

Regulation of heat-induced apoptosis by Mcl-1 degradation and its inhibition by Hsp70

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Cellular stress eliminates irreversibly damaged cells by initiating the intrinsic death pathway. Cell stress is sensed by pro- and antiapoptotic members of the Bcl-2 protein family, which regulate the release of apoptogenic factors, such as cytochrome *c*, from mitochondria. Exposure of cells to hyperthermia results in the activation of the proapoptotic Bcl-2 family protein Bax, which plays an essential role in cytochrome *c* release. Heat directly affects Bax activity *in vitro*; however, antiapoptotic Bcl-2 family proteins, such as Bcl-xL, can suppress this activation, suggesting that a second heat-sensitive step must be breached before apoptosis ensues in cells exposed to hyperthermia. Here we show that heat shock causes the loss of Mcl-1 protein. Depletion of Noxa by short hairpin RNA protected cells from hyperthermia by preventing Mcl-1 degradation. Heat shock caused the dissociation of Noxa from Mcl-1, which allowed binding of the BH3-containing ubiquitin ligase Mule followed by Mcl-1 ubiquitination and degradation. Overexpression of Hsp70, which prevents heat-induced Bax activation, stabilized Mcl-1 protein levels in heat-shocked cells. This resulted from reduced Mule binding and ubiquitination as well as enhanced Mcl-1 expression compared with cells without Hsp70. Our results demonstrate that loss of Mcl-1 is a critical heat-sensitive step leading to Bax activation that is controlled by Hsp70.

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Stress-induced cell death is controlled by pro- and antiapoptotic members of the Bcl-2 protein family. Interactions between these proteins regulate the release of cytochrome *c* from mitochondria, which binds cytosolic Apaf-1 leading to its oligomerization into a caspase-activating complex called the apoptosome. Proteolytic processing of procaspase-3, by apoptosome-associated caspase-9, leads to its activation. This effector caspase cleaves specific proteins that ultimately result in cellular inactivation and apoptotic body formation.¹ The release of cytochrome *c* from mitochondria occurs through channels formed by oligomerization of the proapoptotic Bcl-2 family members Bax and Bak.² This step is inhibited by the antiapoptotic Bcl-2 family members Bcl-2, Bcl-xL, Mcl-1 and A1.³ The ability of these proteins to suppress Bax/Bak activation is negatively regulated by proapoptotic BH3-only-containing Bcl-2 family members, of which there are eight in humans, including Noxa, Puma, Bid, Bim and Bad. The BH3-only proteins act as stress sensors, which when activated either by increased expression or post-translational modification, suppress the ability of the antiapoptotic Bcl-2 family proteins to repress Bax and Bak.⁴ In addition to this indirect mechanism of Bax/Bak activation, some BH3-only proteins (Bim and Bid) are thought to act directly on Bax and Bak by promoting a conformational change necessary for mitochondrial membrane insertion (Bax) and oligomerization (Bax/Bak).⁵

Exposure of lymphoid cells to mild hyperthermia triggers apoptosis through Bax activation.⁶ Bax and Bak are essential for heat-induced apoptosis, as cells lacking both proteins do not release cytochrome *c* when exposed to hyperthermia.⁷ Heating purified mitochondria or recombinant Bax *in vitro* can directly trigger the conformational change in Bax and Bak that leads to oligomerization and cytochrome *c* release. However, the addition of cytosol or the cytosolic antiapoptotic protein Bcl-xL inhibited heat-activated Bax and Bak,⁷ indicating that a second heat-sensitive step must be breached before apoptosis ensues. In support of this, when cells are exposed to hyperthermia, maximal Bax activation and cytochrome *c* release do not occur until several hours after the heated cells are returned to 37°C.⁶ In this study, we sought to determine whether the inactivation of the antiapoptotic protein Mcl-1 was responsible for this second heat-sensitive step. The rationale for this is that unlike Bcl-2 and Bcl-xL, Mcl-1 has a short half-life allowing rapid changes in protein levels in response to stress or survival signaling.⁸

Mcl-1 is essential for the normal development of the immune system. It is often overexpressed in malignant cells including follicular lymphoma and multiple myeloma, in which antisense-mediated downregulation of Mcl-1 was sufficient to induce apoptosis.^{9,10} The expression of Mcl-1 is transcriptionally controlled by growth factor and cytokine signaling pathways. Regulation of Mcl-1 function can occur through

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate hydrate; DEVDase, protease activity toward Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin)); EGS, ethylene glycol bis-(succinimidyl succinate); IP, immunoprecipitation; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; UV, ultraviolet

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alternative splicing to produce an Mcl-1L antiapoptotic isoform and a BH3-only Mcl-1S proapoptotic isoform.¹¹ Caspase cleavage within the N-terminal domain can also generate a proapoptotic protein.¹⁰ Mcl-1 is eliminated by proteasomal degradation in response to genotoxic stress, and this loss of Mcl-1 is essential for Bax/Bak oligomerization and caspase activation.¹² Mcl-1 is targeted for proteasomal degradation by the BH3-containing E3 ligase Mule (aka: Ureb1, Lasu1, HUWE1 and ARF-BP1).¹³ Removal of growth factor signaling can trigger Mcl-1 elimination through glycogen synthase kinase (GSK)-3-mediated phosphorylation of Mcl-1 serine-159.¹⁴ Mcl-1 function has also been shown to be regulated by JNK-catalyzed phosphorylation.¹⁵ Loss of Mcl-1 promotes apoptosis by preventing the sequestration of proapoptotic Bcl-2 family proteins Bak and Bim.^{16,17} The overexpression of Noxa displaces Bim from Mcl-1 and leads to the depletion of Mcl-1 protein.^{18,19} Bim, on the other hand, stabilizes Mcl-1 by preventing its interaction with Mule.^{18,20}

Here we demonstrate a role for Mcl-1 in the regulation of heat-induced apoptosis. The reduction of Noxa protein levels by short hairpin RNA (shRNA) expression stabilized Mcl-1 and protected cells from heat-induced apoptosis. We found that hyperthermia reduced Mcl-1 synthesis, stimulated Mule binding and ubiquitination as well as caspase cleavage of Mcl-1. Overexpression of Hsp70 inhibited heat-induced apoptosis by preventing the loss of Mcl-1. This occurred as a result of a combination of continued Mcl-1 synthesis, inhibition of Mule binding and ubiquitination as well as prevention of caspase cleavage.

Results

Noxa depletion inhibits heat-induced apoptosis. The human acute lymphoblastic T-cell line PEER undergoes apoptosis during incubation at 37°C after exposure to hyperthermia.²¹ Overexpression of Hsp70 prevents heat-induced Bax activation, cytochrome *c* release and caspase activation in these cells.^{6,22,23} To determine the events upstream of Bax activation that are induced by hyperthermia, we examined the role of the BH3-only protein Noxa in heat-induced apoptosis. For this, we produced PEER cell lines stably expressing a Noxa shRNA (clones N12, N14 and NG7) or an irrelevant shRNA (S13) using the pSUPER shRNA expression plasmid. In cells exposed to hyperthermia, Noxa protein levels decreased initially and then returned to higher than basal level by 6 h after the heat-shock treatment (Figure 1a). In the Noxa shRNA cell lines, basal levels of Noxa were undetectable and the level at 6 h after heat shock was greatly reduced compared with the parental or control shRNA cell line. As Noxa has been shown to regulate Mcl-1 levels we also examined the effect of hyperthermia on Mcl-1 in these cell lines. Heat shock caused a decrease in the levels of Mcl-1L in both the parental and control shRNA cell line. In contrast, basal levels of Mcl-1 were elevated in the Noxa shRNA cell lines and these levels were maintained in cells exposed to hyperthermia.

