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REVIEW ARTICLE

Saga of Mcl-1: regulation from transcription to degradation

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Abstract

The members of the Bcl-2 family are the central regulators of various cell death modalities. Some of these proteins contribute to apoptosis, while others counteract this type of programmed cell death, thus balancing cell demise and survival. A disruption of this balance leads to the development of various diseases, including cancer. Therefore, understanding the mechanisms that underlie the regulation of proteins of the Bcl-2 family is of great importance for biomedical research. Among the members of the Bcl-2 family, antiapoptotic protein Mcl-1 is characterized by a short half-life, which renders this protein highly sensitive to changes in its synthesis or degradation. Hence, the regulation of Mcl-1 is of particular scientific interest, and the study of Mcl-1 modulators could aid in the understanding of the mechanisms of disease development and the ways of their treatment. Here, we summarize the present knowledge regarding the regulation of Mcl-1, from transcription to degradation, focusing on aspects that have not yet been described in detail.

Facts

- *MCL1* is the first antiapoptotic gene for which homology to *BCL2* was reported.
- Mcl-1 plays a significant role in the inhibition of apoptosis and demonstrates oncogenic properties.
- Mcl-1 is a short-lived protein, which renders it highly sensitive to changes in its synthesis or degradation.
- Targeting regulatory circuits that control Mcl-1 level could provide a possible therapeutic intervention for cancer treatment.

Open questions

- What mechanisms are most common for Mcl-1 dysregulation during tumor development?
- Is the understanding of the mechanisms of Mcl-1 regulation relevant to comprehend the pathophysiology of non-cancer disorders?
- Can regulation of Mcl-1 posttranslational modifications be exploited as a therapeutic strategy to combat cancer?
- How the development of next-generation sequencing technologies should help in the understanding of mechanisms relevant to the dysregulation of Mcl-1 in patients?

Introduction

In 1993, four genes with a high level of homology were cloned: *MCL1* [1], *BCL2A1* [2] (encoding for A1 or Bfl-1), *BCL2L1* [3] (encoding for Bcl-xL and Bcl-xS), and *BAX* [4]. All shared sequence similarity with *BCL2*, an oncogene that promotes haemopoietic cell survival [5]. Initial experiments demonstrated the opposite roles of the two products of the *BCL2L1* gene in the regulation of cell death: Bcl-xL served as an inhibitor of apoptotic cell death, whereas a smaller Bcl-xS countered antiapoptotic activity [3]. Next, Bax was discovered as a Bcl-2 binding partner, and its overexpression was shown to promote apoptosis [4]. These data pointed to the fact that there was a family of

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Bcl-2-like proteins that positively or negatively regulated apoptotic cell death.

MCL1 is the first gene for which homology to *BCL2* was reported. It was isolated from the ML-1 human myeloid leukemia cell line and accordingly named *MCL1* (myeloid cell leukemia-1). In initial work, Kozopas et al. proposed that the Mcl-1 protein may regulate cell survival [1]. This assumption was later confirmed in numerous studies.

Today, there is no doubt regarding the role of Mcl-1 in the inhibition of apoptosis, as well as the resulting oncogenic properties. A thorough study of the Bcl-2 family was translated into the development of small-molecule inhibitors of its antiapoptotic members, including Mcl-1, and these compounds are now being evaluated in clinical trials [6]. The regulation of apoptosis by members of the Bcl-2 family, as well as the recent advances in targeting various Bcl-2 family proteins, are reviewed elsewhere [6–9].

Meanwhile, the regulation of Mcl-1 is also actively studied, and numerous reviews cover this aspect of Mcl-1 biology [10–14]. As more and more data are accumulated on this issue, systematic analysis is needed to update current understanding of Mcl-1 regulation. Here, we attempted to summarize the data concerning the regulation of Mcl-1 at the transcriptional, translational, and posttranslational levels, giving examples of how these mechanisms could be involved in the modulation of cancer cell survival and how to utilize them for precision medicine.

Mcl-1: functions, structure, and biological significance

Proapoptotic and antiapoptotic members of the Bcl-2 family act through mutual inhibition, controlling permeabilization of the outer mitochondrial membrane, and the escape of various proapoptotic factors into the cytosol [15]. Once such proapoptotic factors invade the cytoplasm, the apoptotic program will be initiated [16].

All antiapoptotic members of the Bcl-2 family have a hydrophobic groove in their structure. This cleft is termed "BH3-binding groove" due to the ability of binding exposed BH3-domains, which represent the main structural features of proapoptotic Bcl-2 family members. In brief, the interactions between BH3-binding grooves and BH3-domains underlie the mutual inhibition of the two subsets of Bcl-2 family members [15]. Based on this knowledge, small molecule compounds that imitate BH3-domains were developed to target antiapoptotic Bcl-2 family proteins. According to the mechanism of action, these agents have been named "BH3-mimetics" [17]. Today, BH3-mimetics are under active clinical trials, and a specific inhibitor of Bcl-2, venetoclax, has been approved by the Food and Drug Administration and the European Medicines Agency for clinical use. As for Mcl-1, four BH3-mimetics to this protein, S64315, AMG-176, AMG-397, and AZD-5991, have entered clinical evaluation in patients with hematological malignancies, either alone or in combination with venetoclax (NCT02979366, NCT 02992483, NCT03672695, NCT02675452, NCT03797261, NCT03465540, and NCT03218683). Nowadays, BH3-mimetics represent the most promising tool for the inhibition of Bcl-2 family antiapoptotic proteins, and the approval of Mcl-1-specific inhibitors is eagerly awaited.

