

Glycogen Synthase Kinase-3 Regulates Mitochondrial Outer Membrane Permeabilization and Apoptosis by Destabilization of MCL-1

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Summary

We investigated the role of glycogen synthase kinase-3 (GSK-3), which is inactivated by AKT, for its role in the regulation of apoptosis. Upon IL-3 withdrawal, protein levels of MCL-1 decreased but were sustained by pharmacological inhibition of GSK-3, which prevented cytochrome c release and apoptosis. MCL-1 was phosphorylated by GSK-3 at a conserved GSK-3 phosphorylation site (S159). S159 phosphorylation of MCL-1 was induced by IL-3 withdrawal or PI3K inhibition and prevented by AKT or inhibition of GSK-3, and it led to increased ubiquitinylation and degradation of MCL-1. A phosphorylation-site mutant (MCL-1^{S159A}), expressed in IL-3-dependent cells, showed enhanced stability upon IL-3 withdrawal and conferred increased protection from apoptosis compared to wild-type MCL-1. The results demonstrate that the control of MCL-1 stability by GSK-3 is an important mechanism for the regulation of apoptosis by growth factors, PI3K, and AKT.

Introduction

In addition to induction of cell growth and proliferation, growth factors maintain cell viability, as lack of appropriate growth factor stimulation will rapidly trigger apoptosis. A central control point for the induction of apoptosis is the permeabilization of the mitochondrial outer membrane, leading to the release of cytochrome c and other proteins residing in the mitochondrial intermembrane space. Mitochondrial outer membrane permeabilization (MOMP) is controlled by the BCL-2 family of proteins,

with proapoptotic BAX and/or BAK required for permeabilization (Wei et al., 2001). This is counteracted by antiapoptotic BCL-2 family members, mainly BCL-2, BCL-xL, and MCL-1, which sequester BAX- or BAK-activating BH3-only proteins BIM and BID (Cheng et al., 2001; Kuwana et al., 2005; Letai et al., 2002). Within a short time, MOMP is followed by apoptosome formation and caspase activation resulting in apoptosis (Green, 2005). Growth factor receptor engagement induces activation of the PI3K/AKT pathway, which prevents the release of cytochrome c (Kennedy et al., 1999).

How AKT prevents MOMP is not fully understood. AKT phosphorylates and inhibits the proapoptotic effects of BAD (Datta et al., 1997; del Peso et al., 1997), Foxo1 and Foxo3a (Brunet et al., 1999; Tang et al., 1999), and human caspase 9 (Cardone et al., 1998). In addition, AKT activates antiapoptotic effects of hexokinase (Gottlob et al., 2001), induces expression of antiapoptotic Bcl-2-family proteins (Song et al., 2004), and engages NF- κ B to block apoptosis (Romashkova and Makarov, 1999). It is likely that AKT may have other antiapoptotic effects as well.

The isoforms of GSK-3, GSK-3 α , and GSK-3 β are inactivated upon phosphorylation by AKT on serine 21 and serine 9, respectively (Cross et al., 1995). In order to phosphorylate its substrates, GSK-3 has a requirement for a priming phosphorylation on the substrate four amino-acids C-terminal of the serine/threonine to be phosphorylated (Fiol et al., 1987). Phosphorylation by AKT at the GSK-3 N terminus generates a “pseudosubstrate,” self-inhibiting GSK-3 by occupying its active center (Frame et al., 2001).

GSK-3 plays roles in metabolic signaling by inactivating glycogen synthase (Embi et al., 1980) and IRS-1 (Eldar-Finkelman and Krebs, 1997), and in cell cycle regulation through its targets β -catenin (Yost et al., 1996), MYC (Sears et al., 2000), cyclin D1 (Diehl et al., 1998), cyclin E (Welcker et al., 2003), and BCL-3 (Viatour et al., 2004). A potential mechanism for the regulation of apoptosis by GSK-3 downstream of PI3K/AKT, however, has not been comprehensively investigated.

A number of recent studies demonstrated the induction of the antiapoptotic BCL-2 family member MCL-1 by a number of growth factors, such as stem cell factor and IL-5 (Huang et al., 2000), IL-6 (Jourdan et al., 2003), VEGF (Le Gouill et al., 2004), GM-CSF, and IL-3 (Wang et al., 1999), as well as through B cell receptor stimulation (Petlickovski et al., 2005). While transcription seems to be a regulatory component in some cases, the exact mechanism for the maintenance of MCL-1 levels preventing MOMP has been elusive. Recently, survival signaling of IL-7 in immature T and B cells has been demonstrated to depend completely on MCL-1 (Opferman et al., 2003). However, IL-7 only transiently induced *mcl-1* transcription, raising the possibility of an alternative mechanism by which the steady-state levels of the short-lived MCL-1 are maintained by growth factors. In the present study, we addressed the maintenance of MCL-1 levels by IL-3 and identified an apoptosis-regulating pathway branching downstream of PI3K/AKT. We show that GSK-3 promotes the induction of apoptosis by

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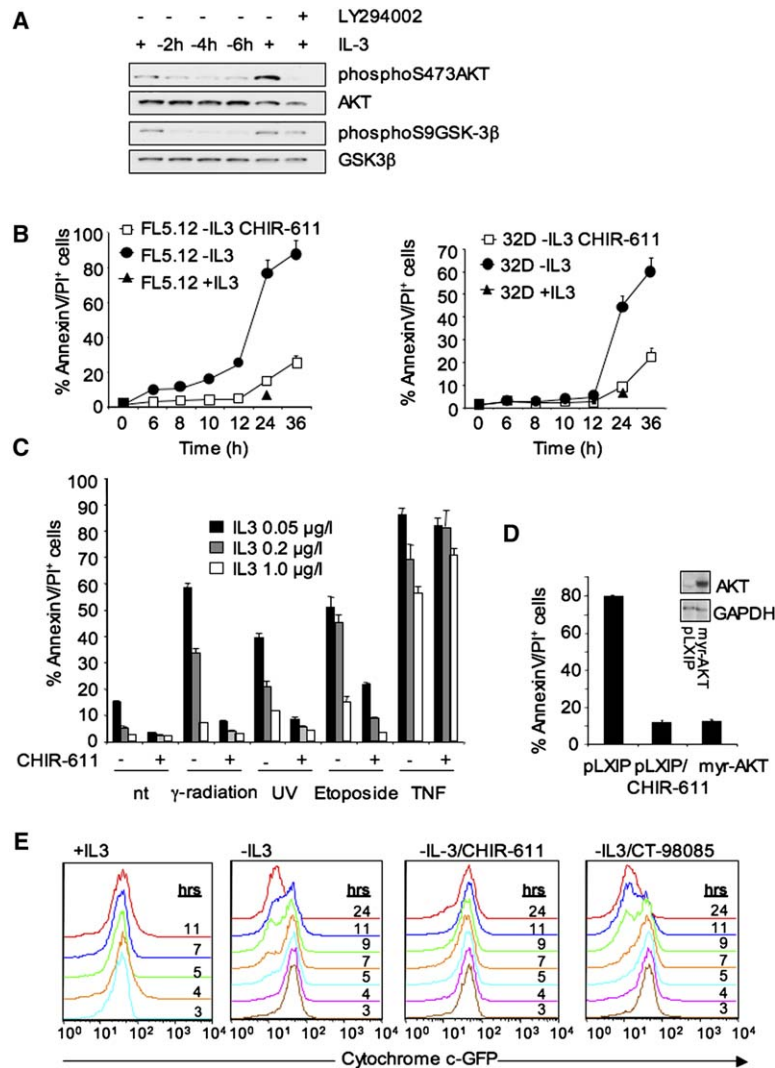


Figure 1. Inhibition of GSK-3 Prevents IL-3 Withdrawal-Induced Cytochrome c Release and Apoptosis of FL5.12 and 32Dcl3 Cells

(A) FL5.12 cells were maintained with (+) IL-3 or were deprived of IL3 for 2, 4, or 6 hr. IL3 was then added back in the presence (+) or absence (-) of LY294002 (20 μ M). S473 phosphorylation of AKT and S9 phosphorylation of GSK-3 β were assessed using the respective phosphospecific antibodies.