Depletion of Noxa therefore prevents the heat-induced loss of Mcl-1. To determine whether this confers increased heat resistance, cells were exposed to hyperthermia and assayed

for protease activity toward Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin) (DEVDase activity) (Figure 1b) and scored for the presence of apoptotic cells (Figure 1c). Compared with the control shRNA cell line, both caspase activation and the apoptotic cell count were reduced in the Noxa shRNA cell lines. Noxa has been shown to play a role in ultraviolet (UV)-induced apoptosis, as Noxa-deficient mouse fibroblasts are resistant to UV treatment.²⁴ The Noxa shRNA cell lines also showed reduced caspase activation in response to UV exposure (Figure 1d). Long-term survival assays demonstrate enhanced resistance to UV or hyperthermia in Noxa-depleted cells (Figure 1e). Noxa reduction therefore does not simply delay apoptosis in the stressed cells.

Noxa depletion inhibits heat-induced Bax activation. Bax activation proceeds through an initial priming step that alters its conformation, allowing mitochondrial membrane insertion followed by oligomerization, producing channels that permit the release of cytochrome *c*. The antiapoptotic Bcl-2 family proteins negatively regulate this oligomerization step. Exposure of cells to hyperthermia induces a conformational change in Bax that permits its translocation and oligomerization in the mitochondrial outer membrane.⁶ We tested whether Noxa depletion could affect either of these steps in heat-induced Bax activation. The stress-induced conformational change in Bax was assessed by immunoprecipitation (IP) with an antibody specific for the active form of Bax followed by western blotting. Bax oligomerization was measured by treating extracts with the chemical cross-linker ethylene glycol bis-(succinimidyl succinate) (EGS) followed by western blotting. Depletion of Noxa significantly reduced Bax activation (Figure 2a) and oligomerization (Figure 2b) in cells exposed to hyperthermia.

Willis *et al.*¹⁷ demonstrated that the binding of Noxa to Mcl-1 caused the displacement of Bak leading to Mcl-1 degradation. We therefore examined the influence of Noxa expression on the interaction of Mcl-1 with Bax and Bak in cells exposed to hyperthermia. Mcl-1 was immunoprecipitated from control and heat-shocked control shRNA- and Noxa shRNA-expressing cells followed by western blotting (Figure 2c). Exposure to hyperthermia altered the composition of Mcl-1 complexes. Increased levels of Bax and BimEL were found associated with Mcl-1 immediately after the heat-shock exposure, and this coincided with a reduced association of Noxa with Mcl-1. The increased Mcl-1/Bax association was only transient with the release of Bax occurring at the same time as Bax oligomerization was taking place. Noxa reassociated with Mcl-1 during post-heat-shock incubation at 37°C with high levels of association occurring after 6 h when the total levels of Noxa were enhanced. The association of Bak and BimEL with Mcl-1 decreased over this time period. In contrast to the control shRNA-expressing cells, when the Noxa shRNA-expressing cells were exposed to heat shock, Mcl-1 maintained higher levels of association with Bak and BimEL.

Hsp70 overexpression prevents the loss of Mcl-1 in heat-shocked cells. As Hsp70 is a potent suppressor of Bax activation,⁶ we next examined the effect of Hsp70

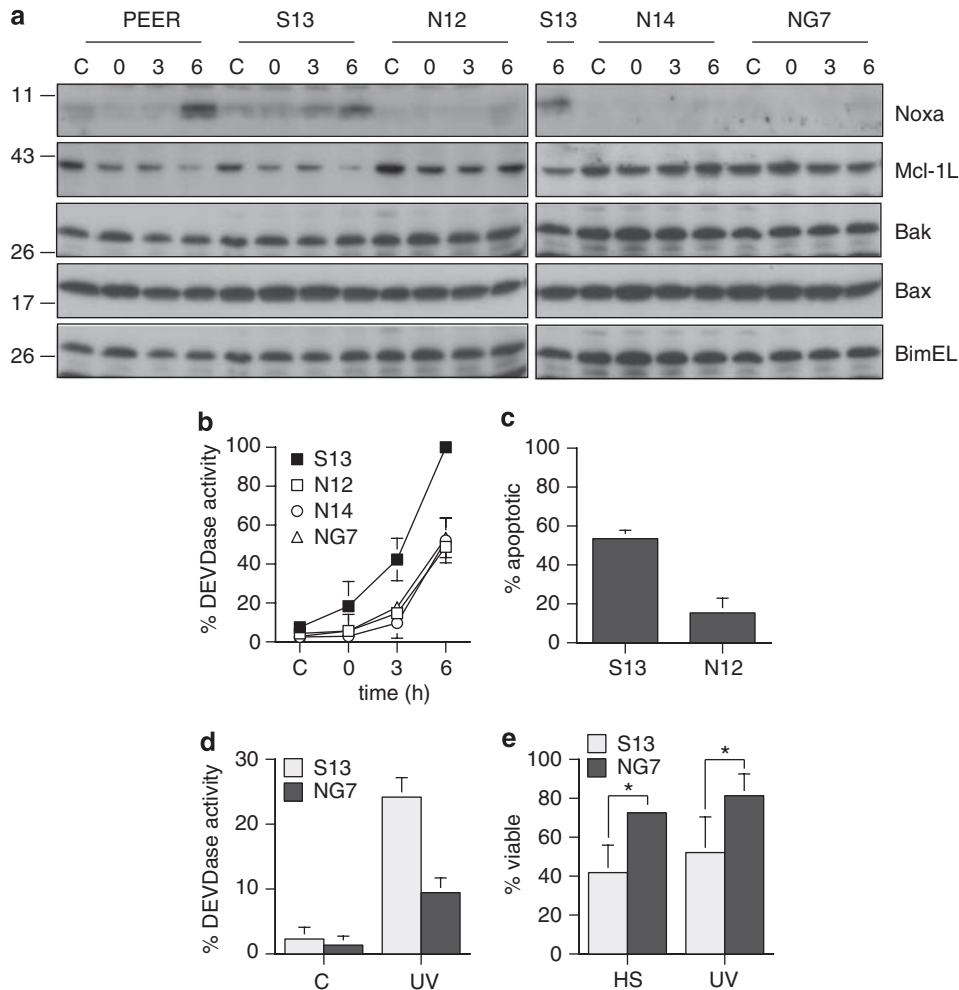


Figure 1 Depletion of Noxa by RNA interference prevents heat-induced apoptosis. (a) Expression of Noxa shRNA reduces basal Noxa protein levels and prevents the heat-induced expression of Noxa. Parental PEER, control shRNA clone S13 and Noxa shRNA clones N12, N14 and NG7 were either untreated (C) or subjected to hyperthermia for 1 h at 43°C and collected immediately (0) or after an additional incubation at 37°C for 3 or 6 h. The abundance of Noxa, Mcl-1, Bak, Bax and Bim was examined in total cell lysates by western blotting. (b) Expression of Noxa shRNA reduces hyperthermia-induced caspase-3 activity. DEVDase activity was examined in lysates of cells from clones S13, N12, N14 and NG7 that were treated as in (a). DEVDase activity is plotted relative to the maximum value (mean \pm S.D., $N=3$). (c) Expression of Noxa shRNA reduces hyperthermia-induced apoptosis. The S13 and N12 clones were exposed to 43°C for 1 h and then incubated at 37°C for 6 h. Cell nuclei were stained with acridine orange and the number of apoptotic and viable cells was counted (mean \pm S.D., $N=2$). (d) Expression of Noxa shRNA reduces UV-induced caspase-3 activation. DEVDase activity was measured in lysates of S13 and NG7 cell clones that were exposed to UV (40 kJ/m²) followed by a 6 h incubation at 37°C (mean \pm S.D., $N=3$). (e) Expression of Noxa shRNA increases long-term viability of cells exposed to UV or hyperthermia. S13 and NG7 clones were exposed to 43°C for 1 h or UV irradiation at 40 kJ/m² and then incubated at 37°C for 72 h. Viability is shown as the mean percentage (\pm S.D., $N=3$) of values for treated cells relative to untreated cells (* $P<0.05$)

