Bmf: A Proapoptotic BH3-Only Protein Regulated by Interaction with the Myosin V Actin Motor Complex, Activated by Anoikis

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Bcl-2 family members bearing only the BH3 domain are essential inducers of apoptosis. We identified a BH3-only protein, Bmf, and show that its BH3 domain is required both for binding to prosurvival Bcl-2 proteins and for triggering apoptosis. In healthy cells, Bmf is sequestered to myosin V motors by association with dynein light chain 2. Certain damage signals, such as loss of cell attachment (anoikis), unleash Bmf, allowing it to translocate and bind prosurvival Bcl-2 proteins. Thus, at least two mammalian BH3-only proteins, Bmf and Bim, function to sense intracellular damage by their localization to distinct cytoskeletal structures.

The BH3-only proteins are proapoptotic members of the Bcl-2 family that share with their relatives only the short BH3 domain, which allows their interaction with prosurvival Bcl-2 family members to trigger apoptosis (*1*). Genetic experiments have demonstrated that they are essential for initiation of programmed cell death in species as distantly related as *Caenorhabditis elegans* and the mouse. EGL-1, the only *C. elegans* BH3-only protein identified thus far, is required for all developmentally programmed death of somatic cells in this organism (*2*). In contrast, multiple BH3-only proteins have been identified in mammals, including Blk, Bad, Bik, Hrk, Bid, Bim, Noxa, and Puma (*1*). Experiments with gene knockout mice have shown that pathways to apoptosis resulting from different damage signals require distinct BH3-only proteins for their initiation (*1*). For example, Bim (*3*) is essential for apoptosis of lymphocytes induced by cytokine withdrawal but dispensable for that induced by phorbol ester (*4*). In contrast, Bid is involved in Fas-induced killing of hepatocytes (*5*). Moreover, distinct BH3-only proteins appear to have tissue-specific roles in programmed cell death. Bim-deficient mice, for example, accumulate abnormal numbers of lymphoid and myeloid cells but have normal erythrocyte levels (*4*).

The proapoptotic activity of BH3-only proteins is controlled by several mechanisms. Expression of *C. elegans* EGL-1 is repressed by the transcription factor TRA-1A in neurons required for egg-laying (*6*). Certain mammalian BH3-only proteins are also subject to transcriptional regulation. For example, *noxa* and *puma* were discovered as p53-inducible genes and are therefore attractive candidates for mediating apoptosis in response to DNA damage (*7*–*9*). Several other mammalian BH3-only proteins can be regulated by different posttranslational mechanisms (*1*). Signaling pathways activated by certain growth factors induce phosphorylation of Bad, allowing 14-3-3 scaffold proteins to bind and sequester it from prosurvival Bcl-2 proteins (*10*). In contrast, Bim is normally sequestered to the microtubular dynein motor complex by binding to the 8-kD dynein light chain (DLC1, also called LC8 or PIN) (*11*). Certain apoptotic stimuli free Bim (together with DLC1), allowing it to translocate and bind to prosurvival Bcl-2 proteins, thereby activating the apoptotic program. This process occurs independently of the cell death executioner cysteine proteases (caspases) and therefore constitutes an upstream signaling event in apoptosis (*11*). These observations demonstrate that by their sequestration to specific sites within cells, different BH3-only proteins are uniquely placed to sense distinct forms of cellular stress.

We used mouse Mcl-1, an antiapoptotic Bcl-2 family member, as bait for a yeast two-hybrid screen of a mouse embryo cDNA library and isolated *bmf* (*bcl-2–modifying factor*; Fig. 1A) (*12*). Using mouse *bmf* cDNA as the probe, we cloned its highly similar human counterpart (87% amino acid identity; Fig. 1A) from a human T lymphocyte cDNA library (*12*). Detailed sequence analysis (*13*) revealed that Bmf has a BH3 domain most similar to that found in Bim, Bik, and EGL-1 (Fig. 1, A and B). In the yeast two-hybrid system, Bmf interacted with Mcl-1 and other prosurvival Bcl-2 proteins (Bcl-2, Bcl-x_L, and Bcl-w) but not with the proapoptotic family members tested (Bax, Bid, and Bad) (*14*). When transiently overexpressed in HEK293T cells, Bmf could be coimmunoprecipitated with prosurvival Bcl-2 family members Bcl-2 and Bcl-w (Fig. 1C), as well as with Bcl-x_L and Mcl-1, but did not bind the proapoptotic protein Bax or the BH3-only protein Bim (*14*). The interaction of Bmf with Bcl-2 or Bcl-w was greatly diminished by mutating the invariant leucine within its BH3 domain to alanine (L138A; Fig. 1C) (*15*). Mutations of conserved residues within the BH1 (G145E) or BH2 (W188A) domain of Bcl-2, which abolish its binding to Bim (*3*) or Bax (*16*), also disrupted binding to Bmf (*14*). Endogenous Bmf could be coimmunoprecipitated with endogenous Bcl-2 from detergent-lysed MCF-7 human breast carcinoma cells (Fig. 1D), excluding the possibility that these proteins associate only when overexpressed.

