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News and Commentary

Unraveling MCL-1 degradation

JT Opferman*,[1]

[1] Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38105, USA
* Corresponding author: JT Opferman, Department of Biochemistry, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105, USA. Tel: +1-901-495-5524; Fax: +1-901-525-8025; E-mail: joseph.opferman@stjude.org

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Growth factor signaling regulates the growth and survival of cells during development and homeostasis. The BCL-2 family of proteins represents a critical control point in the apoptotic pathway residing upstream to irreversible commitment to cell death, where its members control the release of apoptogenic factors from the mitochondria by regulating mitochondrial outer membrane permeabilization. A recent article by Mauer *et al*. (*Mol. Cell* **21**: 749–760, 2006) provides convincing evidence that one important role of growth factor signaling is its regulation of the protein stability of the critical antiapoptotic BCL-2 family member, MCL-1.

Introduction: The Antiapoptotic BCL-2 Family Member MCL-1

Myeloid cell leukemia-1 (MCL-1), originally identified in differentiating myeloid cells, possesses the BCL-2 homology (BH) domains 1–4 like the other antiapoptotic BCL-2 family members (BCL-2, BCL-X_L, BCL-W, and A1).[1] These anti-apoptotic molecules antagonize cell death by sequestering proapoptotic family members including the BH3-only class (BIM, PUMA, BAD, BID, etc.) and multidomain proapoptotics (BAX and BAK).[2] Previously, genetic ablation experiments identified that *Mcl-1* is essential for early embryonic development as *Mcl-1*-deficiency results in a peri-implantation embryonic lethal phenotype.[3] Furthermore, targeted disruption of *Mcl-1* in specific lineages has revealed that MCL-1 also plays obligate roles during the development of the immune system and during early hematopoiesis.[4,5]

MCL-1 expression can be induced by survival and differentiation signals such as cytokines and growth factors. Its expression is highly regulated at the transcriptional, post-transcriptional, and post-translational levels. In response to growth factor stimulation, *Mcl-1* undergoes transcriptional activation downstream of a number of well-known signal transduction pathways (e.g. MAP kinases, PI3K/Akt, and JAK/STAT).[6] Furthermore, like *Bcl-X*, *Mcl-1* has been shown to be post-transcriptionally regulated through alternative splicing to form a proapoptotic molecule (MCL-1_S) made up of exons 1 and 3.[6] MCL-1 is also regulated by phosphorylation in response to a variety of cell stimuli. For example, during oxidative stress, MCL-1 is phosphorylated by JNK at two residues (serine-121 and threonine-163 of human MCL-1) resulting in the loss of its survival function.[7] Conversely, ERK-mediated phosphorylation of human MCL-1 can also occur at threonine-163 in response to differentiation slowing MCL-1 protein turnover.[8] In dying cells, MCL-1 can be eliminated by caspase-mediated cleavage generating a potent proapoptotic protein that can enhance the cell death response.[9] These findings demonstrate that MCL-1 is tightly regulated at several levels, but it remains to be established what the physiological regulation of MCL-1 is during normal development and homeostasis.

The Glycogen Synthase Kinase-3-Signaling Pathway

Although glycogen synthase kinase-3 (GSK-3) was initially discovered in 1980 as an enzyme involved in glycogen metabolism, it has since been linked to a vast myriad of cellular functions.[10] It exists in two closely related isoforms (GSK-3α and GSK-3β) encoded by different genes. The GSK-3 consensus site is [S/T]XXX[S/T℗]in which the second [S/T] is already phosphorylated by another kinase forming a priming site necessary for the docking of GSK-3 to its substrates.[11] In the absence of growth factors, active GSK-3 can dock on substrate proteins that possess the priming phosphate and induce phosphorylation. In the presence of growth factors, signaling cascades activate several kinases (such as Akt) that directly inhibit GSK-3 activity by phosphorylation of a N-terminal serine residue of GSK-3.[12] This generates a 'psuedosubstrate' which self-inhibits GSK-3 by occupying the priming site used to dock on substrate proteins.

GSK-3 kinases exercise control over a broad array of cellular processes; having been demonstrated to phosphorylate more than 40 target proteins involved in pathways including embryogenesis, cellular architecture, motility, metabolism, and cell survival.[11] In most cases the role of GSK-3 activation in cell survival is to render cells more sensitive to cell death stimuli.[13,14] However, this effect may be cell type and pathway specific as paradoxically the lethality observed in the GSK-3β-deficient embryos was due to enhanced liver apoptosis.[15] Although little is known about GSK-3 isoform-specific function, the two family members are not redundant since disruption of GSK-3β resulted in embryonic lethality despite expression of GSK-3α.[15]

Regulation of MCL-1 Half-Life

Evidence suggests that MCL-1 acts as an important short half-life inhibitor in the genotoxic cell death pathway and exerts its function relatively upstream in the apoptotic pathway.[16] Wang and colleagues have demonstrated that in response to genotoxic stress the proteasome-dependent elimination of MCL-1 is required for downstream apoptotic events including