overexpression on Mcl-1 in cells exposed to hyperthermia. For this, we used a stably transfected PEER cell line with tetracycline-regulated expression of Hsp70.²³ The loss of Mcl-1 that was seen when the non-induced cells (OFF) were exposed to hyperthermia was prevented when Hsp70 was overexpressed in the induced cells (ON) (Figure 3a). Loss of Mcl-1 occurred at approximately the same time as Bax activation and caspase-3 processing in the non-induced cells. Mcl-1 is subject to caspase-3-mediated cleavage at Asp127 generating a 28-kDa fragment.²⁵ Hsp70 overexpression, which prevents caspase-3 activation in heat-shocked cells,²² prevented both the loss of Mcl-1L and the generation of the Mcl-1 cleavage product. Noxa protein

levels were reduced immediately after exposure to hyperthermia and then increased to a higher-than-basal level during incubation at 37°C. This occurred in both the non-induced and induced cells; however, the initial loss was somewhat less in the Hsp70-expressing cells.

Heat shock is a potent activator of the stress kinase JNK, which has been shown to phosphorylate and inhibit the activity of Mcl-1.¹⁵ As Hsp70 is able to suppress heat-induced JNK activation,^{22,26} we examined whether the inhibition of JNK by treatment with the specific inhibitor SP600125 could also prevent the loss of Mcl-1 in heat-shocked cells. Similar to Hsp70 overexpression, SP600125-treated cells maintained high levels of Mcl-1 following exposure to hyperthermia

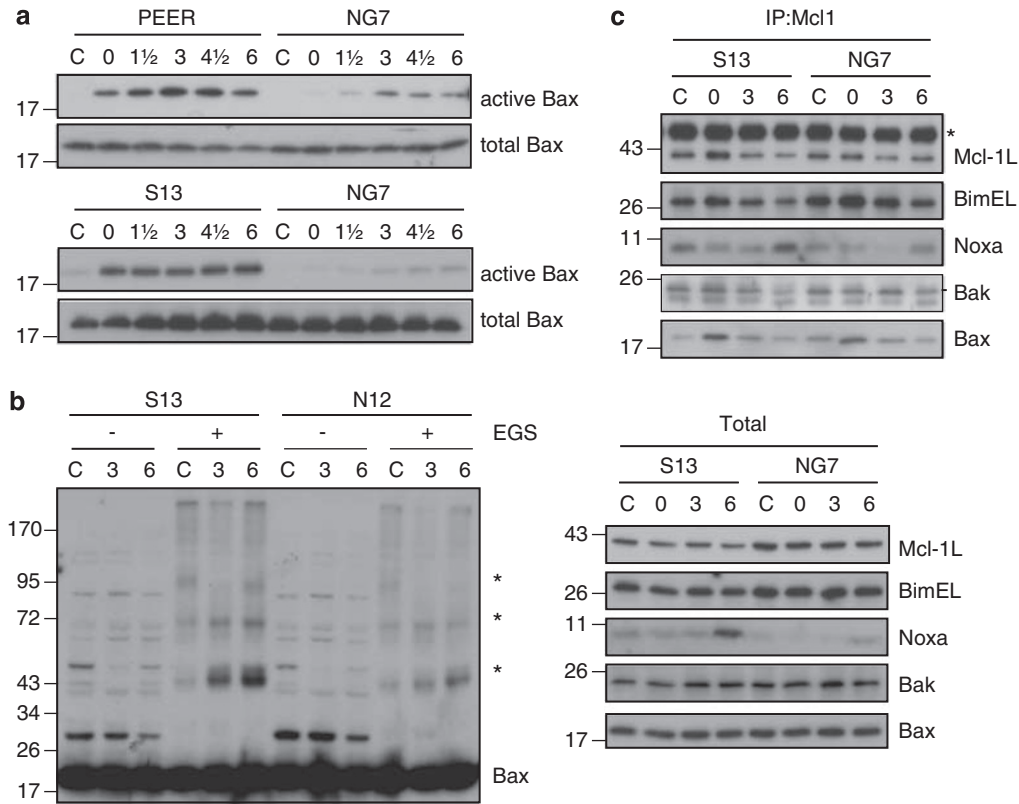


Figure 2 Noxa depletion inhibits heat-induced Bax activation and oligomerization resulting in Mcl-1 stabilization and increased association of Bim and Bak with Mcl-1. (a) Parental (PEER), control shRNA (S13)- and Noxa shRNA (NG7)-expressing cells were exposed to 43°C for 1 h and then incubated at 37°C for the indicated periods of time (h). Bax activation was examined by immunoprecipitation with a conformation-specific antibody. The amount of active Bax and total Bax (total lysate) was analyzed by western blotting with an anti-Bax antibody. (b) Control shRNA (S13)- and Noxa shRNA (N12)-expressing cells were exposed to 43°C for 1 h and then incubated for 3 or 6 h at 37°C. Cells were lysed in CHAPS lysis buffer and treated with EGS to cross-link interacting proteins. Cross-linked (EGS +) and non-treated samples (EGS-) were analyzed by SDS-PAGE and by immunoblotting with an anti-Bax antibody. Asterisks indicate multimeric forms of Bax. (c) Control shRNA (S13)- and Noxa shRNA (NG7)-expressing cells were exposed to 43°C for 1 h and then incubated for 3 or 6 h at 37°C. Cells were lysed in CHAPS lysis buffer and equivalent amounts of protein from each lysate were used for immunoprecipitation with an anti-Mcl-1 antibody. The immunoprecipitated proteins and total extracts were analyzed by SDS-PAGE and by immunoblotting with anti-Mcl-1, Bim, Noxa, Bak and Bax antibodies (*IgG)

(Figure 3b). SP600125 treatment delayed JNK phosphorylation and inhibited caspase-3 activation. These results suggest that the inhibition of Mcl-1 degradation by Hsp70 may be in part attributable to its ability to inhibit JNK activation, although the role of JNK phosphorylation in the regulation of Mcl-1 protein function in heat-shocked cells remains to be determined.

We also examined whether Hsp70 overexpression could prevent Mcl-1 degradation in cells exposed to UV. Exposure to UV resulted in the loss of Mcl-1 protein in both the non-induced and the Hsp70-expressing cells (Figure 3c). Hsp70 overexpression was unable to prevent caspase-3 activation and apoptosis in the UV-exposed cells. This is in contrast to Noxa suppression, which was able to protect cells from either hyperthermia or UV exposure (Figure 1e).

Heat shock causes increased ubiquitination and caspase cleavage of Mcl-1. To determine whether the loss of Mcl-1 was due to proteasomal degradation, we examined Mcl-1 protein levels in cells exposed to hyperthermia in the presence of the proteasomal inhibitor

MG132 (Figure 4a). Treatment with MG132 increased the basal level of Mcl-1 and prevented the loss of Mcl-1 after exposure to heat shock, but did not prevent the generation of the caspase-3-cleaved Mcl-1 fragment. These results show that the loss of Mcl-1L in heat-shocked cells is due to both proteasomal and caspase-mediated degradation. Interestingly, levels of BimEL were also reduced as apoptosis progressed but this was prevented by MG132, illustrating that BimEL is also subject to proteasomal degradation.²⁷ This effect of proteasomal inhibition on BimEL accumulation may explain why caspase-3 activation occurred when the Hsp70-expressing cells were treated with MG132.

