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Review

# Mcl-1; the molecular regulation of protein function

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ABSTRACT

**Apoptosis, an essential and basic biological phenomenon, is regulated in a complex manner by a multitude of factors. Myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic member of the B-cell lymphoma 2 (Bcl-2) family of apoptosis-regulating proteins, exemplifies a number of the mechanisms by which a protein's contribution to cell fate may be modified. The N-terminus of Mcl-1 is unique amongst the Bcl-2 family, in that it is rich in experimentally confirmed and putative regulatory residues and motifs. These include sites for ubiquitination, cleavage and phosphorylation, which influence the protein's stability, localisation, dimerization and function. Here we review what is known about the regulation of Mcl-1 expression and function, with particular focus on post-translational modifications and how phosphorylation interconnects the complex molecular control of Mcl-1 with cellular state.**


## 1. Introduction

The term 'apoptosis' was first coined by Kerr, Wyllie and Currie in 1972, when they used live-cell imaging to make detailed observations of the dynamic behaviour of cells and tissues in culture. The observed morphological changes were identical in all cells undergoing apoptosis, which gave rise to the idea that this form of cell death is genetically programmed in each cell. Apoptosis has since been identified as a crucial process, in physiological terms, for a number of reasons: for the maintenance of tissue homeostasis, for the safe removal of unwanted or damaged cells, for morphogenesis during embryonic development, and for the resolution of inflammation. The culmination of apoptosis involves the activation of a family of cysteine proteases, named caspases, which are responsible for the dismantling of the cell's components, that are then packaged into smaller apoptotic bodies to be cleared by phagocytes. The activation of caspases is tightly regulated at multiple levels, and mammalian cells have evolved mechanisms whereby a cell may intrinsically regulate its own fate, or be extrinsically induced to undergo apoptosis. The two pathways share a number of molecular components, including the B-cell lymphoma 2 (Bcl-2) family – a group of proteins whose role lies in regulating mitochondrial integrity (Fig. 1). There are now 20 or so known family members, which are identified by having sequence homology to the eponymous Bcl-2 protein at one or more regions, named Bcl-2 homology (BH) domains. The family is sub-divided according to the functional contribution that each protein makes to the progression of apoptosis, with both pro-survival, and pro-apoptotic proteins, and it is the balance in activity between these opposing groups which determines a cell's progression towards apoptosis. The current state of knowledge about the Bcl-2 family is reviewed in detail elsewhere [1,2].

## 2. Mcl-1 – discovery and characterisation

Mcl-1 (myeloid cell leukemia 1) is a pro-survival member of the Bcl-2 family that was initially identified as an immediate-early gene expressed during PMA-induced differentiation of ML-1 myeloid leukemia cells [3]. Alignment algorithms identified sequence similarity to the recently discovered pro-survival protein Bcl-2, and so Mcl-1 constituted only the second of this protein family when it was discovered. The Bcl-2 family represented a new class of oncogenes that promoted oncogenesis, not through upregulation

*Abbreviations:* MCL-1, myeloid cell leukemia 1; BCL-2, B-cell lymphoma 2; BH, Bcl-2 homology; BCL-$X_L$, Bcl-2 like protein X; BID, BH3 interacting domain death agonist; PUMA, p53 upregulated modulator of apoptosis; BAX, Bcl-2-associated protein X; BAK, Bcl-2 homologous antagonist killer; BFL-1/A1, Bcl-2 related protein A1; PEST, proline/glutamic acid/serine/threonine; CDK-1, cyclin dependent kinase 1; PCNA, proliferating cell nuclear antigen; CHK-1, checkpoint 1 protein; IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; STAT, signal transducers and activators of transcription; SRE, STAT response element; CRE, cAMP response element; NF-κB, nuclear factor kappa B; CREB, cAMP response element binding protein; HIF-1α, hypoxia-inducible factor 1α; Mir-29b, micro inhibiting RNA 29b; MULE, MCL-1 ubiquitin ligase; β-TrCP, beta transducin-containing protein; GrB, granzyme B; JNK, C-Jun N-terminal kinase; ERK, extracellular regulated protein kinase; Pin-1, peptidyl-prolyl cis-trans isomerase NIMA interacting protein 1; CHX, cycloheximide

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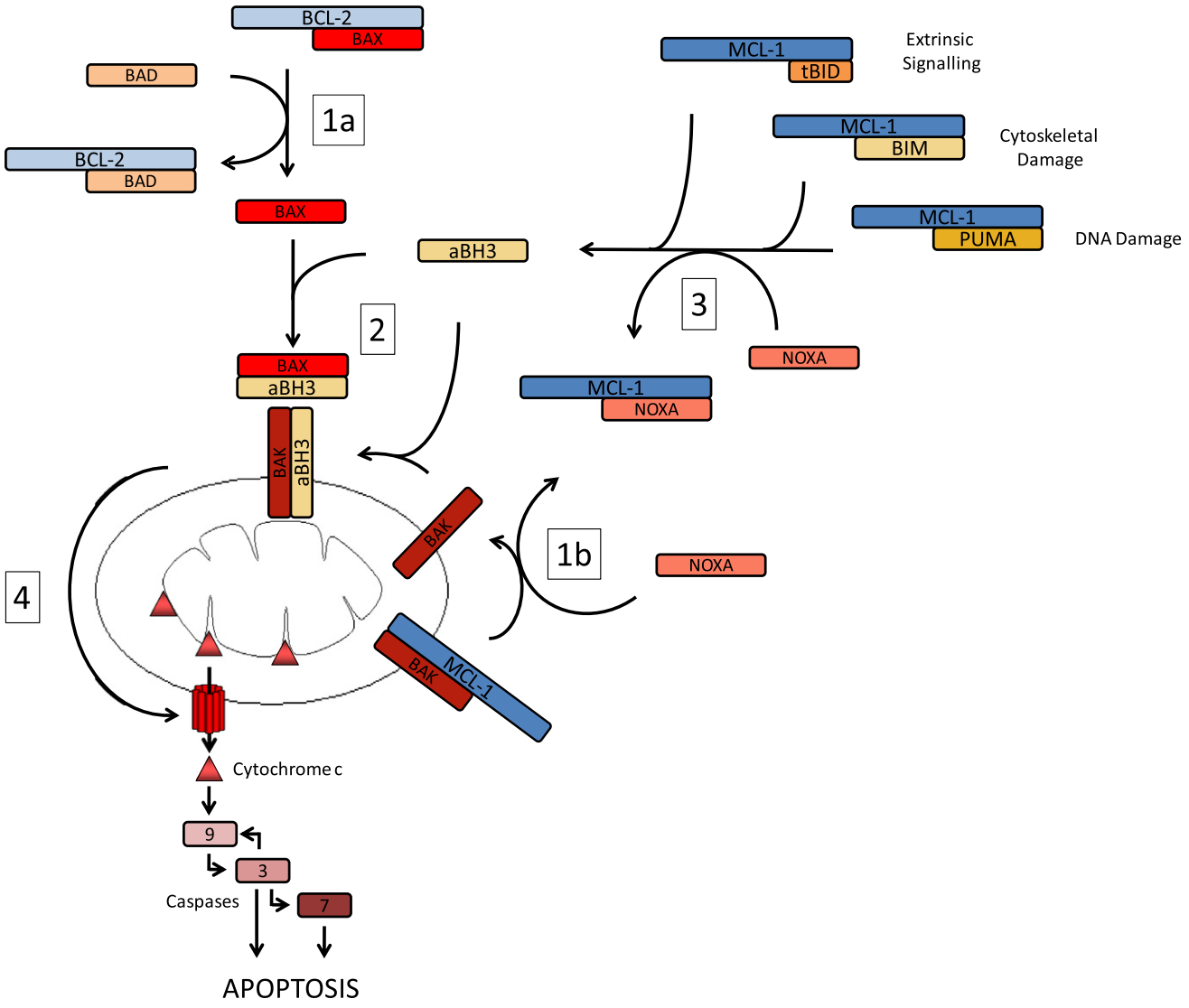


