus are becoming a recurring theme among gene products that affect cell death. Thus, N-terminal caspase cleavage of BCL2 and BCLX removes the BH4 domain and amplifies apoptosis (41), and BCLX also undergoes deamidation near the N terminus (48). The N terminus of BAX undergoes a conformational change or calpain-mediated cleavage, which promotes mitochondrial targeting for apoptosis induction (49, 50). BID is subject to cleavage by caspase as well as by calpain, cathepsins, and granzyme B at nearby sites (51). Alternate splicing of the BIM N terminus yields forms with different pro-apoptotic potencies (63). The N-terminal modification of MCL1 is unique in that it occurs in viable cells. Indeed, the 40-kDa N-terminally truncated form of MCL1 retains viability promoting activity and is prominent in tumor cells. This is reminiscent of the truncation of cyclin E, which occurs at several sites in the N terminus and yields activated forms in breast cancer (64).

The mechanisms through which MCL1 N-terminal truncation affects its degradation remain to be elucidated. Both MCL1 bands are subject to degradation by the proteasome, and MCL1 is known to be targeted by two E3 ubiquitin-protein ligases. These are MULE, which has a BH3 domain that binds MCL1, and SCF-βTrCP, which binds upon GSK3-induced MCL1 phosphorylation (34, 65). It is not known whether MCL1 can be targeted by other E3 ubiquitin-protein ligases or can undergo ubiquitin-independent degradation by the core 20 S proteasome (66). Both the full-length and truncated forms of MCL1 were stabilized upon inhibition of GSK3, suggesting that both are susceptible to degradation through this mechanism. However, truncation might affect degradation by other pathways or interactions with proteins that affect MCL1 stability such as Noxa and Bim (65, 67, 68). MULE and other BCL2 family members bind to MCL1 via their BH3 domains. Therefore, the access or binding of these proteins to MCL1 might be affected by the presence or absence of the N terminus. Both the 42- and 40-kDa forms of MCL1 localize to intracellular membranes, prominently mitochondrial and endoplasmic reticulum membranes, and are anchored there by the highly hydrophobic carboxyl tail (29). Although the C-terminal half of MCL1 consists of a helical fold homologous to BCL2 (62), the extended N-terminal half, which makes MCL1 much longer than BCL2, contains primarily hydrophilic and charged residues. The characteristics of the extreme N terminus are intriguing. Here, MCL1 contains a segment that is mildly hydrophobic (blue and green in Fig. 10A) and has some characteristics of a signal sequence but does not function as such (29). This segment is also reminiscent of the mildly hydrophobic domains present in some outer mitochondrial membrane proteins (69) but does not itself target MCL1 to membranes (29). An additional clue came out of a recent examination of the circular dichroism spectra of a peptide representing the MCL1 N terminus. This peptide was largely unstructured in aqueous solution. However, some α-helical structure became evident in the presence of SDS micelles (supplemental Fig. 2). The hydrophobic-hydrophilic interfaces present in SDS micelles, although nonphysiologic, may mimic some aspects of the membrane environment. This observation therefore warrants future investigation of the possibility that the N terminus of MCL1 might contain a segment that associates with membranes and/or adopts a helical structure in such an environment. Such studies are also warranted based on analysis of MCL1 using a powerful prediction method (Membrane Protein Explorer (47)), which identifies segments likely to associate with the interfacial layer or hydrophobic core of the membrane. In addition to the known carboxyl tail and the central BH1-containing hydrophobic helix, this analysis...
pointed to the N-terminal mildly hydrophobic segment as a potential membrane-associated region. Association of this segment with the interfacial layer is energetically favorable, where this layer contains the lipid headgroups and lies between the membrane hydrophobic core and the external water phase. In one pathway for association with this layer, peptides that are unstructured in the water phase partition to the interfacial phase and then adopt a helical conformation (partitioning-folding coupling (47)). The C terminus targets and anchors MCL1 to membranes, and the above considerations suggest that the N terminus might be capable of partitioning there as well. This might then affect the positioning or conformation of the full-length protein and therefore its accessibility to components of the ubiquitin proteasome pathway or other proteins that affect MCL1 degradation. The N terminus could also itself provide a signal for components that affect degradation. Accordingly, truncation of the N terminus might remove such a signal or result in a conformational change that limits the access/binding of components of the degradation/destabilization machinery. Whatever the case, the identification of multiple modifications that affect MCL1 degradation, and the proteins that target and regulate this process, sets the stage for understanding the complex control of the turnover of this critical antiapoptotic regulator.

The pathways that modulate the antiapoptotic activity of MCL1 are also only beginning to be understood. For example, phosphorylation at Ser\textsuperscript{121} was reported to inhibit antiapoptotic activity, whereas in a different system, phosphorylation at Ser\textsuperscript{64} enhanced activity (42, 43). Another observation that is not yet not understood is that the full-length but not the truncated protein undergoes the phosphorylation/band shift upon G\textsubscript{2}/M arrest, despite that fact that both bands can be shifted by okadaic acid (33, 40). As above, it is possible that the presence of the N terminus has an effect on the positioning of the full-length protein, rendering it accessible to G\textsubscript{2}/M phase kinases. The fact that the 42-kDa but not the 40-kDa MCL1 band undergoes the G\textsubscript{2}/M shift could be another manifestation of the separation between modifications that affect the intrinsic activity of MCL1 (e.g. the phosphorylations involved in the shift) and those that affect its turnover (e.g. N-terminal truncation).

Another area that remains to be explored concerns the in vivo effects of the truncated form of MCL1. This question is important in view of our previous studies that indicate that the effects seen with MCL1 in cells in culture are typically mild (survival extended by ~1 day) and do not predict the profound effects seen in vivo in both knock-out and transgenic animals. Our model for understanding this suggests that expression of MCL1 allows cells to maintain viability as they pass thorough key “windows” of cell fate transition (1). Cells that remain viable during these critical periods can continue to proliferate and differentiate, not requiring MCL1 until a subsequent transition point. In this fashion, short term enhancement of survival during critical periods can profoundly influence the downstream cell populations, and thus tissue differentiation as a whole. Future studies will assess whether this is the case in transgenic mice expressing the truncated form of MCL1.

Sustained, abundant expression of MCL1 promotes survival and chemoresistance in a variety of tumors. However, the mechanisms that underlie this dysregulated expression have been elusive. A possible role for upstream regulatory regions has been controversial, and investigation of the involvement of a variety of signaling pathways is just beginning to yield clues (34, 35, 70). The present findings show that many cancer cells are capable of truncating MCL1 to the more stable 40-kDa form, in fact all but one of the samples we examined expressed MCL1 prominently as the truncated band. Thus, this modification as well as phosphorylation (35) may contribute to constitutive, abundant MCL1 expression in cancer cells. The presence of truncation in cancer cells also suggests this as a possible therapeutic target. Cancer cells may be dependent on BCL\textsubscript{2} family members such as MCL1 (71), and decreasing MCL1 expression can enhance their sensitivity to apoptosis (17, 20–22). Inhibition of the transition to the lower band results in rapid MCL1 turnover, as seen with the cleavage site multiple mutant. Therefore, one approach for interfering with uncontrolled MCL1 expression in cancer might be to inhibit N-terminal truncation to enhance MCL1 degradation and sensitize these cells to apoptosis.

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