Exposure to heat shock caused the accumulation of ubiquitinated proteins; however, this accumulation did not occur in cells overexpressing Hsp70 (Figure 4a). To determine whether ubiquitinated Mcl-1 accumulated in cells exposed to hyperthermia, we immunoprecipitated Mcl-1 under denaturing conditions and probed for ubiquitin. A progressive increase in the amount of polyubiquitinated Mcl-1 was seen in the non-induced cells exposed to heat

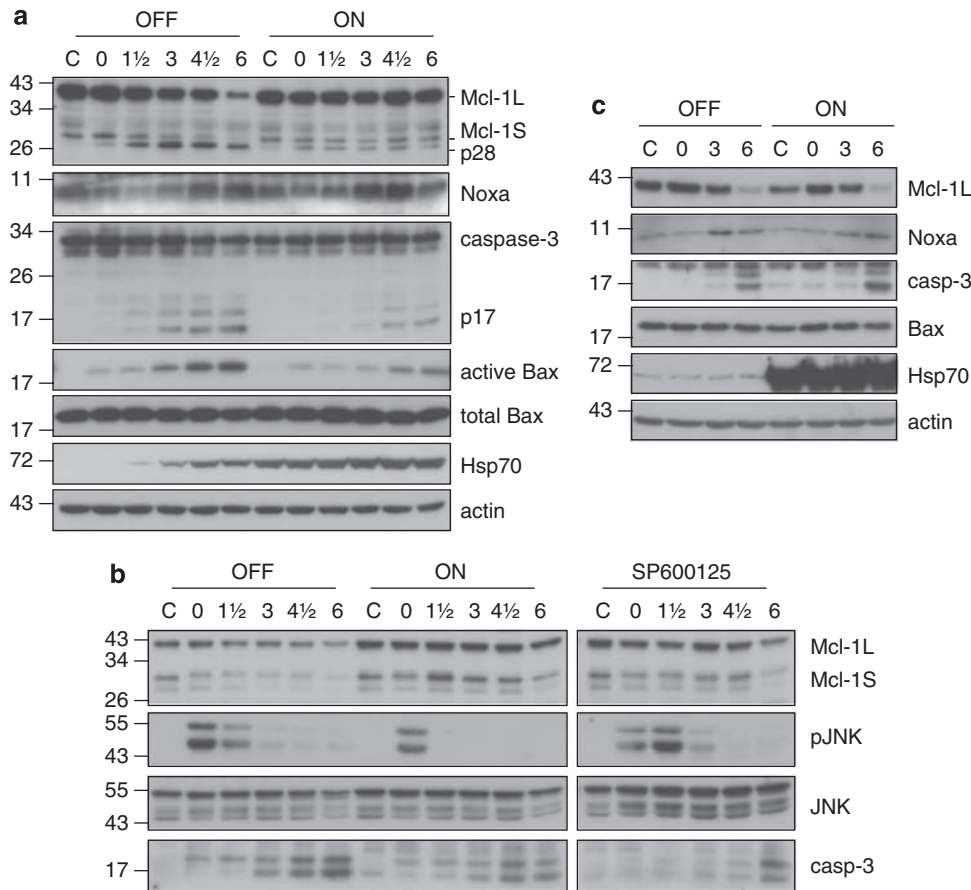


Figure 3 Hsp70 overexpression prevents heat-induced Mcl-1 degradation. **(a)** PERA70 cells with tetracycline-regulated expression of Hsp70 (OFF = non-induced, ON = induced) were exposed to 43°C for 1 h and then incubated at 37°C for the indicated periods of time (h). Lysates containing equivalent amounts of protein were separated by SDS-PAGE and the levels of Mcl-1, Noxa, caspase-3, Bax, Hsp70 and actin were detected by immunoblotting. Active Bax was measured by immunoprecipitation with a conformation-specific anti-Bax antibody followed by western blotting. **(b)** JNK inhibition prevents Mcl-1 degradation. PERA70 cells that were either non-induced, induced to express Hsp70 or treated with the JNK inhibitor SP600125 (20 μM) were exposed to 43°C for 1 h and then incubated at 37°C for the indicated periods of time (h). Lysates containing equivalent amounts of protein were separated by SDS-PAGE and the levels of Mcl-1, pJNK, JNK and caspase-3 (p17 fragment) were detected by immunoblotting. **(c)** Hsp70 overexpression does not prevent Mcl-1 degradation in cells exposed to UV. Non-induced and induced PERA70 cells were exposed to UV (40 kJ/m²) followed by incubation at 37°C for the times indicated (h). Lysates were separated by SDS-PAGE and the levels of Mcl-1, Noxa, caspase-3 (p17 fragment), Bax, Hsp70 and actin were detected by immunoblotting.

shock but not in cells overexpressing Hsp70 (Figure 4b). We next examined whether the rate of Mcl-1 protein turnover was affected by heat-shock treatment. For this experiment, cells were exposed to hyperthermia and then incubated at 37°C for 3 or 6 h in the presence of cycloheximide. The pan-caspase inhibitor zVAD was included to allow the measurement of protein turnover in the absence of caspase cleavage. The half-life of Mcl-1 at 37°C was approximately 3 h in both the non-induced and the induced cells (Figure 4c), similar to what has been reported in other cell lines.²⁸ Some loss of Mcl-1 occurred during the 1 h exposure to 43°C in the absence of cycloheximide (compare HS: C and 0). When the cells were then returned to 37°C and incubated with cycloheximide the rate of Mcl-1 turnover was unaltered in the Hsp70-expressing cells; however, in the non-induced cells, the loss of Mcl-1 was delayed. This is likely due to the excessive load of misfolded and ubiquitinated proteins in the non-induced cells. The ability of Hsp70 to assist in the chaperoning of ubiquitinated proteins

to the proteasome²⁹ allowed for the efficient clearance of ubiquitinated Mcl-1. In the absence of new protein synthesis, actin was also depleted after heat shock in the Hsp70-expressing cells but not in the non-induced cells, illustrating the effect of Hsp70 overexpression on the clearance of ubiquitinated substrates. When cells were incubated at 37°C after heat shock in the absence of cycloheximide, but in the presence of zVAD, Mcl-1 was still lost from the non-induced cells but not from the Hsp70-expressing cells, indicating that heat-shock treatment reduced the rate of new Mcl-1 synthesis in the non-induced cells but not in the cells expressing Hsp70. Hsp70 has been shown to provide transcriptional and translational tolerance to cells exposed to hyperthermia.³⁰ Altogether, these results show that the loss of Mcl-1 in heat-shocked cells is due to the combination of reduced synthesis, ubiquitination/proteasomal degradation and caspase cleavage. Each of these effects is suppressed in cells expressing Hsp70, resulting in the maintenance of Mcl-1 levels.