**Fig. 1.** Interactions of the Bcl-2 family. Bax [1a], a cytosolic protein, and Bak [1b], an integral mitochondrial membrane protein, are retained in monomeric forms by association with various anti-apoptotic Bcl-2 family members. This repression may be relieved by competitive inhibition from 'inactivator' BH3-only proteins, (iBH3) such as Bad and Noxa. Free cytosolic Bax [2], or free mitochondrial-membrane bound Bak require further association with 'activator' BH3-only proteins (aBH3) to induce a conformational change, and in the case of Bax, for effective mitochondrial targeting. aBH3-only proteins [3] are themselves prevented from associating with Bax and Bak by inhibitory association with anti-apoptotic Bcl-2 family members such as Mcl-1. As with Bax and Bak, the aBH3-only proteins may be freed by competitive inhibition from iBH3-only proteins. The conformational change of Bax and Bak [4] permits homo-oligomerisation of the proteins, and the formation of outer-mitochondrial membrane spanning pores. These Bax and Bak pores permit the release of cytochrome c into the cytoplasm, which triggers activation of the caspase cascade, and ultimately apoptosis.

of proliferation, but by maintaining viability through inhibition of apoptosis [4]. As predicted, dysregulation of Bcl-2 protein family expression and function has since been implicated in virtually all malignancies, and a number of other pathologies.

Sequence analysis revealed that while Mcl-1 contained 3 putative BH domains, and experimentally could protect against apoptosis, its large N-terminal region contained potential regulatory motifs that could be predicted to regulate its function (Fig. 2). Mcl-1 differs from its pro-survival relatives in its larger size of 350 residues, as compared to Bcl-2 at 239 residues (Fig. 2) and Bcl-2 like protein X (Bcl-$X_L$) at 233 residues. Residues 170–300 of Mcl-1 share a great deal of structural and functional homology to both Bcl-2 and Bcl-$X_L$, containing its three BH domains (Bcl-2 and Bcl-X possess 4), which confer the ability to heterodimerize with other family members [5]. The three solution structures of Mcl-1 that have been published each use N-terminally truncated forms of the protein, the first study with Mcl-1 as a monomer [6], the second two studies as dimers with the BH3-domains of the pro-apoptotic proteins BH3 interacting domain death agonist (BID) [7] and p53 upregulated modulator of apoptosis (Puma) and Noxa [8], respectively. All three studies show that Mcl-1 has a similar structure to its pro-survival relatives, with a surface-exposed hydrophobic groove for the binding of other BH3-domain containing proteins. Mcl-1 blocks the progression of apoptosis by binding and sequestering the pro-apoptotic proteins Bcl-2 homologous antagonist killer (Bak) and Bcl-2-associated protein X (Bax), which are capable of forming pores in the mitochondrial membrane, allowing the release of cytochrome c into the cytoplasm. In the cytoplasm, cytochrome c induces the activation of a family of cysteine proteases named caspases which are responsible for much of the macromolecular degradation observed during apoptosis. Mcl-1 also binds and sequesters a subset of the BH3-only pro-apoptotic Bcl-2 family members, which act to induce the polymerisation of Bak and Bax. The repression of Bak and Bax polymerisation by Mcl-1 can be relieved both by the degradation of Mcl-1, and by its interaction with a second subset of the BH3-only