(B) FL5.12 and 32Dcl3 cells were cultured without IL-3 in the presence or absence of the GSK-3 inhibitor CHIR-611 (0.75 μ M). Apoptosis was determined by AnnexinV-FITC/PI staining. The average of triplicate samples from one experiment is shown; error bars represent the standard deviation.

(C) FL5.12 cells were cultured in the presence of 1 μ g/l, 0.2 μ g/l, or 0.05 μ g/l IL-3 with or without CHIR-611 (0.75 μ M) for 15 hr. Cells were left untreated (nt) or apoptosis was induced by UV radiation (200J/m²), etoposide (2 μ M), γ radiation (5 Gy), or TNF (5 ng/ml) plus CHX (10 μ g/ml) and assessed by AnnexinV-FITC/PI staining after 4 hr (TNF/CHX) or 8 hr (all others). The average of triplicate samples from one experiment is shown; error bars represent the standard deviation.

(D) FL5.12 cells transduced with control vector (pLXIP) or myristoylated AKT (myr-AKT) were deprived of IL-3. CHIR-611 was added as indicated. Apoptosis was determined after 24 hr by Annexin V/PI staining. The average of triplicate samples from one experiment is shown; error bars represent the standard deviation.

(E) FL5.12 cells expressing cytochrome c-GFP were maintained in the presence (+IL-3) or absence (-IL-3) of IL-3 or deprived of IL3 in the presence of 0.75 μ M CHIR-611 (-IL-3/CHIR-611) or the inactive control compound CT-98085 (-IL-3/CT-98085). Assessment of cytochrome-GFP release from mitochondria was performed by flow cytometry according to the CLAMI protocol at the indicated time points (Waterhouse et al., 2001).

phosphorylation of MCL-1, leading to its degradation by the ubiquitin-proteasome pathway and facilitating cytochrome c release and apoptosis.

Results

IL-3-dependent cell lines undergo apoptosis rapidly when deprived of IL-3. To characterize the role of GSK-3 in growth factor withdrawal-induced apoptosis, we first investigated the phosphorylation of AKT and GSK-3 β in IL-3-dependent FL5.12 cells. GSK-3 β was completely dephosphorylated after IL-3 depletion, which derepresses its kinase activity (Cohen and Frame, 2001). When IL-3 was readded to the FL5.12 cells, AKT and GSK-3 β were strongly phosphorylated, which was prevented by inhibition of PI3K by LY294002 (Figure 1A). To investigate if GSK-3 activity is required for apoptosis induction upon IL-3 withdrawal, we employed small molecule inhibitors of GSK-3 (CHIR-611 and CHIR-911), which inhibit GSK-3 α and GSK-3 β (Ring et al., 2003; Wagman et al., 2004). Strikingly, apoptosis in IL-3-dependent cell lines FL5.12 and 32Dcl3 (Figure 1B) and BA/F3 cells (data not shown) deprived of IL-3 was pre-

vented when GSK-3 was inhibited by CHIR-611 (Figure 1B) and CHIR-911 (data not shown).

Withdrawal of IL-3 sensitizes IL-3-dependent cells to proapoptotic stimuli (Collins et al., 1992). We investigated the contribution of GSK-3 to sensitization to apoptosis induction upon reduced growth factor conditions. FL5.12 cells were treated with UV radiation, etoposide, γ radiation, or TNF plus cycloheximide after culturing them for 15 hr in conventional (1 μ g/l) or reduced concentrations (0.2 and 0.05 μ g/l) of IL-3. As expected, reduced concentrations of IL-3 sensitized the cells to the various proapoptotic stimuli. When cells were cultured in the presence of the GSK-3 inhibitor CHIR-611, the sensitizing effect of low IL-3 concentrations on apoptosis induction by etoposide, UV, or γ radiation was abrogated, while apoptosis induction by TNF plus cycloheximide was unaffected (Figure 1C). This suggests that inhibition of GSK-3 is an important function of IL-3 for the prevention of apoptosis by the mitochondrial pathway engaged by these stressors.

To compare prevention of apoptosis by GSK-3 inhibition to the protection mediated by AKT, we introduced myristoylated AKT into FL5.12 cells and withdrew IL-3

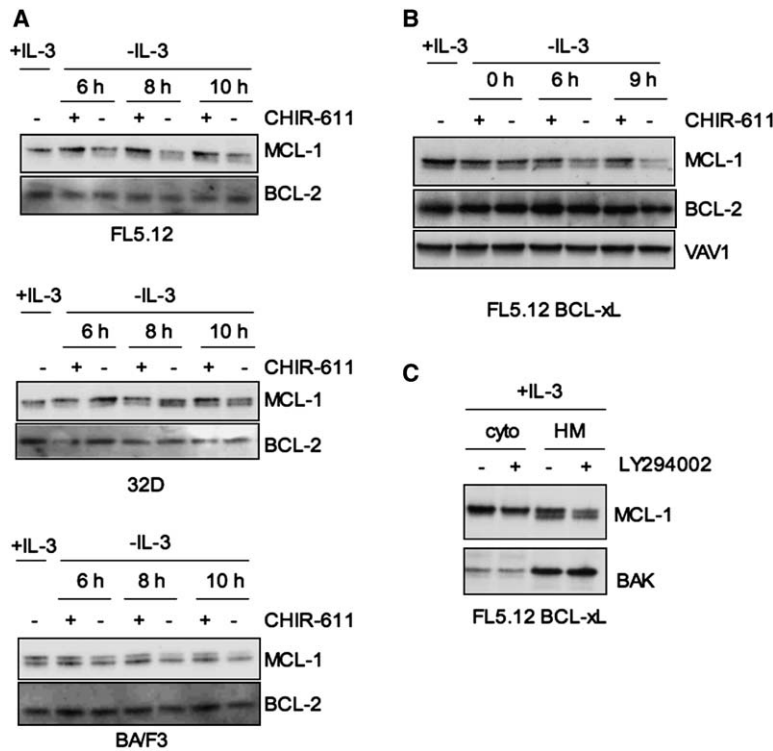


Figure 2. GSK-3 Activity Leads to Reduction of MCL-1 upon IL-3 Withdrawal, which Does Not Depend on Caspase Activity

(A) FL5.12, 32Dcl3, and BA/F3 cells were maintained in the presence (+IL3) or absence of IL-3 (–IL-3) for 6, 8, or 10 hr in the presence of 0.75 μ M CHIR-611 (+) or an equal volume of DMSO (–). Immunoblotting was performed for MCL-1 and BCL-2.

(B) FL5.12 cells stably expressing BCL-xL were maintained in the presence (+IL-3) or absence (–IL-3) of IL-3 for 6 or 9 hr with 0.75 μ M CHIR-611 (+) or DMSO (–). Immunoblotting was performed for MCL-1, BCL-2, and VAV1.

(C) FL5.12 cells expressing BCL-xL were maintained with IL-3 in the presence of 20 μ M LY294002 (+) or DMSO (–) for 4 hr, and subcellular fractionation into heavy membrane (HM) and cytosolic (cyto) fractions was performed as described (Kluck et al., 2000). Immunoblots were probed for MCL-1 and BAK.

for 24 hr. Inhibition of GSK-3 protected the cells from apoptosis to the same degree as did expression of myristoylated-AKT (Figure 1D).