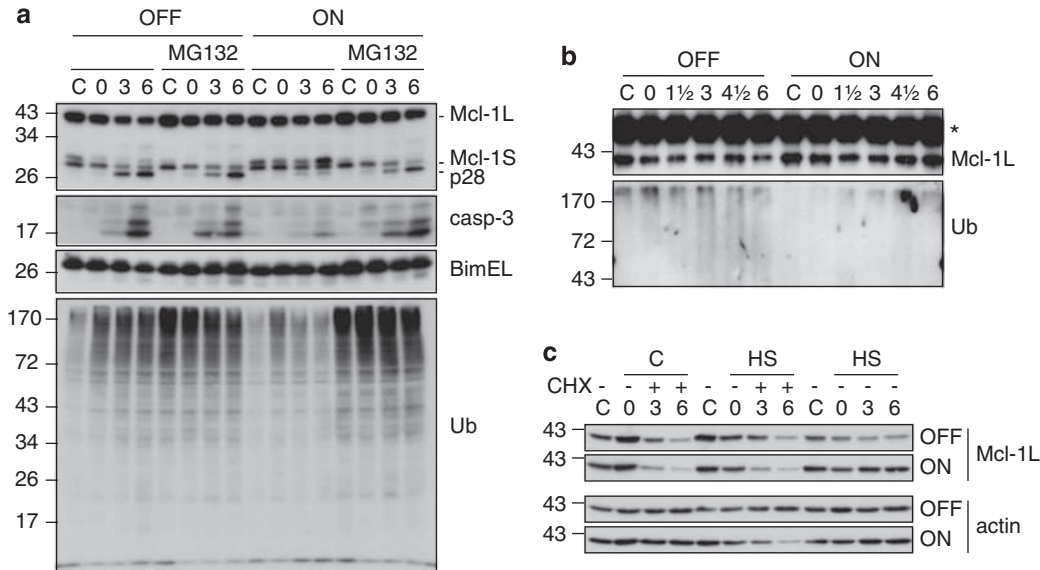


Figure 4 Hsp70 overexpression prevents heat-induced Mcl-1 ubiquitination. (a) Exposure of cells to the proteasome inhibitor MG132 prevents the heat-induced loss of Mcl-1L. Non-induced and induced PE τ A70 cells were treated with 10 μ M MG132 and exposed to 43°C for 1 h and then incubated for 0, 3 or 6 h at 37°C. Lysates containing equivalent amounts of protein were separated by SDS-PAGE and the levels of Mcl-1, caspase-3 (p17 fragment), Bim and ubiquitin (Ub) were detected by immunoblotting. (b) Accumulation of ubiquitinated Mcl-1. PE τ A70 cells were exposed to hyperthermia (43°C for 1 h) and incubated at 37°C for the indicated periods of time (h). Cells were lysed in denaturing IP buffer and equivalent amounts of protein from each lysate were used for immunoprecipitation with an anti-Mcl-1 antibody. The immunoprecipitated proteins were separated by SDS-PAGE and levels of Mcl-1 and ubiquitinated Mcl-1 (Ub) were detected by immunoblotting (*IgG). (c) Measurement of Mcl-1 turnover. PE τ A70 cells were incubated with 10 μ M zVAD for 30 min and then exposed to 43°C for 1 h. Cells were either collected immediately (0) or incubated at 37°C for 3 or 6 h in the presence of 200 μ g/ml cycloheximide. Lysates containing equivalent amounts of protein were separated by SDS-PAGE and the levels of Mcl-1 and actin were detected by immunoblotting

Hsp70 prevents the association of Mule with Mcl-1. We next examined the effect of Hsp70 overexpression on the composition of Mcl-1 complexes in cells exposed to hyperthermia. Mcl-1 was immunoprecipitated from control and heat-shocked cells, and lysates were probed for the presence of Noxa, Bim, Bax and Bak (Figure 5a). Noxa was found to be associated with Mcl-1 in non-stressed cells; however, this complex was disrupted in cells exposed to hyperthermia. Reformation of this complex occurred following the resynthesis of Noxa. Cells expressing Hsp70 showed a similar pattern except that Noxa reassociated with Mcl-1 earlier reflecting the earlier increase in total Noxa levels. The association of Bak with Mcl-1 steadily decreased after heat shock. Although Bax initially showed increased binding to Mcl-1 immediately after exposure to hyperthermia, this association was only transient. Interestingly, cells expressing Hsp70 had higher levels of Bax and Bak bound to Mcl-1, and exposure to heat shock did not result in an increased binding of Bax to Mcl-1 as it did in the non-induced cells. This indicates that Hsp70 might help sequester activated Bax through Mcl-1 binding in control cells and also inhibit the heat-induced conformational change in Bax. Nevertheless, the interaction of Mcl-1 with Bax was transient with the released Bax forming oligomers in the non-induced cells but not in the Hsp70-expressing cells.⁶

As the unligated form of Mcl-1 is a target for Mule binding,²⁰ we next asked whether heat-induced disruption of the complex containing Mcl-1 and Noxa resulted in increased binding of Mule to Mcl-1. This was examined immediately after exposure of cells to hyperthermia. Immunoprecipitation of

Mcl-1 under non-denaturing conditions, to probe for interacting proteins, showed that heat shock increased the association of Mule with Mcl-1 and that this was inhibited in cells expressing Hsp70 (Figure 5b). We also examined these immunoprecipitates for the presence of Hsc70 and Hsp70. Hsc70 was associated with Mcl-1 in heat-shocked cells, whereas Hsp70 was bound in both the control and heat-shocked cells in the Hsp70-expressing cells. To test the specificity of these interactions, we performed IPs in the presence of excess Mcl-1-specific peptide and an irrelevant peptide (Figure 5c). The presence of excess Mcl-1 peptide prevented Mcl-1 IP, resulting in a loss of the Mule, Noxa and Hsc70 signals. Excess of Bax peptide did not prevent Mcl-1 IP and did not affect the co-IP of Mule or Noxa with Mcl-1, although the signal for Hsc70 was somewhat reduced in the presence of excess Bax peptide. Co-IP of Hsp70 with Mcl-1, using extracts from the Hsp70-expressing cells, could not be competed by the presence of excess Mcl-1 peptide, suggesting that this interaction may not be Mcl-1 specific (data not shown).

In summary, these results support a model in which heat shock promotes Bax activation through depletion of Mcl-1 (Figure 5d). Synthesis of Mcl-1 was inhibited in cells exposed to hyperthermia. Mule binding and subsequent ubiquitination inactivated the pre-existing Mcl-1. Depletion of Mcl-1 resulted in Bax activation, and the subsequent caspase activation aided in the complete destruction of Mcl-1. In the presence of excess Hsp70, ubiquitinated Mcl-1 did not accumulate and the heat-induced inhibition of Mcl-1 synthesis was averted as well, leading to the stabilization of Mcl-1 protein levels.