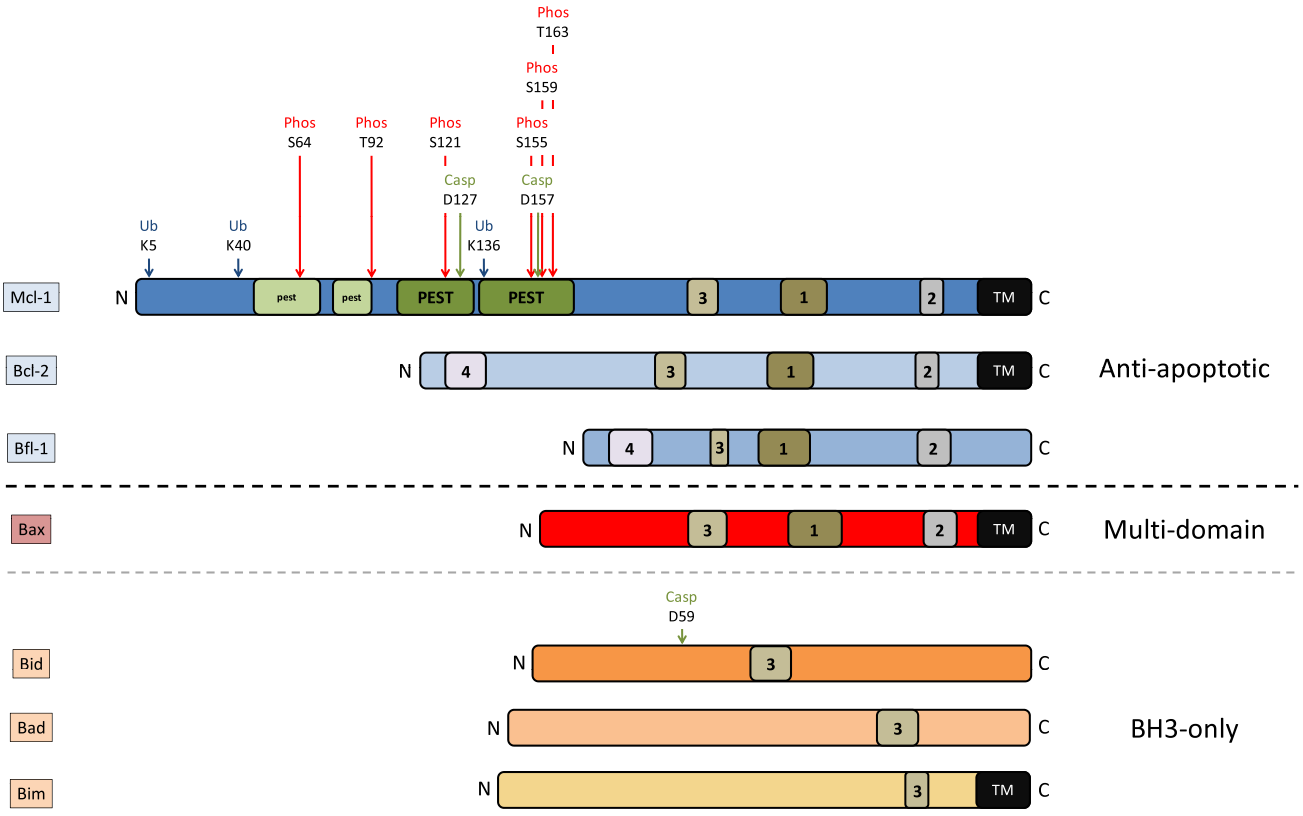


**Fig. 2.** The molecular organization of Mcl-1. A schematic representation of the Mcl-1 protein, to scale, with the relative positions of the functional regions and sites of post-translational modification highlighted. These include the transmembrane domain (TM), the Bcl-2 homology domains (numbered 1–4), two weak (lower case) and two strong (upper case) PEST sequences. Sites of post-translational modification are also shown, including ubiquitination (Ub), caspase cleavage (Casp), and phosphorylation (Phos). A schematic of a representative selection of other members of the Bcl-2 family are shown below, to scale, to compare the relative sizes and molecular organization of the proteins.

pro-apoptotic Bcl-2 family members, which induce dissociation of Bak and Bax from Mcl-1. In the C-terminal portion of Mcl-1 there is also a transmembrane domain, deletion of which blocks membrane insertion and localisation of the protein [9]. Some clues about the molecular basis of the functional non-redundancy of the Bcl-2 family were brought to light using these binding studies, which showed differences in the dimerisation pairs made by the pro-survival family members. Mcl-1, along with Bcl-2 related protein A1 constituted a subset which bound strongly to Noxa but only weakly to Bad, while Bcl-2, Bcl-w and Bcl-$X_L$ bound strongly to Bad, but only weakly to Noxa. Additionally, the BH3-only proteins Bim and Puma bound promiscuously to all the pro-survival proteins, and Mcl-1 and Bcl-$X_L$ have also been shown to preferentially bind Bak, whereas Bcl-2 does not [7]. The fine details of the Bcl-2 family interactions will continue to be uncovered, but the principal of partial non-redundancy is clear, and highlights the complexity of apoptosis regulation in multicellular organisms.

Much less is known about the non-Bcl-2-homologous N-terminal portion of the protein, approximately encompassing residues 1–170, which is predicted to have low-structural complexity, but is rich in confirmed and putative regulatory motifs. Indeed, since its discovery, the molecular characteristics of the N-terminus of Mcl-1 have been under scrutiny, with Kozopas et al. identifying enrichment of this region with proline (P), glutamic acid (E), serine (S) and threonine (T) residues, which earned this half of the protein its name as the 'proline/glutamic acid/serine/threonine (PEST) region' [3]. PEST enrichment and arginine pairs (of which Mcl-1 has four) are common features of labile proteins, and Kozopas et al. later confirmed this prediction in a study using pulse-chase techniques [10]. The constitutive half-life of Mcl-1 is the subject of some uncertainty since the protein's turnover may be shortened or lengthened significantly depending on the cellular conditions, and varies between cell types. Factors which influence the turnover of Mcl-1 are numerous and varied, and an exhaustive list is beyond the scope of this review. As expected however, those agents and conditions which delay Mcl-1 degradation tend to enhance cellular viability – such as trophic factors – and conversely, agents and conditions which accelerate Mcl-1 degradation act to decrease cellular viability – such as trophic factor withdrawal, or DNA damage.

## 3. Mcl-1 – expression and physiological function

Despite an initial appearance of functional redundancy between individual Bcl-2 family members, differences in both tissue distribution and function have been identified, and are reviewed elsewhere [11]. Mcl-1, like its Bcl-2 relatives, is widely expressed but has its own particular tissue distribution [10,12] along with its own specific physiological roles. Numerous, diverse cell-types have been shown to be reliant on Mcl-1 for their survival and development. For example, Mcl-1 deletion is peri-implantation lethal in mouse embryogenesis [13], whereas Bcl-2 deletion is not [14]. Mcl-1 has also been shown to be required for the development and maintenance of B and T-lymphocytes [15], and is also required for neural development [16]. It has also been shown to play a critical role in the regulation of macrophage and neutrophil apoptosis [17,18], and is essential for the survival of haematopoietic stem cells [19] and synovial fibroblasts [20]. Along with its roles in differentiation and apoptosis, Mcl-1 (like its Bcl-2 counterparts [21])

is also known to delay cell-cycle progression through interactions with cyclin dependent kinase 1 (CDK-1) [22], proliferating cell nuclear antigen (PCNA) [23] and checkpoint 1 protein (CHK-1) [24].