The biological activity of Bmf was investigated in transient transfectants of human Jurkat T lymphoma cells and stable transfectants of L929 mouse fibroblasts (Fig. 1E) or interleukin 3 (IL-3)–dependent FDC-P1 mouse promyelocytic cells (Fig. 2B). Expression of Bmf triggered apoptosis in ~80% of Jurkat cells within 24 hours (*14*) and reduced formation of L929 fibroblast colonies by about 65% (Fig. 1E). Bmf-induced apoptosis of Jurkat cells could be blocked by the caspase inhibitor, baculovirus p35, or by coexpression of Bcl-2 or its homologs (Bcl-x_L, Bcl-w, Mcl-1) but not by BH1 (G145E) or BH2 (W188A) mutants of Bcl-2 (*14*). Consistent with its proapoptotic activity, large amounts of Bmf could be expressed stably in FDC-P1 cells only when Bcl-2 (or one of its functional homologs) was also expressed. Such FDC-P1 cells coexpressing Bmf and Bcl-2 died more rapidly than did Bcl-2–expressing cells in response to cytokine withdrawal (Fig. 2B), γ-irradiation, or treatment with etoposide. In all the cell death assays performed, Bmf mutants that lacked the BH3 domain or had the L138A mutation were inert (Figs. 1E and 2B). These results establish that Bmf is a BH3-only protein that binds prosurvival Bcl-2 family members to initiate apoptosis.

The expression pattern of Bmf was investigated by Northern blotting, reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, and by protein immunoblotting. *bmf* mRNA was found in many cell lines of B and T lymphoid, myeloid, or fibroblastoid origin and in mouse embryos at all developmental stages from embryonic day 9 (E9) to birth [Web fig. 1A (*17*)]. Protein immunoblotting of cell lysates with specific antibodies (*18*) detected a single band corresponding to Bmf in many organs, with abundant proteins in pancreas, liver, kidney, and hematopoietic tissues [Web fig. 1B (*17*)]. To assess whether *bmf* expression was induced by apoptotic stimuli, RT-PCR

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analyses were performed of mRNA from thymocytes exposed to various forms of stress, including cytokine deprivation, γ-irradiation, or treatment with dexamethasone or ionomycin. None of these stimuli had any impact on *bmf* expression [Web fig. 1C (*17*)].

The lack of evidence for transcriptional control prompted us to investigate whether Bmf is regulated posttranslationally, perhaps by interacting with other proteins. A yeast two-hybrid screen of a mouse embryo cDNA library with Bmf as bait isolated 14 independent clones of Mcl-1 and more than 40 clones encoding dynein light chain (DLC clones) (*12*). In a previous screen, Bim had isolated exclusively DLC1 (*11*). In contrast, most dynein light chain clones interacting with Bmf encoded the closely related protein DLC2 (*19*). Coimmunoprecipitation experiments in transiently transfected HEK293T cells confirmed the interaction of Bmf with DLC2 (Fig. 2A). Sequence comparison (Fig. 1A) revealed a motif in Bmf (amino acids D^{67}KATQTLSP) that closely resembles the region in Bim (amino acids D^{51}KSTQTPSP) that mediates its binding to DLC1 (*11*, *20*). This appears to be the DLC-binding motif of Bmf, because mutations within it (A69P or D67A,K68A) abrogated the interaction of Bmf with DLC2 in yeast and in mammalian cells (Fig. 2A). Upon IL-3 deprivation or γ-irradiation, FDC-P1 cells coexpressing Bcl-2 and mutants of Bmf that do not bind DCL-2 died much more rapidly than those expressing both Bcl-2 and wild-type Bmf (Fig. 2B). These Bmf mutants also suppressed the formation of L929 fibroblast colonies more potently than did wild-type Bmf (*14*). Hence, interaction with DLC2 negatively regulates the proapoptotic activity of Bmf.