BAX and BAK oligomerization, cytochrome *c* release, and caspase activation. Treatment of cells with proteasome inhibitors could block the elimination of MCL-1 and prolong survival. Interestingly, in these studies the degradation of MCL-1 appeared to be constitutive as the half-life of MCL-1 was the same in the presence or absence of the death stimuli.[16]

To identify the pathway leading to MCL-1 poly-ubiquitylation, Wang and colleagues utilized a biochemical purification strategy to purify a novel E3 ubiquitin ligase (MULE) required for MCL-1 degradation.[17] Expression of a mutant MCL-1, in which the targets of ubiquitin ligation were mutated, or small hairpin RNA-mediated 'knock-down' of MULE increased MCL-1 levels and promoted survival in response to genotoxic death stimuli. MULE belongs to the E6-AP carboxy-terminus (HECT)-domain family of E3 ligases that possess N-terminal domains that allow substrate recognition and conserved C-terminal catalytic HECT domains that can directly transfer ubiquitin to substrates.[18] Surprisingly, MULE possesses a well-conserved BH3 domain similar to that of proapoptotic BAK that allows it to selectively target MCL-1.[17,19] Of note, while MULE may target MCL-1 for degradation it is not only specific for MCL-1 as several other groups independently identified the same E3 ligase and have implicated it in the proteasome degradation of p53, $E3^{Histone}$, and c-Myc.[20–22] Future work will be necessary to unravel how this E3 ligase can regulate such a wide variety of substrates and how this dynamic network is regulated.

Regulation of MCL-1 Stabilization by GSK3

A recent report by Green and colleagues offers insight into how apoptosis is regulated by GSK-3 downstream of PI3K/Akt signaling.[23] Using interleukin-3 (IL-3)-dependent cell lines, they demonstrated that withdrawal of IL-3 resulted in the loss of Akt activity and subsequently decreased the inactive phosphorylated form of GSK-3 prior to the induction of apoptosis. In this model, cell death could be prevented by readdition of IL-3, which rapidly results in Akt activation and subsequent inactivation of GSK-3 by Akt-mediated phosphorylation. Furthermore, either constitutive expression of an active form of Akt (myr-Akt) or treatment of cells with a cell permeable GSK-3 inhibitor (CHIR-611) was capable of preventing the apoptosis induced by IL-3 withdrawal illustrating the importance of the Akt/GSK-3 axis in mediating sensitivity of growth factor withdrawal.

Since MCL-1 is an important antiapoptotic factor induced in response to growth factor signaling, Green and colleagues examined the response of MCL-1 to IL-3 withdrawal. MCL-1 protein levels substantially decreased in a time course (6–10 h) consistent with the onset of mitochondrial outer membrane permeabilization. Loss of MCL-1 expression after IL-3 withdrawal was not a consequence of cell death since overexpression of BCL-X_L, which prevents cell death in response to IL-3 withdrawal, did not prevent MCL-1 elimination. However, treatment of cells with the GSK-3 inhibitor (CHIR-611) substantially maintained MCL-1 expression after IL-3 withdrawal. Of note, although pharmacological inhibition of Akt (with LY294002) in IL-3 cultured cells promoted the loss of MCL-1 expression this was insufficient to induce apoptosis indicating that loss of MCL-1 alone is insufficient to initiate cell death.

Strikingly, the loss of MCL-1 expression after IL-3 withdrawal or GSK-3 inhibition was not due to major effects on *Mcl-1* transcription, but instead altered the half-life of MCL-1 protein. Withdrawal of IL-3 or inhibition of Akt caused a rapid loss of MCL-1 protein whereas addition of GSK-3 inhibitor or constitutive expression of active Akt prolonged MCL-1 expression to near that of IL-3-treated levels. Therefore, MCL-1 protein levels seem to be dynamically regulated by growth factor signaling at the level of protein half-life; positively regulated by Akt and negatively regulated by GSK-3.

MCL-1 protein contains a conserved consensus site for GSK-3 phosphorylation. Unlike wild-type MCL-1, a mutant of human MCL-1 lacking this site (serine-159 mutated to alanine) was not phosphorylated *in vitro* by recombinant GSK-3β. RNAi targeting of either GSK-3α or to a lesser extent GSK-3β decreased S159-phosphoryated MCL-1. Interestingly, a phosphospecific antibody incapable of recognizing mutant $MCL\text{-}1^{S159A}$ only detected the phospho-MCL-1 when cells were treated with a combination of PI3K and proteasome inhibitors (LY294002 and MG132, respectively) indicating that S159-phosphorylated MCL-1 is rapidly degraded. Therefore, Green's group investigated whether MCL-1 phosphorylation may regulate its ubiquitination-dependent degradation. When PI3K signaling was blocked, only wild-type MCL-1, but not $MCL\text{-}1^{S159A}$ mutant was ubiquitinylated and rapidly degraded; this could be blocked by pharmacological inhibition of GSK-3 (CHIR-611). Therefore, GSK-3 phosphorylation regulates MCL-1 protein levels by targeting it for ubiquitin-dependent degradation.