Initial studies of Mcl-1 identified that its expression was growth-factor dependent in many situations, and that it was capable of protecting cells from growth factor withdrawal-induced apoptosis [25]. A growing list of trophic factors have been shown to induce transcriptional upregulation of Mcl-1, including cytokines such as interleukin (IL)-3 [26], IL-5 [27], IL-6 [28], and granulocyte–macrophage colony-stimulating factor (GM-CSF) [29], as well as growth factors such as epidermal growth factor (EGF) [30] and vascular endothelial growth factor (VEGF) [31]. Intracellular regulation of Mcl-1 transcription is mediated by a number of externally activated and constitutively activated transcription factors, notably the signal transducers and activators of transcription (STAT) family. The promoter region of Mcl-1, characterised by Akgul et al. [32], contains an array of putative and confirmed transcription factor binding sites, including consensus STAT response elements, cAMP response elements (CRE), and nuclear factor kappa B binding sites. Both STAT3, in response to IL-6 [33], VEGF [34] and IL-3 [26], and STAT5, in response to Bcr–Abl signalling in chronic myeloid leukemia, have been shown to upregulate Mcl-1 transcription. Indeed, it has been suggested that STAT3 activation by phosphorylation is absolutely required for Mcl-1 mediated macrophage survival [35]. Other confirmed transcription factors known to upregulate Mcl-1 expression include cAMP response element binding protein [26], PU.1 [36] SP1 [37], and under hypoxic conditions, hypoxia-inducible factor 1α [38]. Conversely, Mcl-1 may be down-regulated transcriptionally under a number of conditions, particularly under growth factor withdrawal [29], and on the induction of apoptosis induced by a variety of treatments, including staurosporine [39] and UV exposure [40]. In most cases, down-regulation is mediated by inactivation of the transcription factors stimulating Mcl-1 transcription, but the Mcl-1 promoter is directly repressed by the binding of the E2F-1 transcription factor (Fig. 3) [41].

In addition to transcriptional regulation, Mcl-1 is also subject to post-transcriptional and translational control. The mRNA of Mcl-1 can be alternatively spliced to remove exon 2, which produces a shortened form of Mcl-1, namely Mcl-$1_S$, which lacks BH domains 1, 2 and the transmembrane domain. A second, shorter, splice variant, named Mcl-$1_{ES}$ has also been identified which lacks a portion of exon 1, removing 53 amino acids from the PEST region, but retains all three BH domains and the C-terminal transmembrane domain [42]. These shortened forms are unable to interact and sequester the pro-apoptotic Bcl-2 family members, and in fact induce apoptosis by binding and inhibiting full length Mcl-1 [43]. Upregulation of Mcl-$1_S$ has been identified in macrophages during infection, and this appears to be an important mechanism for the successful resolution of inflammation [44]. The rate of Mcl-1 translation is also tightly regulated, and Mcl-1 mRNA, like the protein, has been shown to have a very short half-life. Mcl-1 mRNA translation is inhibited both by binding of the micro RNA, micro inhibiting RNA 29b [45] and the RNA binding protein CUGBP2 [46] at the 3′-UTR. Overexpression of either of these inhibitors of Mcl-1 translation dampens proliferation and induces apoptosis.

## 4. Contribution to pathophysiology

Well-regulated apoptosis is an essential process in development, tissue homeostasis and the regulation of inflammation, conversely dysregulation of apoptosis has wide-ranging influences in a number of pathophysiologies [47]. Excessive apoptosis is implicated in a number of neurodegenerative diseases such as Alzheimer's [48] and multiple sclerosis [49], while evasion of apoptosis is a cardinal step in oncogenesis [50] and many inflammatory conditions [47]. Mcl-1 overexpression has been reported in several haematological cancers [51–53] and solid tumours [54–56], including chronic myeloid leukemia and hepatocellular carcinoma. It has also been implicated in the chemoresistance of certain malignancies [56–58], notably against the first of a new class of Bcl-2 family targeting compounds, named ABT-737 [59]. Forced overexpression of Mcl-1 in transgenic mice led to a significantly increased incidence of B-cell lymphoma [60], while forced Mcl-1 down-regulation is able to induce apoptosis in a number of cancer cell types [61]. Thus Mcl-1 is an attractive and potential therapeutic target in a number of malignancies, and is the focus of a number of studies (reviewed in [62]).

## 5. Molecular characteristics

As described above, Mcl-1 has a unique tissue expression and specialised roles in physiology and pathology that set it apart from other Bcl-2 family members. It appears that many of these unique properties are determined by the large N-terminal domain of Mcl-1, which contains many motifs that affect its rate of turnover, localisation and phosphorylation status. These post-translational modifications of Mcl-1 provide the protein with the ability to rapidly and reversibly respond to environmental signals, and switch cell fate from survival to apoptosis.

### 5.1. Degradation

The degradation of Mcl-1 was first attributed to the proteasome by Nijhawan et al. in HeLa cells exposed to UV irradiation [40], and later confirmed by our own group and others [63]. The lysine residues targetted for ubiquitination (Fig. 2) were then identified and characterised by Zhong et al., who also discovered a novel E3 ubiquitin-ligase responsible for the constitutive polyubiquitination of Mcl-1, which they named Mcl-1 Ubiquitin Ligase E3 (MULE) [64]. However Mcl-1 degradation during apoptosis may be further accelerated, both by activation of other ubiquitin ligases and cleavage by other proteases. A second E3-ligase which targets Mcl-1 has been identified by Ding et al., namely beta transducin-containing protein (β-TrCP), which polyubiquitinates and targets Mcl-1 for degradation following GSK-3 phosphorylation of Mcl-1 (discussed in greater detail later) [65]. Conversely, Mcl-1 has also recently been shown to be the target of a deubiquitinase, named USP9X, which stabilizes Mcl-1 by reversing its polyubiquitination [66].

Secondly, it was known that Mcl-1 was additionally subject to non-proteasomal degradation, particularly during the progression of apoptosis, and two groups simultaneously characterised the caspase-dependent cleavage of Mcl-1 at two distinct sites within the N-terminus (Fig. 2) [67,68]. Han et al. then identified Granzyme B (GrB) cleavage of Mcl-1 at the same two sites (and one other) during GrB-mediated apoptosis [69]. Cleavage of Mcl-1 at these sites unambiguously impairs the protein's ability to protect against apoptosis by inhibiting dimerisation with its cognate pro-apoptotic partners. However, some studies have reported that cleavage at these sites additionally converts Mcl-1 to a pro-apoptotic protein [67,70], although this has been disputed by others [68,71]. Nevertheless, it is clear that cleavage of Mcl-1 is an important process by which the contribution of Mcl-1 to cell viability may be rapidly regulated.