Through association with DLC1 or DLC2, respectively, Bim and Bmf may be sequestered to different sites within the cell to sense distinct stress signals. Because DLC forms part of the actin-based myosin V motor, as well as the microtubular dynein motor complex (*21*), we wondered whether Bmf and Bim might reside in separate cytoskeletal compartments. Indeed, when we separated cellular proteins into the filamentous actin and the paclitaxel (taxol)-polymerizable microtubular fractions (*22*), Bim and dynein intermediate chain (IC74) largely comigrated with microtubular components (P2), whereas Bmf and myosin V were confined to the filamentous actin–containing P1 fraction (Fig. 3A). Furthermore, treating cells with actin-depolymerizing agents, such as cytochalasin D or *Clostridium difficile* toxin B, released Bmf from the filamentous actin–containing P1 fraction whereas the fractionation of Bim was unaffected (Fig. 3B).

The distinct localization of Bmf and Bim may be determined largely by their preferred dynein light chain partners. Contrary to a previous report (*21*), we found that highly enriched myosin V motor complexes (*22*) contained DLC2 but not DLC1 (Fig. 3C). Thus Bmf, by being preferentially bound to DLC2, might be complexed with myosin V on filamentous actin rather than forming part of the dynein motor complex. Consistent with this notion, incubation of extracts from mouse spleen cells with recombinant Bmf and Bim confirmed that only Bmf associated with myosin V (Fig. 3D). Furthermore, Bmf and Bim showed distinct migration patterns after subcellular fractionation (*22*) of lysates from MCF-7 cells on sucrose gradients (Fig. 3E). Because DLC1 forms homodimers avidly and because it binds Bim and IC74 through the same region (*20*), one partner of a DLC1 homodimer probably interacts with IC74, whereas the other binds Bim, thereby sequestering Bim to the microtubular dynein motor complex. It is likely that DLC2 homodimers similarly sequester Bmf to filamentous actin by binding with one arm to Bmf and with the other to myosin V.

We investigated whether Bmf and Bim are activated by distinct apoptotic stimuli in cells that express both proteins endogenously. Ultraviolet (UV) irradiation of MCF-7 cells released Bim from association with the dynein motor complex. In response to UV irradiation, Bmf translocated from denser to lighter fractions (*22*) in sucrose-gradient centrifugation (Fig. 4A). Treatment of cells with paclitaxel, which polymerizes microtubules, released Bim but not Bmf (Fig. 4A). Consistent with a critical role