To determine if GSK-3 inhibition prevents MOMP, we generated FL5.12 cells stably expressing cytochrome c-GFP, allowing us to follow the release of a cytochrome c-GFP fusion protein upon IL-3 withdrawal (Goldstein et al., 2005). In cells depleted of IL-3, cytochrome c-GFP was released from mitochondria, beginning about 6 hr after IL-3 withdrawal, as indicated by the loss of fluorescence from digitonin-treated cells. Release of cytochrome c from mitochondria was prevented upon inhibition of GSK-3 (Figure 1E), demonstrating a requirement for GSK-3 to facilitate MOMP.

At an early stage in their maturation, lymphocytes depend on MCL-1 for cytokine-mediated protection from apoptosis (Opferman et al., 2003). Therefore, we suspected that MCL-1 might play a role in the protection mediated by IL-3, AKT, or inhibition of GSK-3 of these hematopoietic precursor cell lines. We examined protein levels of MCL-1 following IL-3 withdrawal and observed a substantial decline of MCL-1 levels by 6–10 hr after removal of IL-3 from FL5.12, 32D, and BA/F3 cells. This correlated well with the onset of cytochrome c release and was prevented when GSK-3 was inhibited pharmacologically (Figure 2A). Since MCL-1 is cleaved by caspases during the execution of apoptosis (Han et al., 2004; Herrant et al., 2004) (and data not shown), we sought to prevent caspase activation by using FL5.12 cells stably expressing BCL-xL. Apoptosis of these cells upon IL-3 withdrawal was inhibited (see Figure S1 in the Supplemental Data available with this article online), as shown previously (Rathmell et al., 2000). Nevertheless, when IL-3 was removed from FL5.12 cells overexpressing BCL-xL, MCL-1 protein levels decreased by 6–9 hr,

ruling out MCL-1 degradation as a secondary consequence of apoptosis. Again, when GSK-3 was inhibited, the decrease in MCL-1 was prevented (Figure 2B). Likewise, inhibition of PI3K by LY294002 reduced MCL-1 levels both in the cytosol and in the heavy membrane fraction of FL5.12 cells overexpressing BCL-xL, confirming that stability of MCL-1 is modulated by PI3K signaling, independent of apoptosis induction (Figure 2C). The inhibition of PI3K by itself did not lead to a major induction of apoptosis of FL5.12 cells (Figure S2A), consistent with observations by others for 32D cells (von Gise et al., 2001). Indeed, we observed that, unlike IL-3 deprivation, inhibition of PI3K did not induce the expression of Bim (Figure S2B). In contrast, the MAPK inhibitor UO126 was effective in inducing Bim expression. Since PI3K inhibition decreases MCL-1 levels but does not induce apoptosis, this suggests that downregulation of MCL1 is not sufficient by itself to cause apoptosis but more likely sensitizes cells to apoptosis upon IL-3 deprivation, perhaps by the action of Bim.

To investigate how MCL-1 levels are regulated by GSK-3, we first analyzed *mcl-1* gene transcription by real-time RT-PCR. We did not find major changes of *mcl-1* mRNA dependent on IL-3 withdrawal and GSK-3 inhibition (Figure S3). Instead, we observed that MCL-1 protein half-life depended on GSK-3; inhibition of PI3K decreased MCL-1 protein half-life, which was abrogated by inhibition of GSK-3 (Figure 3A). Consistently, myristoylated AKT, expressed in FL5.12 cells, had the same effect on MCL-1 half-life as did inhibition of GSK-3 (Figure 3B).

MCL-1 contains an evolutionarily conserved GSK-3 site (Figure 4A). Therefore, we suspected that the decrease of MCL-1 protein half-life is a consequence of direct phosphorylation of MCL-1 by GSK-3. To address

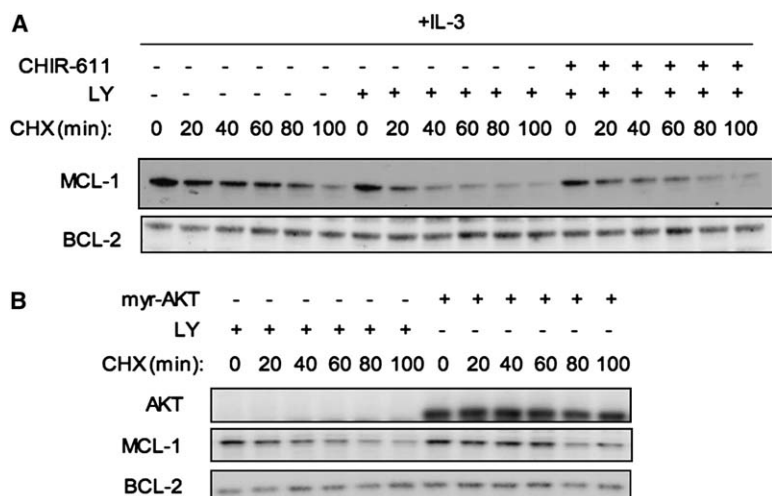


Figure 3. GSK-3 Modulates MCL-1 Protein Half-Life

(A) FL5.12 cells were grown in the presence of IL-3 and preincubated for 1 hr with 0.75 μ M CHIR-611 as indicated. LY294002 (20 μ M) was added for 1.5 hr as indicated, followed by determination of protein half-life upon inhibition of translation with CHX (200 μ g/ml) for the indicated time.

(B) FL5.12 cells or FL5.12 cells expressing constitutively active AKT (myr-AKT) were grown in the presence of IL-3, and 20 μ M LY294002 was added for 1.5 hr as indicated, followed by determination of protein half-life upon inhibition of translation with CHX.

this, we tested the phosphorylation of MCL-1 by GSK-3 in vitro, using recombinant MCL-1 or a protein mutant lacking the GSK-3 site, MCL-1^{S159A}, where alanine was substituted for serine 159. Phosphorylation of GSK-3 substrates has been demonstrated to require a priming phosphorylation four amino acids downstream of the amino acid phosphorylated by GSK-3 through a different, often constitutively active kinase (Cohen and Frame, 2001). For this reason, we expressed His-tagged MCL-1 and the S159A mutant in 293T cells to obtain MCL-1 in its intracellular phosphorylated state and purified the protein by Ni²⁺ affinity. In the presence of recombinant GSK-3 β , wild-type MCL-1 was phosphorylated in vitro, while the mutant was not (Figure 4B). Importantly, other putative GSK-3 sites present in human MCL-1 (which are not evolutionary conserved, however) were not phosphorylated by recombinant GSK-3 β in this assay, indicating the specificity of GSK-3 β for S159. To specifically assess S159 phosphorylation of MCL-1, we generated a phospho-S159-specific antibody, with which we generated results similar to those obtained using ³²P (Figure 4B).

We then investigated MCL-1 phosphorylation in vivo by expressing wild-type MCL-1 or MCL-1^{S159A} in HeLa cells. Wild-type MCL-1 was phosphorylated at S159 upon PI3K inhibition (allowing GSK-3 to be active). This occurred only in the absence of GSK-3 inhibition and, importantly, was only observed when the proteasome was inhibited, suggesting that S159-phosphorylated MCL1 is degraded by the proteasome (Figure 4C). To investigate phosphorylation of MCL-1 in response to changes in cytokine signaling, we introduced human MCL-1 and MCL-1^{S159A} into FL5.12 cells. IL3 withdrawal or inhibition of PI3K led to phosphorylation of MCL-1 at serine 159, which was prevented by GSK-3 inhibition (Figure 4D).