### 5.2. Localisation

Under resting conditions, Mcl-1 is localised to various cellular membranes, including the mitochondria [9,10] and nuclear envelope [72], but it also has prominent cytosolic localisation, presumably as a heterodimer with Bax, and in some studies has been

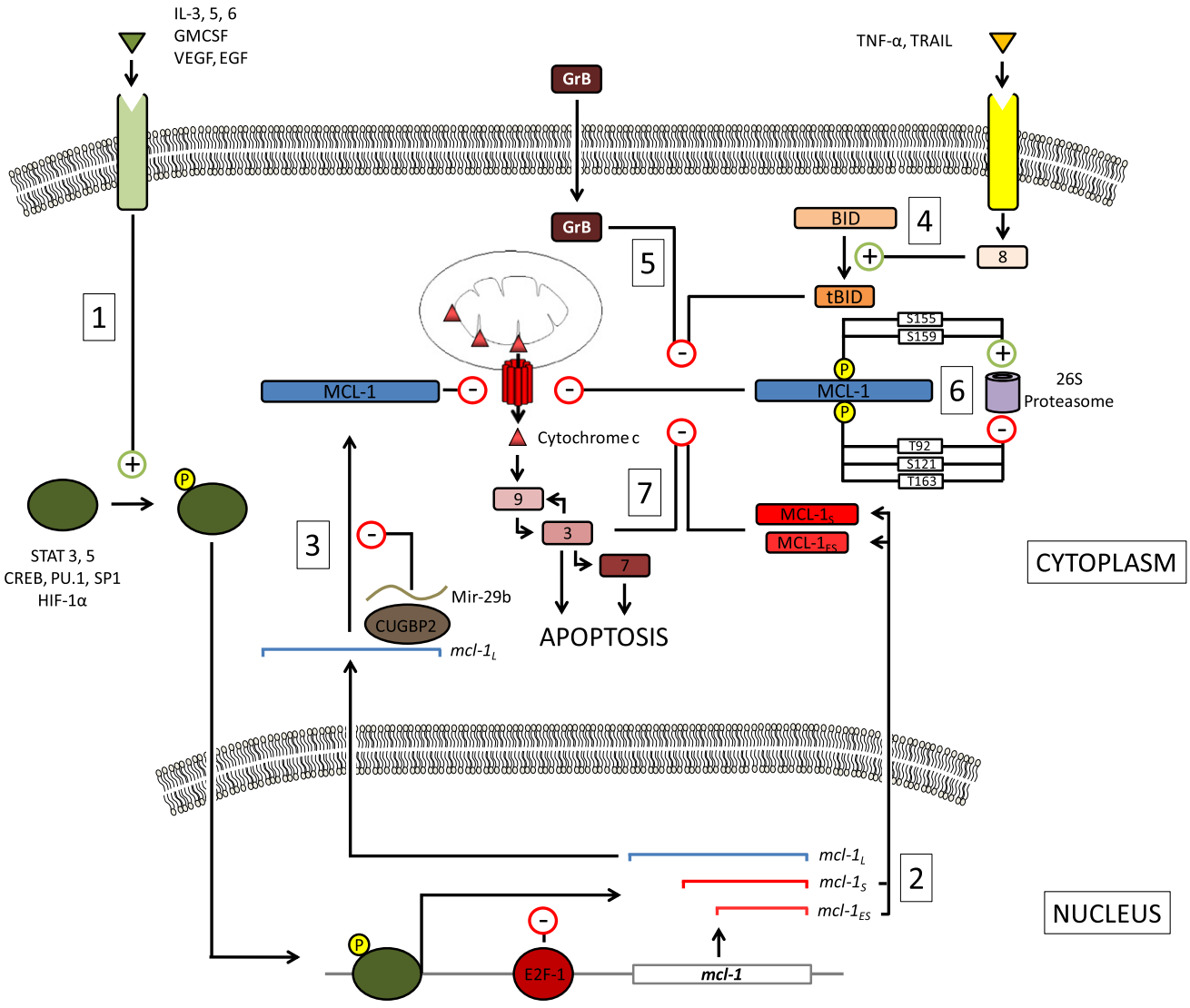


**Fig. 3.** Overview of the molecular regulation of Mcl-1 [1]. Trophic signalling by cytokines and growth factors activates a number of transcription factors which stimulate Mcl-1 transcription, which can be repressed by the E2F-1 transcription factor [2]. Alternative splicing leads to the production of three mRNA species: full length (*mcl-1*$_L$), short (*mcl-1*$_S$) and extra short (*mcl-1*$_{ES}$). The latter two are unable to bind and sequester pro-apoptotic Bcl-2 family members, and instead bind and inactivate full length Mcl-1 [3]. Translation of full length Mcl-1 mRNA is negatively regulated by both the microRNA Mir-29b and the RNA binding protein CUGBP2 [4]. Pro-apoptotic extracellular signalling leads to the activation of caspase-8 which cleaves and activates Bid which binds and inhibits Mcl-1 [5]. Additionally, paracellularly secreted Granzyme B cleaves and inactivates Mcl-1 [6]. Mcl-1 is subject to constitutive degradation by the proteasome, and this may be accelerated or inhibited by phosphorylation at various residues, described in detail in later sections [7]. On the induction of apoptosis, the cascade of caspase activation is amplified by caspase-3 mediated cleavage and inactivation of Mcl-1.

localised to the nucleus [22,23,72]. Efficient targeting of Mcl-1 to the mitochondria has been shown to be dependent on both the first 79 residues of Mcl-1 [73], an internal EELD sequence (at residue 124–127), interaction with the Tom70 receptors [74] and the C-terminal transmembrane domain [9]. Indeed, the ability of Mcl-1 to localise to the mitochondria is necessary for it to counter the activity of the pro-apoptotic proteins Bak and Bax [73]. Nuclear localisation of Mcl-1 has been attributed to interactions with CDK-1 and PCNA, though the molecular regulation of these interactions and transport of Mcl-1 across the nuclear membrane is, as yet, unknown.

### 5.3. Phosphorylation

In the preceding part of this review, we have detailed the majority of mechanisms by which the function of Mcl-1 is regulated, and these mechanisms are themselves regulated by the phosphorylation of Mcl-1. Phosphorylation is an almost universal, rapid and reversible modification of proteins that has wide-ranging influences on protein behaviour, including protein localisation, structure, protein–protein interactions, stability, the regulation of other protein modifications and protein function. Thus, phosphorylation serves to integrate a complex array of biological processes that may be complimentary, or in opposition to each other. The PEST region of Mcl-1 is rich in putative phosphorylation sites, and a number of these have been experimentally confirmed, and their influence on Mcl-1 behaviour have, in part, been elucidated. In the final part of this review, we detail those phosphoresidues of Mcl-1 that have been confirmed experimentally as well as describing their influence on Mcl-1 function (Figs. 4 and 5).