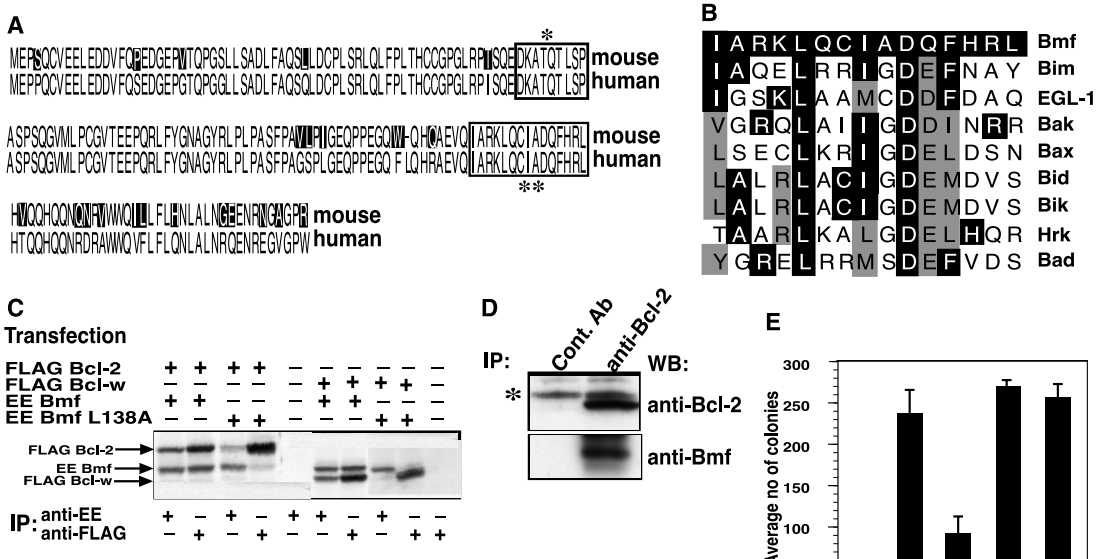

Fig. 1. Bmf, a novel mammalian BH3-only protein. (**A**) Predicted amino acid sequence of mouse and human Bmf. The conserved dynein light chain-binding motif is indicated by a box marked with a single asterisk (*). The short BH3 region, identified by hidden Markov modeling (*13*), is indicated by a box marked with two asterisks (**). (**B**) Alignment of the BH3 region of Bmf with that of some other proapoptotic Bcl-2 family members. Black boxes indicate identical amino acids; gray boxes indicate similar residues. (**C**) Wild-type Bmf, but not a BH3 mutant, binds to Bcl-2 and Bcl-w. HEK293T cells were transiently cotransfected with expression constructs for FLAG-tagged Bcl-2 (or Bcl-w) and EE (Glu-Glu)-tagged Bmf or L138A mutant Bmf. Twenty-four hours after transfection, the cells were metabolically labeled overnight with [^{35}S]methionine. Volumes of cell lysates with equivalent trichloroacetic acid (TCA)–precipitable ^{35}S counts were used for immunoprecipitations with mAbs to the FLAG or EE epitope tags. (**D**) Interaction of endogenous Bmf with Bcl-2 in MCF-7 cells. Lysates from 10^7 MCF-7 cells were immunoprecipitated with either Bcl-2-100 mAb (antibody against human Bcl-2) or an isotype-matched control mAb coupled to Sepharose. Bound proteins were eluted, size fractionated on SDS–polyacrylamide gel, subjected to electrophoresis (SDS-PAGE), and transferred onto nitrocellulose filters. Protein immunoblotting was performed with human Bcl-2 mAb or a rat Bmf mAb (9G10) (*18*). The asterisk (*) indicates the light chain of the mAb used for immunoprecipitation. (**E**) Wild-type Bmf, but not a BH3 mutant, kills L929 fibroblasts. L929 fibroblasts were transfected with empty vector, expression constructs for hygromycin resistance alone, or with wild-type Bmf, a BH3 mutant (L138A) of Bmf, or Bmf lacking its BH3 domain. Transfected cells were plated in medium containing hygromycin, and the resulting drug-resistant colonies were counted after 10 to 14 days. Values are means (±SD) of three independent experiments.

for Bim in this pathway to apoptosis, Bim-deficient thymocytes are abnormally resistant to the cytotoxic effects of paclitaxel (*4*). On the other hand, anoikis (absence of cell attachment and integrin signaling), an apoptotic stimulus that affects the actin cytoskeleton (*23*), resulted in the release of Bmf but not Bim (Fig. 4A). Because these experiments were conducted in the presence of the broad-spectrum caspase inhibitor zVAD-fmk at a concentration (50 μM) sufficient to block caspase activation, the release of Bmf or Bim, or both, is likely to represent an initiating event in apoptosis signaling rather than a consequence of apoptotic changes. Most important, endogenous Bmf (together with DLC2) released during anoikis could be coimmunoprecipitated with endogenous Bcl-2 isolated from mitochondria (Fig. 4B). In contrast, little if any Bmf was found complexed with Bcl-2 isolated from mitochondria of healthy cells.

Our data demonstrate that Bmf and Bim transduce distinct death signals caused by different forms of cell stress. They seem to represent sentinels mounted on the main cytoskeletal structures to monitor the well-being of the cell. Deregulated expression of Bcl-2 can promote tumorigenesis (*24*), thus it is possible that abnormalities in proapoptotic BH3-only proteins