S159-phosphorylated MCL-1 in HeLa and FL5.12 cells was only detected upon inhibition of the proteasome (Figure 4C and data not shown). Likewise, degradation of MCL-1 upon IL-3 withdrawal or inhibition of PI3K was prevented by proteasome inhibition (Figure S4). This suggests that a proteasomal degradation mechanism is governed by S159 phosphorylation. To explore this further, wild-type and mutant MCL-1 were expressed in HeLa cells along with HA-tagged ubiquitin. Ubiquitiny-

lation of wild-type-MCL-1, but not of MCL-1 S159A, was detected upon PI3K inhibition. This was prevented when GSK-3 was inhibited pharmacologically. S159-phosphorylated MCL-1 was detected as a high-molecular-weight-migrating species, paralleling the appearance of polyubiquitinated MCL-1 (Figure 5A). Next, we employed RNA interference to knock down GSK-3 α and GSK-3 β (Yu et al., 2003) and evaluated the effects on phosphorylation of MCL-1. Knockdown of both GSK-3 α and, to a lesser extent, GSK-3 β in NIH3T3 cells diminished the appearance of S159-phosphorylated high-molecular-weight MCL-1 species. Phosphorylation of S159 was abolished when myr-AKT was coexpressed (Figure 5B, left panel). Reduction of either GSK-3 isoform had an effect on MCL-1 phosphorylation in response to PI3K inhibition, while knockdown of GSK-3 α had a more pronounced effect than knockdown of GSK-3 β . However, this may have been the consequence of a more efficient knockdown of GSK-3 α than that of GSK-3 β . We suspect that net GSK-3 reduction is the main reason for the diminished MCL-1 modification we observed (Figure 5B, lower panel).

Next, we compared the half-life of MCL-1 and the S159A mutant when overexpressed in HeLa cells. Upon inhibition of PI3K, wild-type MCL-1 had a significantly shorter half-life compared to the S159A mutant. The difference between wild-type and mutant was completely abrogated, however, when GSK-3 was inhibited, extending the half-life of wild-type MCL-1 to that of MCL-1^{S159A} (Figure 5C).

In order to investigate this in hematopoietic cells, we expressed human MCL-1 in murine FL5.12 cells and probed for the introduced MCL-1. Consistently, when we analyzed the half-life of MCL-1 versus MCL-1^{S159A} in FL5.12 cells, we found a decrease of wild-type MCL-1 protein stability upon PI3K inhibition, which was prevented by GSK-3 inhibition. Again, the stability of the MCL-1^{S159A} mutant did not depend on the activity of GSK-3 (Figure 5D).

Since the phospho-S159-antibody was raised against the human MCL-1 sequence, we employed Jurkat cells to investigate the phosphorylation of endogenous MCL-1. When cells were treated with PI3K inhibitor, a high-molecular-weight species was detected with the

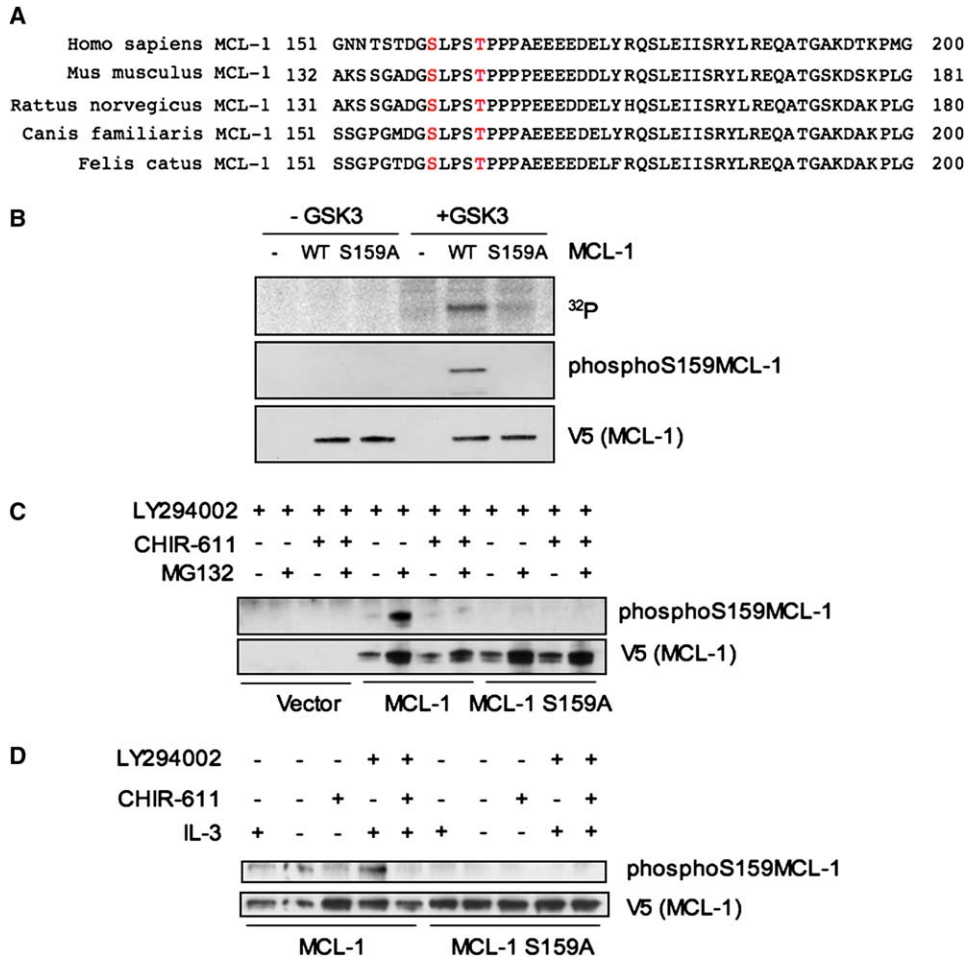


Figure 4. Phosphorylation of MCL-1 by GSK-3

(A) Comparison of MCL-1 amino acid sequences from various species. The GSK-3 site is indicated in red.
 (B) V5/His-tagged MCL1 and MCL1^{S159A} were expressed and purified from 293T cells and incubated for 20 min in the presence of 20 μ Ci 32P γ -ATP with or without recombinant GSK3 β (500 U). The blot was assessed using a PhosphorImager and subsequently probed with a MCL1 phospho-S159-specific antibody.
 (C) V5/His-tagged MCL1, MCL1^{S159A}, or empty vector control was introduced into HeLa cells and incubated for 6 hr with 0.75 μ M CHIR-611, 20 μ M MG132, and 20 μ M LY294002 as indicated. After pull-down with Ni²⁺ beads, phosphoS159-MCL-1 was assessed with the anti-phospho-S159 antibody, and the blot was reprobed for MCL-1 (V5).
 (D) V5/His-tagged MCL-1 or MCL-1^{S159A} was expressed by retroviral transduction of FL5.12 cells. After 1 hr of preincubation with 10 μ M MG-132, cells were maintained in the presence or absence of IL-3 for 3 hr with or without 0.75 μ M CHIR-611 or in the presence of IL-3 and LY294002 with or without 0.75 μ M CHIR-611 for 2 hr. After pull-down with Ni²⁺ beads, Western blots were probed with the anti-phospho-S159 antibody and for V5.

phospho-S159-specific antibody after immunoprecipitation of MCL-1, which was diminished by GSK-3 inhibition. While MCL-1 with the expected molecular weight of about 40 kDa was immunoprecipitated, the phospho-specific antibody did not detect any signal at this molecular weight of MCL-1 (data not shown), suggesting that endogenous phospho-MCL-1 is rapidly ubiquitinated. With an anti-MCL-1 antibody, we also detected high-molecular-weight MCL-1 species upon PI3K inhibition, confirming that these high-molecular-weight species are MCL-1 (Figure 5E). Again, this strongly suggests that, upon PI3K inhibition, MCL-1 is phosphorylated on S159 and rapidly ubiquitinated.