#### 5.3.1. Serine 64

This phosphoresidue was identified and confirmed in vivo by Kobayashi et al. [75] using mass spectrometric analysis of a threonine 163 to alanine (T163A) mutant of Mcl-1. The residue was found to be phosphorylated by CDKs 1 and 2, and by C-Jun N-ter-

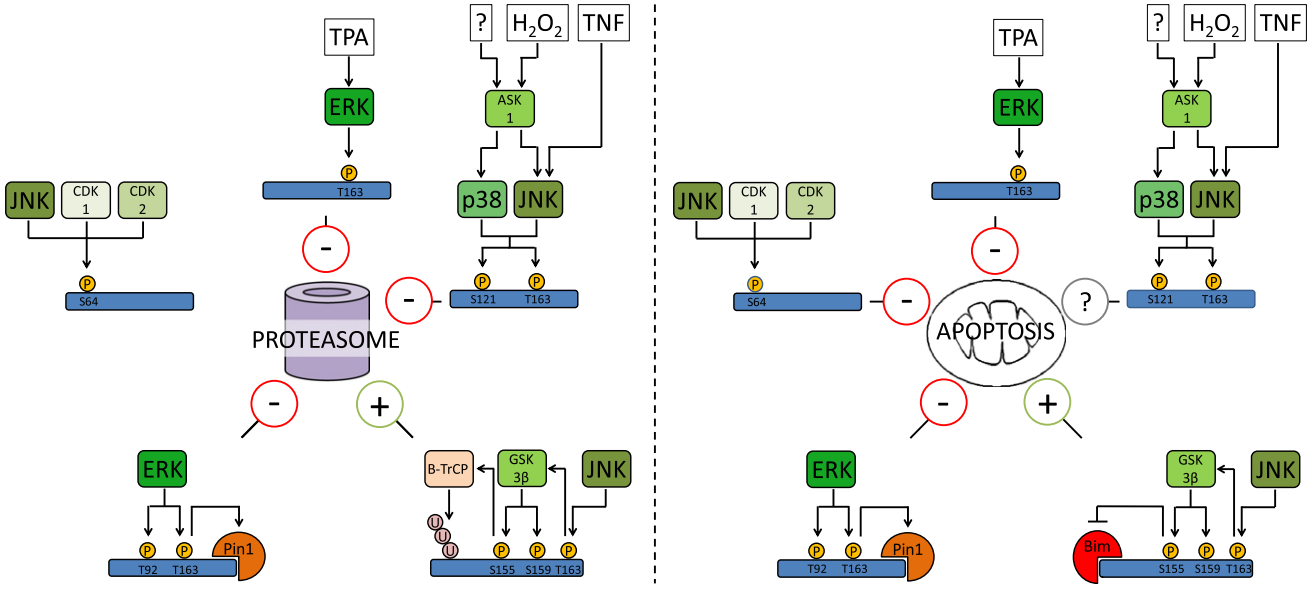


**Fig. 4.** Signalling pathways and Mcl-1 phosphorylation. A schematic diagram representing the confirmed signalling pathways and kinases that leads to the phosphorylation of Mcl-1. The influence that phosphorylation of each residue has on the stability of Mcl-1 (left panel) and the progression of apoptosis (right panel) is also shown, whether positively (+) or negatively (−) or if this is currently unknown (?).

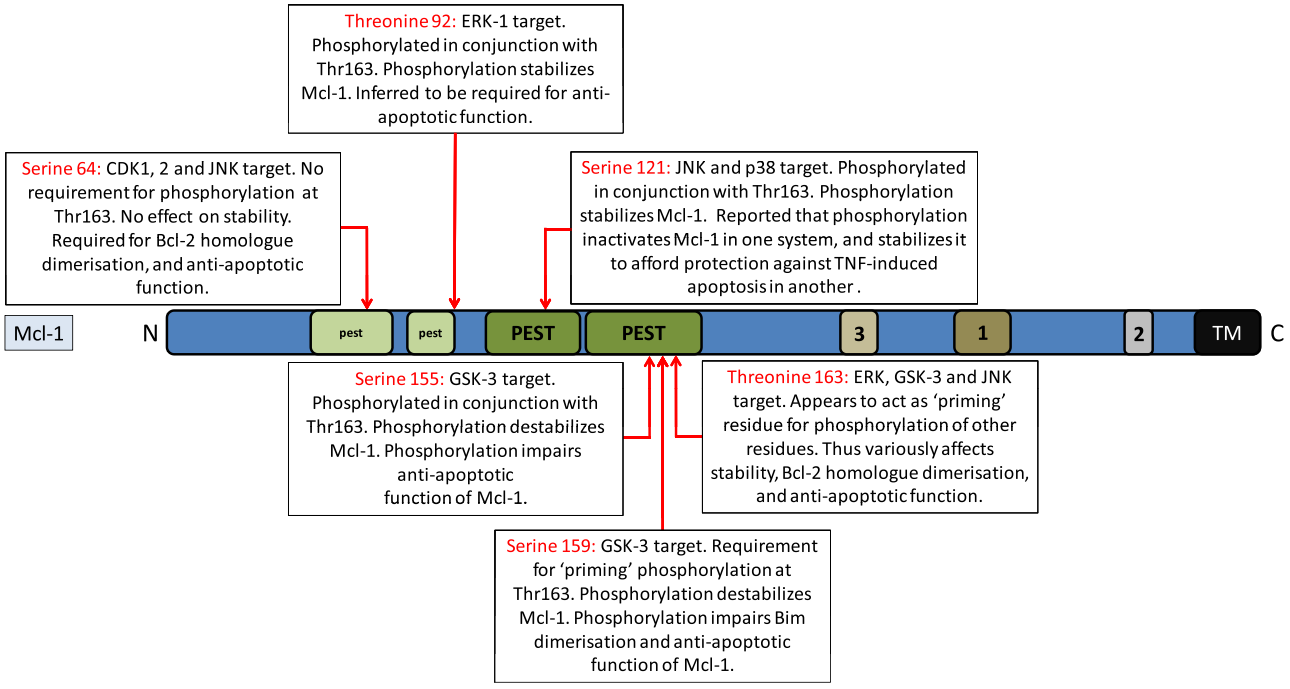


**Fig. 5.** The phosphoresidues of Mcl-1. A schematic diagram of the Mcl-1 protein, to scale, summarising the experimentally confirmed phosphoresidues of Mcl-1, and the influence that each phosphorylation has on protein stability, dimerization and function.

minal kinase (JNK), and also to be more strongly phosphorylated in the $G_2$/M phase of the cell-cycle than at the $G_1$/S boundary, suggesting that there is a cell cycle-dependency for $Ser^{64}$ phosphorylation. No significant differences in protein stability were found between wild-type Mcl-1 and phosphonegative (S64A) or phosphomimic (S64E) forms, suggesting that this residue does not influence the rate of Mcl-1 turnover. It was demonstrated that the S64A mutant of Mcl-1 had lower binding affinity for various pro-apoptotic members of the Bcl-2 family compared to the S64E phosphomimic mutant, and that this translated to a decreased ability to counter TRAIL-induced apoptosis.