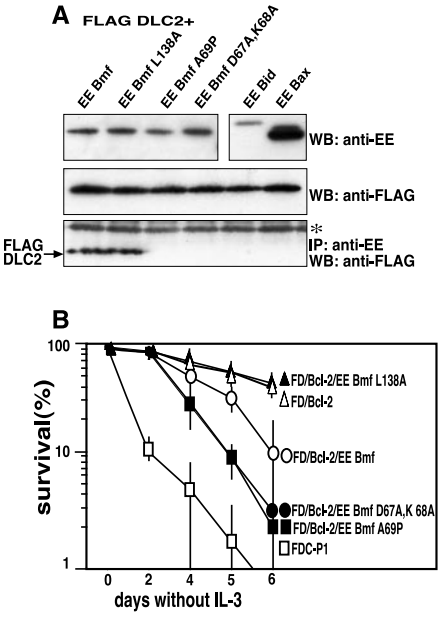

Fig. 2. Regulation of Bmf by interaction with DLC2. (**A**) Binding of Bmf to DLC2 through its dynein light chain–binding region. Coimmunoprecipitation experiments were done as described (Fig. 1C) with lysates of HEK293T cells transiently expressing FLAG-tagged DLC2 and EE-tagged wild-type (wt) Bmf, a BH3 mutant (L138A) of Bmf, dynein light chain–binding region mutants of Bmf (A69P or D67A,K68A), Bid, or Bax. The asterisk (*) indicates the light chain of the mAb used for immunoprecipitation. (**B**) Interaction with DLC2 regulates the proapoptotic potency of Bmf. FDC-P1 cells stably expressing Bcl-2 plus EE-tagged wt Bmf, a BH3 mutant (L138A) of Bmf or dynein light chain–binding region mutants of Bmf (A69P or D67A,K68A) were deprived of IL-3 for 1 to 6 days. Cell viability was assessed by propidium iodide staining and flow cytometric analysis. Values are means (±SD) of three independent experiments done with four independent clones of each genotype.

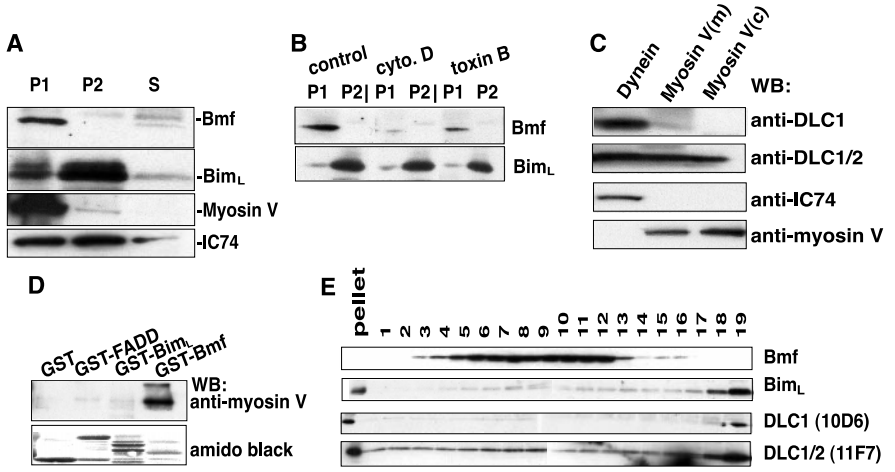

Fig. 3. Association of Bmf with the actin-based myosin V motor complex via DLC2. (**A**) Lysates from 10^7 MCF-7 cells were separated into F-actin–enriched P1, dynein-enriched P2, and supernatant fractions (*22*). Proteins from each fraction were size-fractionated by SDS-PAGE, transferred onto nitrocellulose, and probed with mAbs specific to Bmf (*18*), Bim$_L$ (*27*), myosin V (*29*), or dynein intermediate chain IC74 (Sigma). (**B**) MCF-7 cells were treated for 3 hours with cytochalasin D (10 μM), or toxin B (10 ng/ml), then fractionated and processed as described in (A). Presence of IC74 and Bim$_L$ in the F-actin–enriched P1 fraction represents contamination with microtubular components as evidenced by the fact that some Bim$_L$ can still be found in the P1 fraction even after treatment with cytochalasin D or toxin B. (**C**) Myosin V is associated mostly with DLC2. Cytoplasmic dynein was enriched from MCF-7 cells (*28*) and myosin V from mouse spleen (m) or chicken brain (c) (*30*). These fractions were analyzed by protein immunoblotting using rat mAb 11F7 or 10D6, which can be used to distinguish between DLC1 and DLC2 [Web fig. 2 (*17*)]. Nitrocellulose membranes were probed with antibodies to myosin V or IC74 (Sigma) to demonstrate purity of the myosin and dynein motor fractions. (**D**) Extracts from mouse spleen cells (200 μg of protein) were incubated for 3 hours at 4°C with recombinant glutathione *S*-transferase (GST) or GST-tagged FADD, Bmf, or Bim$_L$ proteins. Bound proteins were recovered on glutathione beads, eluted, size-fractionated by SDS-PAGE, and electroblotted onto nitrocellulose membranes, which were probed with an antibody to myosin V (*29*). The nitrocellulose membrane was stained with amido black (bottom panel) to document that comparable amounts of proteins were used in the pull-down experiments. (**E**) Lysates from 10^7 MCF-7 cells were fractionated through a 5 to 20% sucrose gradient (*11*). The pellet and soluble fractions were analyzed by protein immunoblotting for the presence of Bmf, Bim, DLC1, or DLC2.