Our results suggested that expression of the phosphorylation-defective mutant of MCL-1 may mediate increased protection from apoptosis in the presence of suboptimal IL-3. To test this, we introduced MCL-1 and the S159A mutant into FL5.12 cells and maintained

the cells at a low IL-3 concentration (which by itself did not induce apoptosis) for 15 hr. When IL-3 was removed subsequently, a marked protection of cells expressing the MCL-1^{S159A} mutant was observed (Figure 6A). While both forms of MCL-1 were expressed at similar levels in the presence of conventional amounts of IL-3 as assessed by intracellular staining of MCL-1, at low IL-3 concentrations, MCL-1^{S159A} levels were consistently higher than those of wild-type MCL-1 (Figure 6B).

We had observed that FL5.12 cells showed markedly increased apoptosis upon UV radiation when maintained at a low concentration of IL-3 for 15 hr prior to irradiation (Figure 1C). We therefore asked if IL-3 concentration influences the protection from UV-induced apoptosis conferred by wild-type MCL-1 or MCL-1^{S159A}. FL5.12 cells expressing either form of MCL-1 were maintained with conventional or reduced concentrations of IL-3 for 15 hr. Upon induction of apoptosis by UV radiation,

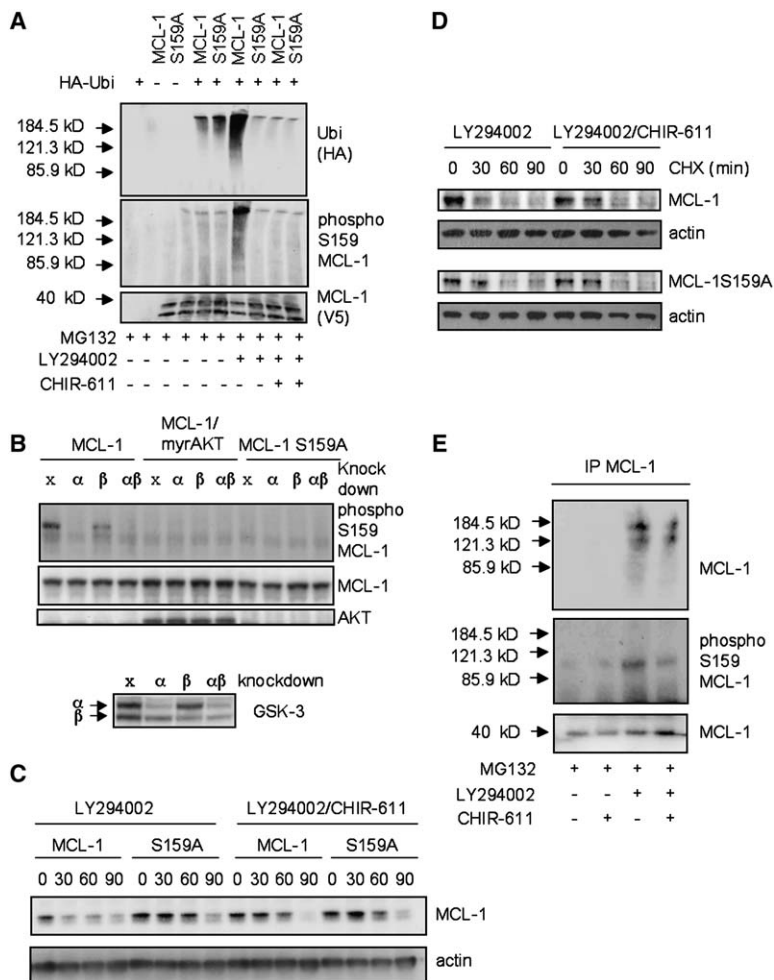


Figure 5. GSK-3 Regulates MCL-1 Half-Life through S159 Phosphorylation

(A) MCL-1 but not MCL-1^{S159A} is ubiquitinated upon PI3K inhibition, which is prevented by inhibition of GSK-3. His-tagged MCL-1, MCL-1^{S159A}, or empty vector, together with HA-tagged ubiquitin as indicated, were expressed in HeLa cells and incubated with 20 μM MG132, 20 μM LY294002, and 0.75 μM CHIR-611 as indicated. After pull-down with Ni²⁺ beads, the blot was probed with anti-HA (upper panel), anti-phospho-S159 MCL-1 (middle panel), and anti-V5 (lower panel).

(B) NIH3T3 cells were transfected with plasmids encoding shRNA complementary to *Xenopus* ASH (x), mouse GSK-3α (α), mouse GSK-3β (β), or combined (αβ), along with plasmids encoding human MCL-1 or MCL-1^{S159A} and myrAKT as indicated. After 72 hr, LY294002 (20 μM) and MG132 (20 μM) were added for 1.5 hr (upper panel). In a parallel experiment, NIH3T3 cells were transfected with plasmids encoding shRNA as indicated and a plasmid encoding puromycin resistance. After 12 hr, 10 μM puromycin was added to eliminate nontransfected cells. After 72 hr, disappearance of GSK-3 isoforms was analyzed by immunoblotting (lower panel).

(C) MCL-1 or MCL-1^{S159A} was expressed in HeLa cells for 18 hr, and then 0.75 μM CHIR-611 and 20 μM LY294002 were added for 60 min, followed by addition of 500 μg/ml CHX. Cells were harvested at the indicated time points after CHX addition.

(D) Human MCL-1, MCL-1^{S159A}, and empty vector were expressed in FL5.12 cells for 18 hr, and then 0.75 μM CHIR-611 and LY294002 were added for 1 hr as indicated. MCL-1 half-life after addition of CHX was assessed as above.

(E) Jurkat cells were preincubated with MG132 (10 μM) for 1 hr, and then CHIR-611 (0.75 μM) and LY294002 (20 μM) were added as indicated for 3 hr. After immunoprecipitation of endogenous MCL-1, the blot was probed as indicated.

protection from apoptosis by MCL-1^{S159A} was markedly increased compared to the protection by wild-type MCL-1 (Figure 6C). This effect was particularly evident when low amounts of DNA encoding MCL-1 were introduced into FL5.12 cells, as, under these conditions, changes in protein stability were not masked by high levels of synthesis. Also, relatively greater protection by MCL-1^{S159A} was observed in the presence of low IL-3. Interestingly, MCL-1^{S159A} conferred the same protection in the presence of low IL-3 as did wild-type MCL-1 at a conventional IL-3 concentration (Figure 6C). Taken together, these experiments provide strong evidence defining MCL-1 as a bona fide GSK-3 target according to the criteria outlined by Cohen and Frame (2001).

The proapoptotic BH3-only protein BIM is induced upon removal of IL-3 (Dijkers et al., 2000), stabilized in the absence of growth factor signaling (Luciano et al., 2003), and required for apoptosis due to growth factor deprivation (Bouillet et al., 1999). We therefore asked if MCL-1, “rescued” by GSK3 inhibition after IL-3 withdrawal, contributes to inhibition of MOMP by binding and sequestering BIM. As shown in Figure 6D, BIM was induced after IL-3 withdrawal. BIM was not coim-

munoprecipitated with MCL-1 from cells grown in the presence of IL-3, and little BIM was detectable in MCL-1 coimmunoprecipitations from cells grown without IL-3, due to the low amount of MCL-1 present after IL-3 withdrawal. However, upon IL-3 withdrawal and in the presence of GSK3 inhibitor, abundant amounts of BIM were associated with the rescued MCL-1, suggesting that MCL-1 prevented BIM from inducing MOMP by its sequestration (Figure 6D, right panel).