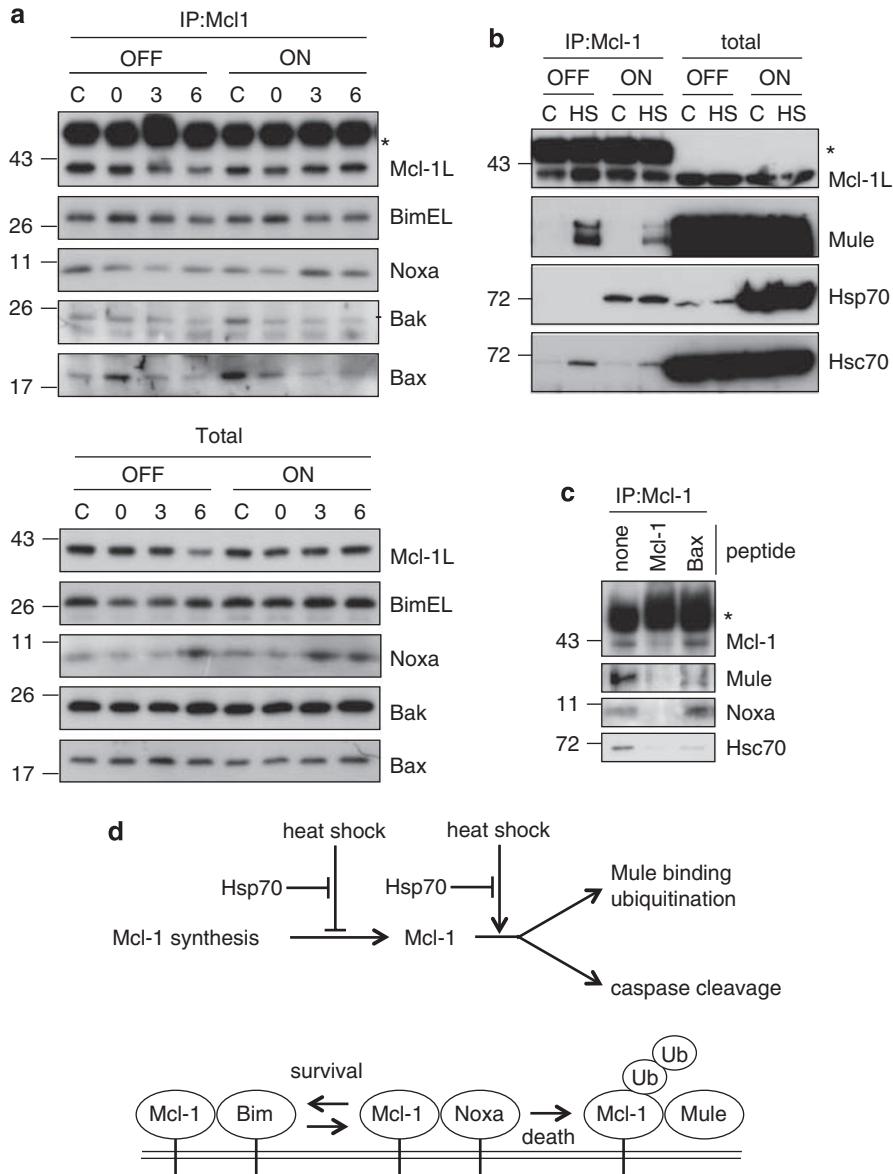


Figure 5 Heat-induced disruption of Noxa from Mcl-1 leads to Mule binding, ubiquitination and degradation of Mcl-1. **(a)** PERTA70 cells were exposed to 43°C for 1 h and then incubated for 3 or 6 h at 37°C. Cells were lysed in CHAPS lysis buffer and equivalent amounts of protein from each lysate were used for immunoprecipitation with an anti-Mcl-1 antibody. The immunoprecipitated proteins and total extracts were analyzed by SDS-PAGE and by immunoblotting with anti-Mcl-1, Bim, Noxa, Bak and Bax antibodies (*IgG). **(b)** Control and heat-shocked (43°C for 1 h) cells were lysed in CHAPS buffer and immunoprecipitated with anti-Mcl-1 antibody. The immunoprecipitated proteins and total extracts were analyzed by SDS-PAGE and immunoblotting with anti-Mcl-1, Mule, Hsc70 and Hsp70 antibodies. **(c)** Extracts from non-induced cells that were exposed to heat shock (43°C for 1 h) were immunoprecipitated in the presence of a fivefold excess of an antigen-specific peptide (Mcl-1) or a nonspecific peptide (Bax). The immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with anti-Mcl-1, Mule, Noxa and Hsc70 antibodies (*IgG). **(d)** Model showing Mcl-1 synthesis and the effect of hyperthermia on Mcl-1 protein levels (see text for details)

Discussion

Understanding the mechanisms controlling stress-induced apoptosis is central to the understanding of how cells and organisms respond to their environment. A universally conserved mechanism for providing adaptive tolerance to hyperthermia is the heat-shock response.³¹ Stress-induced expression of the heat-shock proteins, which assist in protein conformational homeostasis, allows cells to survive under conditions that threaten the folding integrity of cellular

proteins, which can occur during environmental insult, disease and aging.³² The major heat-inducible protein, Hsp70, plays an important role in stress resistance.³¹ How hyperthermia causes apoptosis and how Hsp70 suppresses this are not clear. Earlier, we have shown that the exposure of lymphoid cells to hyperthermia leads to the activation of Bax, a key step in the mitochondrial apoptotic pathway, and that Hsp70 overexpression inhibits heat-induced Bax activation and cell death.⁶ *In vitro* experiments have shown that conditions affecting protein stability, such as non-ionic

detergents, altered pH or hyperthermia can also activate Bax. Whether these physical treatments participate in the direct activation of Bax *in vivo* is not known. Nevertheless, the ability of heat-treated Bax to permeabilize mitochondria *in vitro* can be inhibited by the inclusion of cytosol or purified Bcl-xL.⁷ This suggests that a second heat-sensitive step, involving an antiapoptotic member of the Bcl-2 family, must be breached for hyperthermia to trigger Bax activation. Our results reveal that this second step is the degradation of Mcl-1, which allows Bax oligomerization to occur.

Elimination of Mcl-1 is an essential step in the initiation of apoptosis in response to DNA damage or growth factor withdrawal.^{14,33,34} Our results indicate that loss of Mcl-1 is also essential for apoptosis to proceed in response to proteotoxic stress. For both genotoxic stress and growth factor withdrawal, the rapid loss of Mcl-1 was due to the relatively short half-life of the Mcl-1 protein. Exposure to UV or etoposide resulted in an inhibition of Mcl-1 mRNA and protein synthesis, such that apoptosis ensued when the existing pool of Mcl-1 turned over without any affect on the rate of this turnover.³⁴ The inhibitory effect of hyperthermia on protein synthesis also contributed to the loss of Mcl-1 in heat-shocked cells. Hsp70 overexpression, which is capable of providing translational thermotolerance,³⁰ allowed for the continued replacement of the degraded Mcl-1 by new synthesis.

Mcl-1 protein turnover is regulated by phosphorylation. Following IL-3 deprivation, the loss of Mcl-1 was attributable to an increased rate of Mcl-1 protein degradation.¹⁴ This increase in proteasomal degradation was regulated by Mcl-1 phosphorylation by GSK-3, which was activated as a result of reduced Akt phosphorylation in growth factor-deprived cells. Phosphorylation of Mcl-1 serine-159 by GSK-3 was required for Mcl-1 ubiquitination and proteasomal degradation. Ubiquitination of Mcl-1 is mediated by the E3 ligase Mule (ARF-BP1), which interacts with Mcl-1 through its BH3 domain.¹³ Our results show that heat shock caused an enhanced binding of Mule to Mcl-1 followed by the accumulation of ubiquitinated Mcl-1 and that both of these events were inhibited in cells expressing Hsp70. Whether the interaction of Mule with Mcl-1 is also controlled by Mcl-1 phosphorylation is not known; however, this is a common mechanism of substrate recognition by the HECT E3 ligases.³⁵ Interestingly, GSK-3 requires a priming phosphorylation to recognize a phosphorylatable target residue. In Mcl-1, this priming site, threonine-163, has been shown to be phosphorylated by JNK in response to oxidative stress.¹⁵ JNK is activated by proteotoxic stress and this activation can be suppressed by Hsp70 overexpression.^{22,26} We found that suppression of JNK with SP600125 was as effective as Hsp70 overexpression in the prevention of Mcl-1 turnover in heat-stressed cells. It will be interesting to determine whether JNK-mediated phosphorylation controls the ability of Mcl-1 to interact with Mule.