*5.3.2. Threonine 92*

This phosphoresidue was identified by Ding et al. [76] using an in vitro extracellular regulated protein kinase-1 (ERK-1) kinase assay, with purified GST-tagged Mcl-1 as the substrate, followed by mass spectrometric analysis, and both $Thr^{92}$ and $Thr^{163}$ were found to be simultaneously phosphorylated. It was found that a double T92/163A phosphonegative mutant of Mcl-1 was significantly less stable than the wild-type, and a double T92/163D phosphomimic mutant was significantly more stable. The authors then discovered that siRNA knockdown of the peptidyl-prolyl cis/trans isomerase, peptidyl-prolyl cis–trans isomerase NIMA interacting protein 1

(Pin-1), abrogated any increase in Mcl-1 stability induced by Erk-1 activation, which suggests that Erk-1 phosphorylation of these residues stabilizes Mcl-1 by promoting association with Pin-1. No direct assessment of the anti-apoptotic function of $Thr^{92}$ phosphomutants was made, only inferentially using Pin1 siRNA knock-down, which led to a small increase in constitutive apoptosis, but also strongly sensitized the cells to Taxol and 5-fluorouracil induced apoptosis.

*5.3.3. Serine 121*

This phosphoresidue was identified by Inoshita et al. [77] in a study investigating the influence of oxidative stress on apoptosis, and also by Kodama et al. [78]. Inoshita et al. identified that phosphonegative mutation at $Ser^{121}$ and $Thr^{163}$ abrogated the phosphorylation-dependent electrophoretic mobility shift of Mcl-1, induced by $H_2O_2$ treatment. Similarly, Kodama et al. discovered that the same phosphonegative mutation abrogated Ser/Thr phosphorylation of Mcl-1 in primary mouse hepatocytes. JNK was identified in both studies as the kinase responsible for phosphorylation at this residue. While Inoshita et al. did not detect any changes in Mcl-1 stability when $Ser^{121}$ (in conjunction with $Thr^{163}$) was mutated, Kodama et al. found that in their model, a double S121A/T163A mutant of Mcl-1 was significantly less stable than the wild-type. Kodama et al. concluded that JNK phosphorylation of Mcl-1 at $Ser^{121}$, in conjunction with $Thr^{163}$, stabilizes Mcl-1 and affords protection against TNFα-induced apoptosis. However, Inoshita et al. demonstrated in their study that a double phosphonegative S121A/T163A mutant of Mcl-1 was significantly more potent at inhibiting apoptosis induced by $H_2O_2$ treatment.

*5.3.4. Serine 155*

This phosphoresidue was identified by Ding et al. [65] in a study investigating the role of Glycogen Synthase Kinase (GSK) 3 in tumour apoptosis. Initially, they identified that GSK-3 inhibition, through either mutation or chemical means, led to an increase in Mcl-1 expression whereas expression of constitutively active GSK-3 decreased Mcl-1 expression. Using sequence analysis, the authors then identified two potential consensus GSK-3β phosphorylation motifs ($^{150}$SGGNN$^{154}$T and $^{155}$STDG$^{159}$SLPS$^{163}$T), but only phosphonegative mutation of the second, named Mcl-1-3A, abolished GSK-3β mediated phosphorylation in an in vitro kinase assay. Phosphorylation at $Ser^{155}$ was then confirmed using phospho-specific antibodies (to $Ser^{155}$, $Ser^{159}$ and $Thr^{163}$), and it was found that GSK-3β phosphorylated all three residues in the sequence, while under the same conditions ERK-1 phosphorylated only $Thr^{163}$. The authors also found that phosphonegative mutation of $Ser^{155}$ (in conjunction with $Ser^{159}$ and $Thr^{163}$) in the Mcl-1-3A mutant, stabilised Mcl-1 compared to the wild-type. Finally, the authors showed that the Mcl-1-3A mutant of Mcl-1 was significantly more potent at inhibiting the apoptosis of MCF-7 breast cancer cells induced by 5-fluorouracil, cisplatin and taxol, as compared to the wild-type.

*5.3.5. Serine 159*

The phosphorylation of serine 159 has been identified by three groups, Ding et al. [65] in the work described above, by Maurer et al. [79], and by Morel et al. [80]. All three studies identified GSK-3 as the kinase targetting this residue, and discovered that phosphorylation of this residue led to significant decrease in Mcl-1 protein expression. Morel et al. also identified that an initial 'priming' phosphorylation of $Thr^{163}$ (by JNK) was required for $Ser^{159}$ phosphorylation – a characteristic of other GSK-3 targets [81] – using sequential in vitro JNK and GSK-3 kinase assays. Both Morel et al. [80] and Maurer et al. [79] found that phosphonegative mutation of $Ser^{159}$ stabilized Mcl-1, following UV-irradiation or cycloheximide (CHX) treatment respectively. Ding et al. showed that ubiquitination of Mcl-1 following $Ser^{159}$ phosphorylation was mediated by the E3 ubiquitin ligase β-TrCP, rather than MULE. Finally, Maurer et al. and Morel et al. both demonstrated that phosphonegative mutation of Mcl-1 at $Ser^{159}$ affords better protection against IL-3 withdrawal-induced apoptosis and UV irradiation-induced apoptosis respectively. Maurer et al. additionally showed that in the absence of IL-3 (in order to initiate apoptosis and a Bim:Mcl-1 interaction), Bim was only co-immunoprecipitated with Mcl-1 following treatment with the small-molecule inhibitor of GSK-3, CHIR-611. In conclusion, GSK-3 phosphorylation at $Ser^{159}$ both destabilizes Mcl-1 and inhibits the interaction of Mcl-1 with the pro-apoptotic protein, Bim, thus impairing its anti-apoptotic function.