Discussion

In this study, we present an apoptosis-regulatory mechanism downstream of PI3K and demonstrate that inactivation of GSK-3 is an important function of PI3K- and AKT-mediated protection from apoptosis. Further, we found that stabilization of MCL-1 is a vital consequence of inactivation of GSK-3. Ultimately, this results in the sequestration of BIM and prevention of the permeabilization of the outer mitochondrial membrane in hematopoietic cells.

PI3K is involved in IL-7-mediated (Barata et al., 2004) as well as IL-3-mediated survival (Songyang et al., 1997).

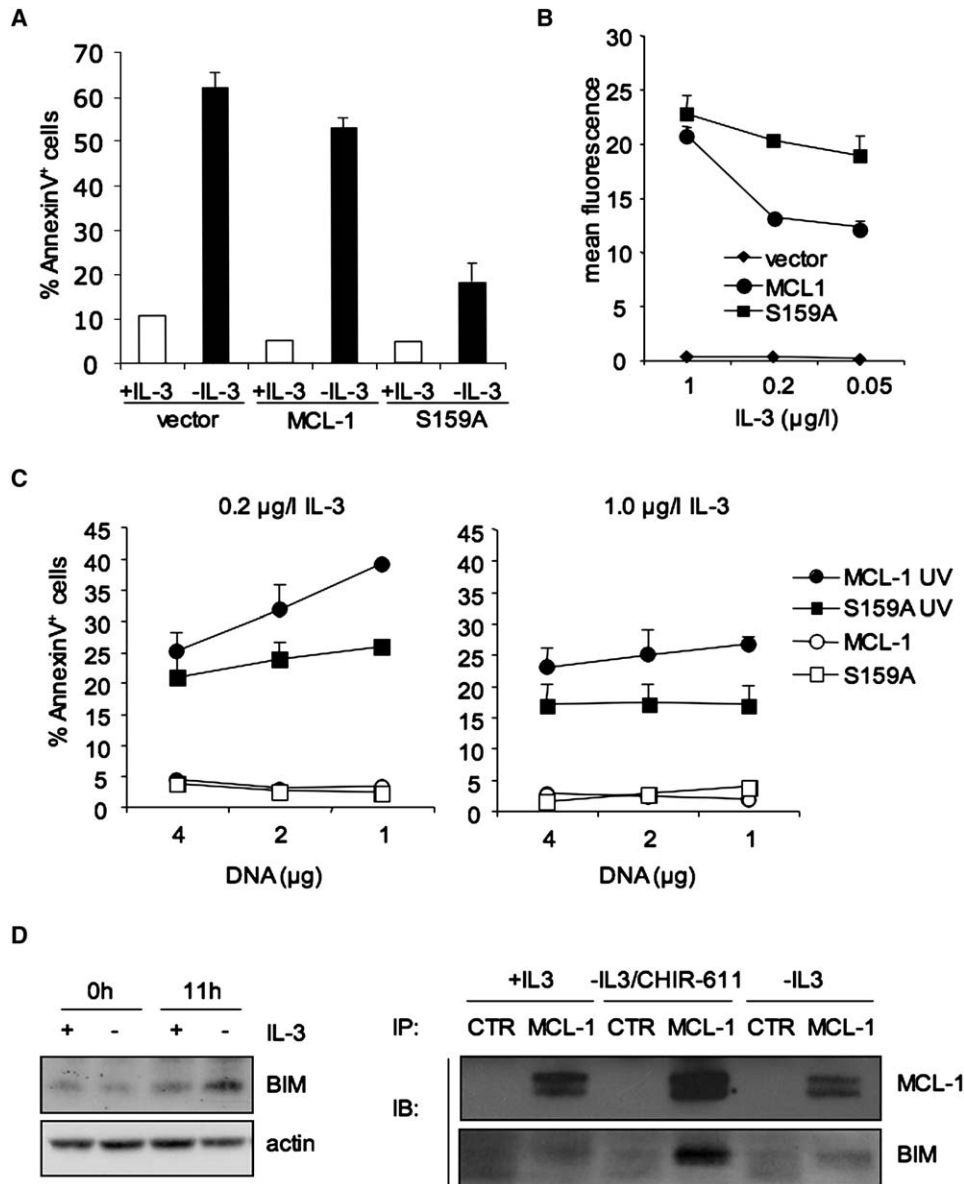


Figure 6. Stable MCL-1 Provides Increased Protection from Cell Death and Increased BIM Sequestration

(A) Plasmids encoding MCL-1 and MCL-1^{S159A} (4 µg) were introduced together with a plasmid encoding spectrin-GFP (10 µg) into FL5.12 cells. Cells were maintained at a reduced concentration of IL-3 of (0.05 µg/l) for 15 hr and subsequently maintained at reduced (+IL-3) or without (-IL-3) IL-3. Apoptosis of bright green fluorescent cells was determined by Annexin V-APC staining after 9 hr. The average of triplicate samples from one experiment is shown; error bars represent the standard deviation.

(B) FL5.12 cells were transfected with 4 µg of plasmid encoding human MCL-1, MCL-1^{S159A}, or empty vector, together with a plasmid encoding spectrin-GFP (10 µg). Cells were then maintained for 15 hr in media containing 0.05 µg/l, 0.2 µg/l, or 1 µg/l IL-3. Levels of human MCL-1 in bright green cells were determined by intracellular staining and flow cytometry. The average of triplicate samples from one experiment is shown; error bars represent the standard deviation.

(C) Plasmids (1 µg, 2 µg, and 4 µg) encoding MCL-1 or MCL-1^{S159A} were introduced together with a plasmid encoding spectrin-GFP (10 µg) into FL5.12 cells. Cells were maintained at conventional (1 µg/l) or decreased (0.2 µg/l) concentrations of IL-3 for 15 hr and subsequently UV irradiated (200 J/m²). Apoptosis of bright green fluorescent cells was determined by Annexin V-APC staining after 9 hr. The average of triplicate samples from one experiment is shown; error bars represent the standard deviation.

(D) FL5.12 cells were grown in the presence (+IL3) or absence (-IL3) of IL-3 with or without CHIR-611 (0.75 µM) for 11 hr, and induction of BIM was assessed by Western blotting (left panel). Immunoprecipitation was performed with anti-MCL-1 (MCL-1) and control antibody (CTR), and the immunoprecipitates were probed for MCL-1 and BIM.

Likewise, MCL-1 is absolutely required for IL-7-mediated survival of immature T and B cells as well as hematopoietic stem cells (Opferman et al., 2003, 2005). Our data suggest that these are requirements for components of the same pathway. In addition to lymphocyte

apoptosis, the mechanism observed in our system may also explain a suggested role for GSK-3 in the programmed cell death of erythroid progenitors (Somerville et al., 2001). Thus, regulation of MCL-1 by GSK-3 is likely to be an important switch in the delicate

life-or-death decisions made in immature hematopoietic cells on their way to maturation.

An interesting aspect of GSK-3 is its inactivation by phosphorylation, while most kinases are activated by phosphorylation; GSK-3 can therefore function in a manner opposite to that of most other kinases. For example, while ERK phosphorylates and thereby destabilizes proapoptotic BIM upon growth factor stimulation (Ley et al., 2003; Luciano et al., 2003), GSK-3 phosphorylates and destabilizes antiapoptotic MCL-1 when growth factor is low or withdrawn. The pharmacological inhibition of GSK-3 with highly specific small molecules provided us with the advantage that both GSK-3 α and GSK-3 β activity was abrogated simultaneously with high specificity (Wagman et al., 2004) and facilitated the finding that GSK-3 has a major role in the regulation of apoptosis by IL-3.