Noxa appears to play a role in the regulation of Mcl-1 stability. Overexpression of Noxa results in the depletion of Mcl-1,¹⁸ suggesting that Noxa binding targets Mcl-1 for degradation. This is supported by our finding that Mcl-1 is present in a complex with Noxa in non-stressed cells and that depletion of Noxa by shRNA increased Mcl-1 protein levels. Heat shock appears to directly displace Noxa from Mcl-1, allowing Mule binding and ubiquitination to occur. Displace-

ment of Noxa from Mcl-1 also resulted in an increased binding of BimEL to Mcl-1. This was followed, during incubation at 37°C after the heat treatment, by the release of BimEL due in part to displacement with newly synthesized Noxa. The reassociation of Noxa with Mcl-1 seen at 6 h after heat treatment would inhibit the ability of the remaining Mcl-1 to prevent Bax/Bak activation or, by preventing sequestration of Bim, allow Bim to directly activate Bax/Bak. Heat shock also caused increased synthesis of Noxa in the Hsp70-expressing cells; however, as Mcl-1 levels were maintained, there was sufficient Mcl-1 to inhibit Noxa and Bim. We were unable to demonstrate a direct interaction between Bim and either Bax or Bak, similar to the findings of Willis *et al.*³⁶ and so we cannot conclude that the declining levels of Mcl-1 result in an increased pool of free Bim able to directly activate Bax and Bak. However, our results do show that heat shock leads to the loss of Mcl-1 through a combination of reduced synthesis, inactivation by Mule binding and ubiquitination as well as caspase cleavage and that overexpression of Hsp70 prevents each of these effects resulting in Mcl-1 stabilization. The overall outcome is that Bax is not activated in cells expressing Hsp70. It should be noted that our results do not rule out the potential contribution of Bad and the Bad-interacting proteins Bcl-2, Bcl-xL and Bcl-w in heat-induced apoptosis.

Noxa is unique among BH3-only proteins in its high degree of specificity for Mcl-1 and A1.³⁷ Overexpression of Noxa is not an efficient inducer of cell death unless Bad, which binds to Bcl-2, Bcl-xL and Bcl-w, is also overexpressed. In leukemia cells, the sequestered Bim can be released by a combination of ABT-737, a BH3 mimetic that binds Bcl-2, Bcl-xL and Bcl-w, and treatments that lead to Mcl-1 downregulation, as ABT-737 does not inhibit Mcl-1.³⁸ Pharmacological modulators of Mcl-1 stability include proteasomal inhibitors such as bortezomib, the cyclin-dependent kinase inhibitor seliciclib and the synthetic retinoid *N*-(4-hydroxyphenyl) retinamide.^{39–41} Our results suggest that ABT-737 in combination with hyperthermia would provide an effective means to target all of the antiapoptotic Bcl-2 family proteins and promote apoptosis in tumor cells. However, our results also caution that the effectiveness of this regime would be limited in cells that overexpress heat-shock proteins, as often occurs in tumor cells.³¹ Therefore, targeting heat-shock protein function in tumor cells may also be required.

Materials and Methods

Cells and treatments. The human acute lymphoblastic T-cell line, PEER, with tetracycline-regulated expression of Hsp70 (PErTA70) has been described.²³ For the generation of Noxa shRNA cell lines, PEER cells were transfected with the pSUPER plasmid (obtained from Dr. Reuven Agami) containing inserts targeting human Noxa mRNA (NM021127.2: AGAAGTAATTATTGACACA) or an irrelevant sequence that does not target any human gene product (TTCTCCGAA CGTGTCACGT) as a control. The plasmid was modified to include the mRFP gene under the control of the CMV promoter to assist in stable clone selection. Cells were maintained at 37°C in a 5% CO₂ humidified incubator in RPMI medium with 10% fetal bovine serum (Invitrogen Inc., Burlington, Canada). Cells were heat shocked by the immersion of log-phase cells in a circulating water bath maintained at 43 ± 0.1°C. After the hyperthermic treatments, the cells were either diluted in fresh 37°C medium, transferred to a culture flask and returned to the 37°C incubator or placed directly on ice and washed in cold phosphate-buffered saline (PBS) for collection. For UV exposure, cells suspended in PBS were exposed to 40 kJ/m² in

an open petri dish using a UV cross-linker chamber (Ultra-LUM; Fischer Scientific, Ottawa, Canada). After exposure, the cells were diluted to 0.5×10^6 cells/ml with fresh medium and incubated at 37°C for 6 h before collection. Control cells were treated in the same way except that they were not exposed to UV light. For proteasomal inhibition studies, cells were pretreated with 10 μ M MG132 (BIOMOL International, Plymouth Meeting, PA, USA) for 30 min before exposure to hyperthermia. Following the heat-shock treatment, cells were pelleted and resuspended in fresh medium containing MG132 and incubated at 37°C until collection. Mcl-1 protein turnover was measured by treating cells with cycloheximide (200 μ g/ml) in the presence of the pan-caspase inhibitor zVAD (10 μ M) and incubation at 37°C for 3 and 6 h.

Cell viability assays. Caspase activity was evaluated by measuring DEVDase activity in cell extracts using the substrate Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin); BIOMOL International) as described earlier.⁶ The relative fluorescence units of AMC released per min per μ g protein were calculated for each sample and plotted relative to the maximum value. Viable/apoptotic cell counts were determined by assessing plasma membrane integrity and nuclear morphology using plasma membrane-permeant (acridine orange) and -impermeant (ethidium bromide) DNA-intercalating dyes.²² Long-term viability was determined by measuring the reduction of Alamar Blue as described earlier.⁴² Briefly, control and treated cells were seeded at a concentration of 2.5×10^5 cells/ml in 12-well microplates and incubated at 37°C. At daily intervals, cell suspensions (100 μ l in triplicate) were seeded into a 96-well plate with 100 μ l of media containing 50 μ M resazurin (Sigma-Aldrich, Oakville, Canada) and incubated at 37°C for 5 h. Fluorescence generated from resazurin reduction was measured in a microplate fluorescence reader (Ex516/20, Em590/35). Viability is expressed as a percentage of the control values measured after 3 days of culture.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting experiments were performed as described earlier.⁶ Briefly, for IP, cells were lysed in 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate hydrate (CHAPS) IP lysis buffer (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 2% CHAPS) containing phosphatase and protease inhibitors (2 μ g/ml each of pepstatin, leupeptin and aprotinin) and phosphatase inhibitors (50 mM sodium fluoride and 1 mM sodium vanadate) for 30 min on ice. Lysates (300 μ g protein) were incubated overnight at 4°C on a rotator with 1 μ g of anti-Mcl-1 (sc-819; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Bax (N20; Santa Cruz Biotechnology) antibody. The N20 Bax antibody is conformation specific and immunoprecipitates only active Bax. Immunoprecipitates were collected by the addition of 10 μ l of washed protein A agarose beads (EZview Affinity Gel; Sigma-Aldrich) and incubation for 1 h at 4°C on a rotator. Complexes were collected by centrifugation and washed four times with lysis buffer. Finally, the pelleted beads were resuspended in 1 \times sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β -mercaptoethanol) and heated at 95°C for 5 min for analysis by SDS-polyacrylamide gel electrophoresis and western blotting. The specificity of the co-IP results was tested by performing the Mcl-1 IPs in the presence of a fivefold excess of the Mcl-1 peptide used to generate the antibody (sc-819P: Santa Cruz Biotechnology) or an irrelevant peptide (Bax N20 peptide, sc-493P: Santa Cruz Biotechnology). Immunoprecipitation of denatured Mcl-1 was performed as above except that the cells were lysed in denaturing lysis buffer (40 mM Tris, 1% SDS, 1% β -mercaptoethanol, 100 mM DTT), sonicated and heated to 95°C for 10 min. The lysates were cleared by centrifugation, diluted 1:10 with RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 10 mM Tris (pH 7.4), 0.1% SDS, 1 mM PMSF) and used for IP with Mcl-1 antibody. The following antibodies were used in western blotting experiments: Actin (ACTN05; NeoMarkers, Fremont, CA, USA), Bak (556396; BD Biosciences, Mississauga, Canada), Bax (N20; Santa Cruz Biotechnology), Bim (AAP-330; Stressgen/Assay Designs, Ann Arbor, MI, USA), Caspase-3 (SA-320-0100; BIOMOL International), Hsc70 (1B5; Stressgen), Hsp70 (C92F3A-5; Stressgen), Mcl-1 (sc-819; Santa Cruz Biotechnology), Mule (aka Lasu1/Urb1, A300-486A; Bethyl Laboratories, Montgomery, TX, USA), Noxa (114C307.1; Alexis Biochemicals, San Diego, CA, USA) and Ubiquitin (P4D1; Cell Signaling Technology, Danvers, MA, USA). For the detection of Noxa, we used 0.2- μ m PVDF membranes (Immobilon PSQ) instead of 0.45 to enhance binding of low-molecular-weight proteins and performed the incubations in Hikari signal enhancer solution (Nacalai USA Inc., San Diego, CA, USA). Chemical cross-linking with EGS before western blotting to assess Bax oligomerization was performed as described.⁶