*5.3.6. Threonine 163*

This residue was the first experimentally confirmed phosphoresidue of Mcl-1, in a study by Inoshita et al. [77], and is also the most highly investigated, having been confirmed twice by Ding et al. [65,76] and also by Domina et al. [82]. Inoshita et al. first identified $Thr^{163}$ as a strong candidate phosphoresidue from sequence analysis, and this was later repeated by Domina et al., who noted that the residue lies within a complete consensus MAP kinase phosphorylation sequence, and is highly conserved across species. Domina et al. identified this residue as a target for ERK-1 in response to TPA stimulation of CHO and BL41-3 cells, using $^{32}$P incorporation assays, as well as utilising inhibitors of ERK. This observation was later confirmed in a study by Ding et al. using an in vitro ERK-1 kinase assay [76]. The influence of single $Thr^{163}$ phosphorylation on Mcl-1 stability was inferred by Domina et al. from two experiments. Firstly, TPA treatment significantly increased the stability of endogenous Mcl-1 in BL41-3 cells following treatment with CHX at 25 μg/mL (88 μM). Secondly, since mutation of threonine 163 to alanine abolished TPA-induced phosphorylation of Mcl-1, it was concluded that TPA-induced phosphorylation of $Thr^{163}$ stabilizes Mcl-1. It must be noted that this is seemingly contradicted by the results published by Inoshita et al. who concluded that phosphorylation of $Thr^{163}$ by JNK destabilized Mcl-1, though Domina et al. address this and point out that Inoshita et al. used a double S121A/T163A mutant in their stability experiments, which confounds any conclusions that may be drawn about $Thr^{163}$ phosphorylation alone. While Domina et al. made no investigation as to the influence of $Thr^{163}$ on the anti-apoptotic function of Mcl-1, other reports have done so, but only in conjunction with phosphorylation of other residues. Ding et al. inferred in one report that co-phosphorylation of $Thr^{163}$ with $Thr^{92}$ by ERK-1 improves Mcl-1 function [76], and showed in a second that co-phosphorylation of $Thr^{163}$ with $Ser^{155}$ and $Ser^{159}$ by GSK-3 impairs its anti-apoptotic function [65]. Inoshita et al. similarly show that co-phosphorylation of $Thr^{163}$ with $Ser^{121}$ 'inactivates' Mcl-1, and impairs its anti-apoptotic function in response to $H_2O_2$ treatment [77].

## 6. Discussion

Evidence is accumulating that Mcl-1 is a protein whose contribution to cell fate is rapidly and acutely regulated, through changes in transcription, localisation, stability and its ability to form dimers with Bcl-2 homologues and other proteins. Much of this flexibility in expression and anti-apoptotic function appears to be due to its unique (among the Bcl-2 family) and extensive N-terminal portion which contains a large number of modifiable residues, and therefore appears to act as a regulatory region. With such complex regulation of protein function at multiple levels, it is highly likely that these regulatory processes are context-based. For example, the regulatory mechanisms that operate in different cells and tissues

under varying conditions of stress or other environmental factors could lead to differential activation of kinase/phosphatase cascades, or alternative proteolytic pathways (e.g. calpains, proteasome, or caspases). It is also important to bear in mind that some regulatory mechanisms may be cell-cycle dependent. Thus, while the range of methodological approaches used to study Mcl-1 function may sometimes yield conflicting data, genuine variance in the mechanisms of Mcl-1 regulation may exist in cells and tissues under different pathophysiological conditions.

The published data detailed in this review highlights some important problems when attempting to piece together an accurate picture of the molecular regulation of Mcl-1 by phosphorylation. The gold-standard technique for confirmation of phosphorylation at a particular residue is mass-spectrometry. However, since sample preparation can be complicated, particularly for a low abundance protein such as Mcl-1, most groups resort to in vitro kinase assays of immunoprecipitated exogenously-expressed tagged forms of the protein. The second choice for confirmation of phosphorylation is with the use of phospho-specific antibodies, which have the added benefit of being usable for the detection of changes in relative levels of phosphorylation by immunoblotting. Finally, autoradiographic detection of $^{32}P$ incorporation into mutant forms of Mcl-1 is useful and has been widely used, but suffers from being a less direct and specific approach than either mass-spectrometry or phospho-specific antibodies.

More difficulties arise when comparing the data regarding the influence of phosphoresidues on the stability of Mcl-1. None of the stability experiments detailed here share the same experimental conditions, with wide variations in the cell-types, molecular characteristics of the Mcl-1 being assessed, expression systems, chemical treatments and data analyses used. While transient transfection of mutant forms of Mcl-1 is widely used and bypasses the technical challenges of creating stable transfectants, there is often no control for transfection efficiency, and sometimes no means of distinguishing between exogenously and endogenously expressed Mcl-1. The use of the translation inhibitor CHX is common, and yet the quoted working concentrations vary from 5 μg/mL to 5 mg/mL. This variation confounds comparison of the data from different studies, since there is a concentration-dependent effect of CHX treatment on the expression of labile proteins, and issues of cytotoxicity at higher concentrations (>30 μg/mL). In addition, most studies use doubly or triply mutated forms of Mcl-1, making it hard to elucidate the contribution of individual residues.

Nevertheless, some principals are clear from the literature. Firstly that phosphorylation at $Thr^{163}$ contributes to the stabilization and destabilization of Mcl-1 depending on the activity of other kinases. The literature currently highlights $Thr^{163}$ as one of, if not the key phosphoresidue in regulating the fate of Mcl-1, since it appears to be a necessary 'priming' site for phosphorylation of, or at least to be phosphorylated in conjunction with all other experimentally confirmed phosphoresidues, with the exception of $Ser^{64}$. Secondly, phosphorylation by GSK-3 at multiple residues is a key modification for the rapid degradation of Mcl-1. Thirdly that constitutive Erk-1 phosphorylation at $Thr^{163}$ stabilizes Mcl-1 under resting conditions. The influence of JNK phosphorylation is less clear, since it appears to be required at $Thr^{163}$ for the destabilizing phosphorylation of $Ser^{159}$ and $Ser^{155}$ by GSK-3, while also itself phosphorylating and stabilizing Mcl-1 at $Ser^{121}$. Next, two studies have identified that phosphorylation of certain residues affects the degree of Mcl-1 ubiquitination [65,79], and thus it would appear that modification by phosphorylation leads to exposure or masking of lysine residues for binding and modification by ubiquitin ligases. And finally, while the N-terminal portion of the protein is predicted to have low-structural complexity, it evidently must play a role in regulating the binding of Mcl-1 to other proteins. Abrogating the phosphorylation of $Ser^{159}$ diminished the ability of Mcl-1 to associate with the BH3-only protein Bim, while the same treatment of $Thr^{92}$ inhibited the association of Mcl-1 with Pin-1. Currently however, the only published solution structures of Mcl-1 are of N-terminally truncated forms of the protein [6,8], and so the exact steric behaviour of this portion of Mcl-1 is still unknown, though this will be of importance in understanding how its modification can influence Mcl-1 dimerization. Phosphorylation must also play a role in regulating the influence of Mcl-1 on the cell cycle – an exciting but underinvestigated feature of the Bcl-2 protein family. Phosphorylation of $Ser^{64}$ was found to have a cell-cycle dependency, and since Mcl-1 has been reported to bind to CDK1, PCNA and CHK-1 in vivo [22], it is tempting to hypothesise that phosphorylation of this residue may regulate the timing of these dimerisations.

These data highlight the multifactorial nature of the regulation of cell fate, and how proteins such as Mcl-1 intersect opposing and complimentary signalling cascades, and can effect changes in the balance between these signals.

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