Our findings add to other well-established mechanisms downstream of growth factor stimulation governing apoptosis: the phosphorylation of the BH3-only protein BAD, as well as the transcription factors FOXO1 and FOXO3a, being transcriptional inducers of FasL and BIM, mediate their retention in the cytoplasm by 14-3-3 and hence inactivation (Downward, 2004). PIM, a short-lived kinase induced by IL-3, has been shown to protect cells from apoptosis (Fox et al., 2003). Other reports demonstrated a glucose-dependent AKT-mediated protection and showed that hexokinase activity confers protection downstream of AKT (Gottlob et al., 2001). Together, the integration of these signals governs the response of the cell to a given growth factor environment.

While enforced high expression of BCL-xL can provide long-term protection from apoptosis, AKT does not seem to provide as much long-term protection (Edinger and Thompson, 2002; Plas et al., 2001). Likewise, prevention from apoptosis by GSK-3 inhibition was incomplete at later time points, even upon readdition of CHIR-611. This may reflect the impact of AKT and inhibition of GSK-3 on turnover of MCL-1, rather than sustained expression of this antiapoptotic BCL-2 family protein.

Our results support the idea that IL-3 signaling through PI3K regulates MCL-1 protein stability rather than *mcl-1* mRNA transcription. Induction of *mcl-1* mRNA by growth factors has been shown to be transient upon growth factor stimulation (Opferman et al., 2003). In our experiments, when we readded IL-3 directly after washing the cells, only a slight mRNA induction by 1 hr was observed, which did not explain the sustained differences in the steady-state levels of this short-lived protein, dependent on IL-3 withdrawal and GSK-3 activity. Also, GSK-3 inhibition did not induce MCL-1 mRNA, while having a substantial effect on MCL-1 protein half-life and therefore steady-state levels of the protein. Interestingly, when separated by SDS-PAGE, MCL-1 often appears as a doublet, and, upon IL-3 withdrawal, the upper band disappears before the lower. This may indicate that the upper band is the GSK-3 phosphorylated form. However, since we only observe phospho-S159 MCL-1 when the proteasome is inhibited, it is more likely that this upper band represents MCL-1 that has been phosphorylated at the "GSK-3 priming site" (T163).

The stability of MCL-1 may reflect the intensity of the growth factor signal, as the decreased protection by wild-type MCL-1 compared to the S159A mutant was

more pronounced when the concentration of IL-3 was decreased. Therefore, inhibition of GSK-3 by growth factor signaling may constitute a gatekeeper function by elevating steady-state levels of MCL-1. We observed that, upon IL-3 withdrawal, the decrease of the steady-state MCL-1 levels correlated well with the induction of cytochrome c release (beginning after about 6 hr after cytokine depletion in FL5.12 cells), suggesting that decrease of MCL-1 is a requirement for cytochrome c release, which is prevented by MCL-1 stabilization due to GSK-3 inhibition. This is consistent with a report that MCL-1 elimination is a prerequisite for BAX/BAK activation following UV radiation, preceding mitochondrial translocation of BAX and BCL-xL (Nijhawan et al., 2003).

The regulation of MCL-1 by GSK-3 resembles the regulation of MYC, which is a labile protein that is further destabilized upon phosphorylation by GSK-3 (Sears et al., 1999, 2000). In contrast, phosphorylation of MYC at the GSK-3 priming serine resulted in increased stability. Interestingly, MCL-1 similarly shows increased stability when phosphorylated on threonine 163, which represents the GSK-3 priming phosphorylation in this protein (Domina et al., 2004). Thus, phosphorylation of either MYC or MCL-1 at the GSK-3 priming site stabilizes the protein, while phosphorylation by GSK-3 promotes its accelerated degradation. However, as MYC and MCL-1 are labile proteins in the absence of GSK-3-mediated phosphorylation, additional mechanisms are responsible for their turnover. Other modifiers of MCL-1 stability have been described: translationally controlled tumor protein (TCTP) enhances MCL-1 stability (Liu et al., 2005), whereas the BH3-only protein Noxa was described to promote MCL-1 degradation (Willis et al., 2005).

Recently, Mule/ARF-BP-1, a 482 kDa protein containing a BH3 domain, was identified as a major MCL-1 ubiquitin ligase. Among the evolutionary conserved MCL-1 lysine residues, Mule was shown to primarily mediate ubiquitinylation of lysines 136, 194, and 197 (Zhong et al., 2005). These lysines are located close to the GSK-3 phosphorylation site S159 in the MCL-1 PEST and the BH3 domain of MCL-1 (aa 204–226), which was shown to associate with the BH3 domain of Mule. It is therefore tempting to speculate that MCL-1 ubiquitinylation by Mule is enhanced by S159 phosphorylation through GSK-3. Since the biological consequence of Mule activity, degrading MCL-1 and p53 (Chen et al., 2005), can be divergent, it is conceivable that substrate-specific levels of regulation exist.

An apparent paradox regarding the proapoptotic role of GSK-3 is the requirement of GSK-3 β for survival. Homozygous deletion of GSK-3 β in mice resulted in embryonic lethality during midgestation due to liver failure resulting from reduced NF- κ B activity, an effect that was not rescued by the remaining GSK-3 α (Hoefflich et al., 2000). It is conceivable however, that GSK-3 β may play a dual role resulting in survival (in liver cells through NF- κ B) or death (in hematopoietic cells through MCL-1 elimination). This is consistent with our finding that GSK-3 inhibition did not protect against apoptosis induced by TNF- α .

In addition to the hematopoietic system, regulation of MCL-1 by GSK-3 might also play a role in neuronal

apoptosis. Overexpression of GSK-3 β can induce apoptosis of neuronal cells (Bijur et al., 2000; Crowder and Freeman, 2000; Hetman et al., 2000; Pap and Cooper, 1998). While these reports did not clearly show that GSK-3 actually takes part in apoptotic signaling itself, they suggested that GSK-3 may have a role in apoptosis downstream of PI3K/AKT. Likewise, GSK-3 inhibitors have been demonstrated to inhibit apoptosis of cerebellar granule neurons (Cross et al., 2001). Since expression levels of MCL-1 were associated with neuroprotection (Mori et al., 2004), this raises the intriguing possibility that this PI3K/AKT/GSK-3/MCL-1 pathway is important for the regulation of neuronal cell survival as well as that of hematopoietic cells.

Experimental Procedures

Cell Culture

FL5.12 and FL5.12 BCL-xL (kindly provided by Jeff Rathmell, Duke University, Durham, North Carolina), 32Dcl3 (ATCC), BA/F3 cells (kindly provided by Abelardo López-Rivas, Instituto de Parasitología y Biomedicina, Granada, Spain) were grown in RPMI with 10% FCS supplemented with the antibiotics penicillin/streptomycin and 1 μ g/l recombinant interleukin-3 or GM-CSF (PeproTech, Rocky Hill, New Jersey). For some experiments, cells were kept at low IL-3 concentrations of 0.2 μ g/l or 0.05 μ g/l for 15 hr prior to apoptosis induction. To deplete cultures of IL-3 or GM-CSF, cells were washed twice in PBS and once in RPMI and grown in RPMI with 10% FCS. FL5.12 BAF3 and 32D were cultured at less than 5×10^5 cells/ml. HeLa cells were grown in DMEM with 10% FCS. LY294002, UO126, and MG132 were from Calbiochem, San Diego, California; Cycloheximide was from Sigma, St. Louis, Missouri. UV irradiation was performed using a Stratalinker (Stratagene, San Diego, California).