Expression of the $MCL\text{-}1^{S159A}$ rendered IL-3-dependent cell lines resistant to cell death induced by growth factor withdrawal. Even when cultured in low amounts of growth factor, MCL-1 protein levels were much higher in $MCL\text{-}1^{S159A}$-expressing cells than in cells expressing wild-type MCL-1. Indeed, $MCL\text{-}1^{S159A}$ cells demonstrated increased resistance to cell death induced by UV irradiation consistent with the prolonged stability of the mutant. How does MCL-1 prevent cell death in response to growth factor withdrawal? The proapoptotic BH3-only family member BIM is induced by growth factor withdrawal; however, when IL-3 is withdrawn MCL-1 is phosphorylated by GSK-3 and degraded. When GSK-3 was inhibited during IL-3 withdrawal, MCL-1 was stabilized and readily sequestered large amounts of BIM preventing cell death. Therefore, phosphorylation may target MCL-1 for elimination preventing its antagonism of proapoptotic molecules and fostering cell death.

How does Phosphorylation Regulate MCL-1 Degradation?

It has been suggested that in some cell types that MCL-1 turnover may be a constitutive process independent of cell death signaling.[16] In contrast, Green and colleagues suggest a growth factor-dependent regulation of MCL-1 stability.[23]

Perhaps, the difference in cell types lie at the heart of this discrepancy; in growth factor-dependent cell lines, the regulation of MCL-1 levels may be under a different level of control than in fibroblasts.

It is intriguing to hypothesize how the phosphorylation of MCL-1 may regulate proteasome-dependent degradation. Ubiquitin conjugation can often be regulated by extracellular signaling pathways. Phosphorylation can target proteins for rapid degradation by allowing the substrates to be recognized by the ubiquitination machinery or modulate the activity of the E3 ligase itself. The best examples are the RING E3s known as Skp1-CUL1-F box protein complexes that target phosphorylated proteins for proteasome degradation.[18] HECT E3 ligases can also be regulated by phosphorylation downstream of signaling pathways.[18] Therefore, it will be interesting to address whether phosphorylation of MCL-1 by GSK-3 may regulate the direct recognition of MCL-1 by the HECT-family member MULE or perhaps other E3 ligases. Furthermore, it remains to be established what kinase(s) are responsible for the priming phosphorylation (threonine 163 in human MCL-1) necessary for the docking of GSK-3 to MCL-1. Interestingly, phosphorylation of human MCL-1 at threonine-163 has been observed by two other groups to be catalyzed by JNK and ERK in response to extracellular cues.[7,8] These findings suggest an additional level at which MCL-1 stabilization may be regulated upstream of GSK-3.

In healthy cells, MCL-1 is bound to proapoptotic molecules such as BAK preventing their activation (Figure 1).[24] However, upon cell death, it can be displaced by BH3-only family members (such as BIM, NOXA, PUMA, etc.) promoting BAK activation and allowing MCL-1 to be targeted by MULE for proteasome-dependent degradation.[17,19,25] Interestingly, Green and colleagues demonstrate that withdrawal of IL-3 combined with inhibition of GSK-3, promoted the interaction between MCL-1 and proapoptotic BIM.[23] These data imply that phosphorylation of MCL-1 (at human serine-159) may hinder its interaction with proapoptotic BCL-2 family members perhaps further sensitizing cells to apoptotic stimuli.

Concluding Remarks

MCL-1 plays several obligate roles during normal development and homeostasis, but the basis of its regulation is relatively unknown. While only further genetic studies will determine the physiological relevance of MCL-1 ubiquitin-mediated degradation in response to phosphorylation by GSK-3, the recent studies by Green and others have identified several potential pathways for controlling the expression of this important antiapoptotic player.

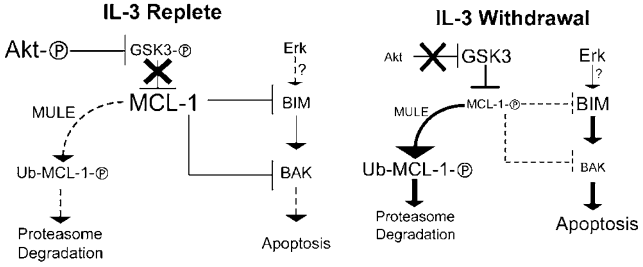

Figure 1 In growth factor-stimulated cells (IL-3 replete), active Akt (phosphorylated) inactivating GSK-3 by phosphorylation on its N-terminal serine. Inactive (phosphorylated) GSK-3 cannot phosphorylate MCL-1, thus preventing its ubiquitylation by the E3 ligase (MULE). This stabilizes MCL-1 levels promoting antagonism of proapoptotic molecules (BIM, BAK, etc.) and preventing cell death. Upon growth factor withdrawal (IL-3 withdrawal) Akt is inactive preventing the phosphorylation of GSK-3. Thus, active GSK-3 can phosphorylate MCL-1, targeting it for ubiquitylation by MULE leading to the loss of MCL-1 protein. While merely losing MCL-1 expression may not be enough to induce apoptosis, it may sensitize cells by reducing the antagonism of proapoptotics. Growth factor withdrawal also activates other signaling pathways, some of which may induce the expression of proapoptotic molecules (such as BIM) tipping the balance towards the induction of cell death

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