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1. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* 2008; **9**: 231–241.
2. Antonsson B, Montessuit S, Sanchez B, Martinou JC. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* 2001; **276**: 11615–11623.
3. Leber B, Lin J, Andrews DW. Embedded together: the life and death consequences of interaction of the Bcl-2 family with membranes. *Apoptosis* 2007; **12**: 897–911.
4. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007; **26**: 1324–1337.
5. Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* 2008; **18**: 157–164.
6. Stankiewicz AR, Lachapelle G, Foo CP, Radicioni SM, Mosser DD. Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. *J Biol Chem* 2005; **280**: 38729–38739.
7. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tu S, Beere HM *et al*. The multidomain proapoptotic molecules Bax and Bak are directly activated by heat. *Proc Natl Acad Sci USA* 2005; **102**: 17975–17980.
8. Opferman JT. Unraveling MCL-1 degradation. *Cell Death Differ* 2006; **13**: 1260–1262.
9. Derenne S, Monia B, Dean NM, Taylor JK, Rapp MJ, Harousseau JL *et al*. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood* 2002; **100**: 194–199.
10. Michels J, Johnson PW, Packham G. Mcl-1. *Int J Biochem Cell Biol* 2005; **37**: 267–271.
11. Bae J, Leo CP, Hsu SY, Hsueh AJ. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. *J Biol Chem* 2000; **275**: 25255–25261.
12. Nechushtan AR, Smith CL, Lamensdorf I, Yoon SH, Youle RJ. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J Cell Biol* 2001; **153**: 1265–1276.
13. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* 2005; **121**: 1085–1095.
14. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 2006; **21**: 749–760.
15. Inohita S, Takeda K, Hatai T, Terada Y, Sano M, Hata J *et al*. Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. *J Biol Chem* 2002; **277**: 43730–43734.
16. Han J, Goldstein LA, Gastman BR, Froelich CJ, Yin XM, Rabinowich H. Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events. *J Biol Chem* 2004; **279**: 22020–22029.
17. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI *et al*. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 2005; **19**: 1294–1305.
18. Czabotar PE, Lee EF, van Delft MF, Day CL, Smith BJ, Huang DC *et al*. Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proc Natl Acad Sci USA* 2007; **104**: 6217–6222.
19. Han J, Goldstein LA, Hou W, Rabinowich H. Functional linkage between NOXA and Bim in mitochondrial apoptotic events. *J Biol Chem* 2007; **282**: 16223–16231.
20. Warr MR, Accoca S, Liu Z, Germain M, Watson M, Blanchette M *et al*. BH3-ligand regulates access of MCL-1 to its E3 ligase. *FEBS Lett* 2005; **579**: 5603–5608.
21. Mosser DD, Martin LH. Induced thermotolerance to apoptosis in a human T lymphocyte cell line. *J Cell Physiol* 1992; **151**: 561–570.
22. Mosser DD, Caron AW, Bourget L, Denis-Larose C, Massie B. Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. *Mol Cell Biol* 1997; **17**: 5317–5327.
23. Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI *et al*. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol Cell Biol* 2000; **20**: 7146–7159.
24. Naik E, Michalak EM, Villunger A, Adams JM, Strasser A. Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly via the BH3-only protein Noxa. *J Cell Biol* 2007; **176**: 415–424.
25. Clohessy JG, Zhuang J, Brady HJ. Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells. *Br J Haematol* 2004; **125**: 655–665.
26. Gabai VL, Meriin AB, Mosser DD, Caron AW, Rits S, Shifrin VI *et al*. Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. *J Biol Chem* 1997; **272**: 18033–18037.
27. Meller R, Cameron JA, Torrey DJ, Clayton CE, Ordonez AN, Henshall DC *et al*. Rapid degradation of Bim by the ubiquitin–proteasome pathway mediates short-term ischemic tolerance in cultured neurons. *J Biol Chem* 2006; **281**: 7429–7436.
28. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. *Oncogene* 2004; **23**: 5301–5315.
29. Esser C, Alberti S, Hohfeld J. Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochim Biophys Acta* 2004; **1695**: 171–188.

30. Liu RY, Li X, Li L, Li GC. Expression of human hsp70 in rat fibroblasts enhances cell survival and facilitates recovery from translational and transcriptional inhibition following heat shock. *Cancer Res* 1992; **52**: 3667–3673.
31. Mosser DD, Morimoto RI. Molecular chaperones and the stress of oncogenesis. *Oncogene* 2004; **23**: 2907–2918.
32. Morimoto RI. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes Dev* 2008; **22**: 1427–1438.
33. Cuconati A, Mukherjee C, Perez D, White E. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev* 2003; **17**: 2922–2932.
34. Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F *et al*. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev* 2003; **17**: 1475–1486.
35. Gao M, Karin M. Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. *Mol Cell* 2005; **19**: 581–593.
36. Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE *et al*. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* 2007; **315**: 856–859.
37. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG *et al*. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005; **17**: 393–403.
38. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA *et al*. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* 2006; **9**: 351–365.
39. Podar K, Gouill SL, Zhang J, Opferman JT, Zorn E, Tai YT *et al*. A pivotal role for Mcl-1 in Bortezomib-induced apoptosis. *Oncogene* 2008; **27**: 721–731.
40. MacCallum DE, Melville J, Frame S, Watt K, Anderson S, Gianella-Borradori A *et al*. Seliciclib (CYC202, R-Roscovotine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. *Cancer Res* 2005; **65**: 5399–5407.
41. Kang MH, Wan Z, Kang YH, Spoto R, Reynolds CP. Mechanism of synergy of N-(4-hydroxyphenyl)retinamide and ABT-737 in acute lymphoblastic leukemia cell lines: Mcl-1 inactivation. *J Natl Cancer Inst* 2008; **100**: 580–595.
42. Lachapelle G, Radicioni SM, Stankiewicz AR, Mosser DD. Acute acidification or amiloride treatment suppresses the ability of Hsp70 to inhibit heat-induced apoptosis. *Apoptosis* 2007; **12**: 1479–1488.