Pharmacological Inhibition of GSK3

The GSK-3 inhibitor CHIR-611 (also designated as CT98014) and CHIR-911 (also known as CT99021), low-molecular-weight (MW 486 and 465, respectively) small molecules, are ATP competitive inhibitors of GSK-3 β , giving an IC₅₀ of 1 and 5 nmol/l, respectively, in a cell-free binding assay (Wagman et al., 2004). These inhibitors are approximately equipotent against both isoforms of GSK-3 (α/β). GSK-3 inhibitors of this class exhibit >2500-fold selectivity versus a panel of 16 other protein kinases, including CDC2 and ERK2, which are the closest homologs of GSK-3. The concentration of GSK-3 inhibitor causing half-maximal glycogen synthase stimulation (EC₅₀) in CHO-IR cells was 75 nmol/l for CHIR-611 and 766 nmol/l for CHIR-911 (Wagman et al., 2004). The inhibitors were diluted in DMSO. In all experiments, the corresponding amount of DMSO was present in exposed and control cells. In some experiments, a small molecule compound, CT98085, which is structurally related to CHIR-611 but inactive for inhibition of GSK-3, was included as an additional control.

Apoptosis Assays

Apoptosis was determined by staining with Annexin V-FITC (Caltag, Burlingame, California) and propidium iodide. GFP-expressing cells were analyzed by labeling with Annexin V-APC (Caltag, Burlingame, California).

Cytochrome c Release

Cytochrome c-GFP release from mitochondria was determined by the cell-lysis-and-mitochondria-intact (CLAMI) assay as previously described (Waterhouse et al., 2001). Briefly, after having transduced cells with cytochrome c-GFP-fusion construct as described (Goldstein et al., 2005), the cell membrane was lysed by digitonin in CLAMI buffer (250 mM sucrose, 80 mM KCl, 50 μ g/ml digitonin) on ice for 5 min, then cells were diluted in 200 μ l of CLAMI buffer and GFP content was analyzed by flow cytometry.

Constructs, Transfection, and Retroviral Infection

Human MCL-1 was amplified by PCR and cloned in pcDNA3.1 (Invitrogen, San Diego, California) using the following primers: MCL-1S,

5'-ATGTTTGGCCTCAAAGAAGACGC-3', and MCL-1AS, 5'-TCTTAT TAGATATGCCAAACAGC-3'. For experiments investigating protection by MCL-1 or MCL-1^{S159A}, the C-terminal HIS/V5 was removed by introducing a stop codon using the QuikChange Mutagenesis Kit (Stratagene) according to the manufacturer, using the following primers: STOPMCL-1 S, 5'-GCATATCTAATAAGATAGGG CAATTCTG-3', and STOPMCL-1 AS, 5'-CAGAATTGCCCTATCTTA TTAGATATGC-3'. The S159A mutant was generated using the following primers: S159A, S 5'-ACGGACGGGGCACTACCCTCGA-3', and S159A AS, 5'-TCGAGGGTAGTGCCTCCGTCGT-3'. Myristoylated Akt was cloned in pLXIN (BD Clontech), from which the neomycin resistance encoding sequence was removed and replaced by a puromycin resistance encoding sequence. HeLa, 293T, and NIH3T3 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. FL5.12 cells were transfected by electroporation as described by others (Shimamura et al., 2000). Producer cell supernatants containing retroviruses were added to FL5.12 and 32Dcl3 cells in the presence of IL-3/GM-CSF and 5 μ g/ml polybrene, spun for 20 min at room temperature (RT), and then cultured at 37°C for 1 hr, followed by addition of media. Constructs encoding shRNA against *Xenopus*-ASH transcription factor (control), GSK-3 α , and GSK-3 β were kindly provided by David Turner (University of Michigan, Ann Arbor, Michigan).

Western Blot

Cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8]), 1% NP-40, 1 mM NaVO₄, 20 mM NaPO₄ [pH 7.6], 10 mM NaF, 3 mM β -glycerol phosphate, and 5 mM sodium pyrophosphate (all from Sigma, St. Louis, Missouri); 100 μ M MG-132 (Calbiochem); and complete protease inhibitor cocktail (Roche, Indianapolis, Indiana). Protein (10–30 μ g) was separated by SDS-PAGE, transferred on nitrocellulose membranes, and probed with anti-phospho-S9GSK-3 β , anti-phospho-S473AKT, anti-AKT (Cell Signaling, Beverly, Massachusetts), anti-BIM (Sigma or Stressgen, Ann Arbor, Michigan) anti-GSK-3 β (Upstate, Charlottesville, Virginia), anti-BCL-2 (Santa Cruz, Santa Cruz, California), anti-mouse MCL-1 (Rockland Immunochemicals, Gilbertsville, Pennsylvania), anti-human MCL-1 (Pharmingen), anti-Actin (MP Chemicals, Aurora, Ohio), anti-phospho-S159-MCL-1 (custom made by Zymed, San Francisco, California), anti-V5 (Invitrogen), or anti-HA (Santa Cruz).

Immunoprecipitation

Cells (1×10^8) are lysed as above except using 1% CHAPS as a detergent. IP was performed with 1 μ g of antibody for 4 hr; then 20 μ l washed protein A agarose beads were added and incubated for 30 min. Beads were washed with lysis buffer, lysis high salt (400 mM NaCl), and again lysis buffer and separated by SDS-PAGE.

Intracellular Staining of MCL-1

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT, blocked with 1% BSA in PBS for 15 min at RT, permeabilized with 0.05% saponin 1% BSA in PBS for 30 min 4°C, stained with mouse anti-human MCL-1 (Pharmingen) or IgG ctrl 1:100 in PBS 0.05% saponin 1% BSA for 30 min at 4°C, washed twice with PBS, and stained with APC-conjugated anti-mouse (1:500) in PBS 0.05% saponin 1% BSA for 30 min at 4°C. After washing, cells were suspended in 200 μ l of PBS 1% BSA and analyzed by flow cytometry. For analysis, cells expressing high levels of spectrin-GFP (cotransfected along with human MCL-1) were gated. Cells were stained for MCL-1, and mean fluorescence under each condition was determined by subtraction of the isotype control.

Affinity Pull-Down

Lysis was performed as above, and lysates were incubated with 20 μ l washed Ni²⁺ beads for 2–3 hr and washed with lysis buffer, lysis buffer containing high salt (500 mM NaCl), and lysis buffer containing 10 mM imidazole followed by a final lysis buffer wash. To produce MCL-1 for in vitro phosphorylation experiments, His-tagged MCL-1 or MCL-1^{S159A} was transfected in 293T cells. The following day, media was replaced with media containing 0.75 μ M of CHIR-611, and cells were harvested after 36 hr total time of expression.

Phosphorylation Assay

Protein-loaded beads (20%) produced as described above were put in a kinase reaction according to the manufacturer with recombinant GSK3 (New England Biolabs, Beverly, Massachusetts) and 20 μ Ci adenosine 5'-(γ -³²P)triphosphate (Amersham, Buckinghamshire, United Kingdom) for 20 min, washed as above, and analyzed by SDS-PAGE, autoradiography, and Western blot.

Real-Time RT-PCR

Relative expression of MCL-1 was determined by real-time RT-PCR in comparison to the L32 housekeeping gene as described elsewhere (Droin et al., 2003). Primer sequences were the following: mMcl-1S, 5'-GTGGAGTTCCTCCACGTACAGGA-3', and mMcl-1AS, 5'-AGCAACACCCGCAAAGC-3'.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at <http://www.molecule.org/cgi/content/full/21/6/749/DC1/>.

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