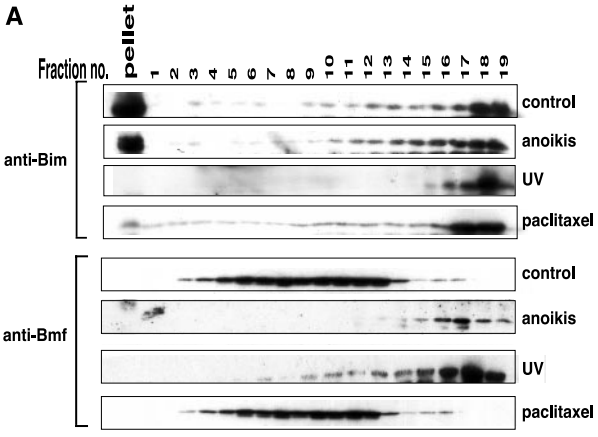

Fig. 4. Release of Bmf and Bim from their sequestration sites in response to distinct apoptotic stimuli. (**A**) MCF-7 cells were cultured in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (50 μM). Lysates from control (untreated) cells were compared with those from cells subjected to various apoptotic stimuli, including anoikis (culturing cells for 24 hours in suspension on poly-hema–coated bacteriological Petri dishes), UV irradiation (100 J/m^2), 1 μM paclitaxel. Lysates of 10^7 cells were fractionated through sucrose gradients (*22*). The sedimented (pellet) and soluble fractions were collected and analyzed by protein immunoblotting for Bmf and Bim. (**B**) During anoikis, Bmf translocates to mitochondria and binds to Bcl-2. Mitochondria were purified as described (*11*) from 2×10^8 healthy MCF-7 cells or cells subjected to anoikis. Mitochondrial proteins were extracted in lysis buffer containing 1% Triton X-100 (*11*). Immunoprecipitations were performed with mAb against human Bcl-2 bound to Sepharose beads. Bound proteins were eluted, size-fractionated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and probed with mAbs to Bcl-2, Bmf, DLC1, or DLC1 plus DLC2.

might also cause cancer. It is intriguing that the gene for human *bmf* is located on chromosome 15q14, the site of a candidate tumor suppressor gene lost in many metastatic, but not primary, carcinomas (*25*). Anoikis has been implicated as a barrier against metastatic tumor growth (*26*), raising the possibility that metastatic tumors harboring 15q14 mutations might have abnormalities in the expression or function of Bmf.

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12. The cDNA libraries from day 17 mouse embryos or from mouse embryos from embryonic day 9 to day 1 postpartum were prepared in pAD-GAL4-2.1 (HybriZAP-2.1 kit, Stratagene). The bait vector was made by cloning mouse *mcl*-1 lacking the sequences encoding its hydrophobic COOH-terminus into pGBT-9 (Clontech). Yeast transformation and plasmid rescue were performed as previously described (*11*). Of the 7×10^5 clones screened, one positive clone was obtained. Interaction between Mcl-1 and the novel protein was confirmed by β-galactosidase staining (*11*). Sequence analysis revealed that the clone was a partial one lacking the 5′ end. This partial clone was used as the probe to isolate full-length clones by screening a cDNA library derived from the $p53^{-/-}$ KO52DA20 thymoma cell line. Human *bmf* was isolated by screening a human, activated T cell cDNA library using mouse *bmf* as probe. To screen for Bmf-interacting proteins, mouse *bmf* was subcloned into a pGBT-9 derivative harboring the gene for chloramphenicol acetyltransferase as the selection marker. Out of 5×10^6 clones screened, 60 positive clones were initially selected.
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18. Rat monoclonal antibodies (mAbs) to dynein light chains or Bmf were generated as described (*27*). Antibodies were purified either on a protein-G column (Amersham Pharmacia) or on a Sepharose column conjugated with MAR 18.5 antibody (monoclonal mouse secondary antibody to rat Igκ). Monoclonal antibody 11F7 (rat γ2a/κ) recognizes mouse and human DLC1 and DLC2, whereas 10D6 (rat μ/κ) detects mouse and human DLC1 but not DLC2 [Web fig. 2 (*17*)]. Monoclonal antibodies 9G10 and 12E10 (both rat γ2a/κ) detect endogenous mouse and human Bmf by Western blotting and immunoprecipitation. To generate polyclonal antibodies against Bmf, New Zealand White rabbits were immunized with recombinant mouse Bmf. Sera were purified over a Sepharose column conjugated with recombinant mouse Bmf protein.
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22. For subcellular fractionation, MCF-7 cells (5×10^6) were lysed in 500 μl of extraction buffer containing 1% Triton X-100 (*28*). Cell debris and nuclei were removed by centrifugation at 2000*g*. The supernatant was centrifuged for 60 min at 4°C at 140,000*g* to obtain the filamentous actin–enriched P1 pellet fraction. The remaining supernatant was incubated for 13 min at 37°C with 2 mM paclitaxel and 5 U apyrase (Sigma). This mixture was then loaded on top of a 0.5-ml cushion of 7.5% sucrose (made in the extraction buffer) and centrifuged at 140,000*g* for 30 min at 30°C. Sedimented material was saved as the dynein-enriched P2 fraction and the supernatant was saved as the S fraction. Fractionation of cell extracts on sucrose gradients was done as described (*11*). Heavier fractions contain cytoskeletal proteins; lighter fractions contain soluble and intracellular membrane-associated proteins.
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Role of the Nonsense-Mediated Decay Factor hUpf3 in the Splicing-Dependent Exon-Exon Junction Complex

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Nonsense-mediated messenger RNA (mRNA) decay, or NMD, is a critical process of selective degradation of mRNAs that contain premature stop codons. NMD depends on both pre-mRNA splicing and translation, and it requires recognition of the position of stop codons relative to exon-exon junctions. A key factor in NMD is hUpf3, a mostly nuclear protein that shuttles between the nucleus and cytoplasm and interacts specifically with spliced mRNAs. We found that hUpf3 interacts with Y14, a component of post-splicing mRNA-protein (mRNP) complexes, and that hUpf3 is enriched in Y14-containing mRNP complexes. The mRNA export factors Aly/REF and TAP are also associated with nuclear hUpf3, indicating that hUpf3 is in mRNP complexes that are poised for nuclear export. Like Y14 and Aly/REF, hUpf3 binds to spliced mRNAs specifically (~20 nucleotides) upstream of exon-exon junctions. The splicing-dependent binding of hUpf3 to mRNAs before export, as part of the complex that assembles near exon-exon junctions, allows it to serve as a link between splicing and NMD in the cytoplasm.

Eukaryotic cells have a conserved surveillance mechanism that serves to ensure that only correctly processed mRNAs will be translated to produce proteins (*1–4*). An important example of this is NMD, which selectively degrades mRNAs that contain premature termination codons, thus avoiding the production of potentially deleterious COOH-terminal–truncated proteins. For NMD, cells must have the capacity to distinguish premature stop codons from legitimate, wild-type stop codons. In mammalian cells, the legitimate stop codon is almost always found on the last exon in the mRNA. If translation terminates more than ~50 to 55 nucleotides (nt) upstream of the last exon-exon junction, the mRNA is subject to rapid decay. This suggests that a mechanism must exist to define exon-exon junctions on mRNAs in the cytoplasm. Such a mechanism would be established by splicing in the nucleus and would persist on the mRNA in the cytoplasm at least through the first round of translation, because translation is required to trigger NMD. Although nucleus-associated NMD has also been suggested (*5–9*), it is likely that mRNA degradation takes

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