Although Mcl-1 possesses a structure similar to that of other antiapoptotic Bcl-2 family proteins, its size is much larger since Mcl-1 contains an extended N-terminal regulatory domain. One of the most important features of this region is the abundance of proline [P], glutamic acid [E], serine [S], and threonine [T] residues [11]. Commonly, PEST sequences serve as signals for rapid protein degradation [18]. In line with this concept, Mcl-1 is characterized by a high turnover rate and a short half-life (usually <1 h) [19]. This feature makes Mcl-1 extremely sensitive to perturbations in its synthesis and/or degradation.

It should be mentioned that for Mcl-1 additional splicing variants with proapoptotic activity have been described [20, 21]. Here, however, we focus on only the most abundant antiapoptotic isoform of Mcl-1.

Like other antiapoptotic proteins of the Bcl-2 family, Mcl-1 plays an important role in the survival of various types of normal cells, including hematocytes [22], neurons [23], cardiomyocytes [24], and others. Like other antiapoptotic proteins of the Bcl-2 family, Mcl-1 is also often abused by cancer cells to evade apoptosis. The expression of Mcl-1 in tumor cells was found in a high proportion of patients with acute myeloid leukemia (AML) [25], hepatocellular carcinoma [26], non-small cell lung cancer [27], breast cancer [28], and other malignancies [29]. Mcl-1 is associated with resistance of tumor cells to various anticancer agents, [17, 30–32] while Mcl-1 dependence serves as a predictor of worse response to BH3-mimetic venetoclax in AML patients [33]. Moreover, Mcl-1 upregulation was found at the time of relapse in chemotherapy-treated leukemia patients, thus highlighting the role of Mcl-1 in tumor biology [34]. Hence, Mcl-1 is both an essential regulator of survival in normal cells and a promising target for cancer therapy.

Regulation of Mcl-1

Numerous modulators finely regulate Mcl-1 level by providing rapid protein-level changes in response to internal and external signals. *MCL1* gene expression is controlled at transcriptional, posttranscriptional, and translational levels. Moreover, various posttranslational modifications

determine the stability and functional activity of Mcl-1. Dysregulation of Mcl-1 may result in various pathological processes, including carcinogenesis. Thereby, understanding the molecular mechanisms that underlie the regulation of Mcl-1 is of great therapeutic significance.

Transcriptional control of Mcl-1

Different cytokines, growth factors, and other extracellular and intracellular stimuli, including IL-6 and IFN-α in multiple myeloma cells [35], hepatocyte growth factor in primary human hepatocytes [36], epidermal growth factor (EGF) in esophageal carcinoma [37] and breast cancer cells [38], can control *MCL1* transcription. *MCL1* is transcriptionally modulated in response to different cellular stresses, such as microtubule disruption [39], ER stress [40], and hypoxia [41]. Importantly, the dysregulation of *MCL1* transcription could be utilized by cancer cells to develop apoptosis resistance. In general, transcriptional regulation represents an important node in the complex regulation of Mcl-1. Table 1 summarizes the data regarding transcriptional factors, which were found to bind to the promoter region of human *MCL1*.

It is noteworthy to mention that several studies focused on the binding of transcription factors to the promoter of the mouse *Mcl1*, but not of its human counterpart. Although both promoters have a certain degree of homology, the binding of transcription factors to the mouse *Mcl1* promoter, apparently, should not always be extrapolated to human *MCL1*. Thus, activating transcription factor 5 (ATF5) was shown to be a regulator of Mcl-1 in mouse neuroblastoma cells [42]. However, no significant correlation between Mcl-1 and ATF5 levels in patient samples was observed [42], and further study failed to prove the role of ATF5 in the regulation of Mcl-1 transcription in human cells [43]. Whether ATF5 can transcriptionally activate human *MCL1* remains to be elucidated. In general, transcription factors found to regulate non-human *Mcl1* should be confirmed with the human gene to avoid possible misinterpretation.

Posttranscriptional control of Mcl-1

Posttranscriptional control of Mcl-1 includes pre-mRNA splicing and regulation of mRNA levels. In addition to the Mcl-1 protein, the corresponding mRNA has a very short half-life (~2–3 h) [39]. The turnover of Mcl-1 mRNA is modified by several RNA-binding proteins and also by multiple regulatory RNAs. The RNA-binding proteins and microRNAs (miRNAs) participating in Mcl-1 posttranscriptional control, as well as splicing regulators, were recently reviewed in detail [44]. Hence, these aspects are not covered here.

In addition to miRNAs, several long non-coding RNAs (lncRNAs) were found to modulate Mcl-1 mRNA stability. LncRNAs represent a class of RNA molecules more than 200 nucleotides in length and devoid of protein-coding ability. LncRNAs are implicated in the regulation of gene expression by diverse mechanisms [45], one of which is acting as decoys in order to prevent the binding of miRNAs to their targets. Since this mechanism of regulation could be readily assessed via bioinformatics analysis of complementary sequences, most of the described lncRNAs that modulate Mcl-1 mRNA stability regulate its levels in this way. Table 2 contains information about lncRNAs that have been found to control Mcl-1 expression.

Translational control of Mcl-1

As a short-lived protein, Mcl-1 is highly sensitive to alterations in the translational activity of a cell. Hence, the modulation of eukaryotic initiation factors, which are important regulators of translation, controls Mcl-1 levels. Many cellular stresses converge on the phosphorylation of eIF2 at Ser51 in order to block general translation. ER stress, UVC, elevated osmotic pressure, and arsenite treatment decrease Mcl-1 levels through eIF2-mediated translational suppression [46]. Perhaps a more selective way to control Mcl-1 synthesis is the regulation of cap-dependent translation (CDT), a common translational mechanism controlled by the eIF4F protein complex, the assembly of which is positively regulated by the mammalian target of rapamycin complex 1 (mTORC1). eIF4F availability differently influences the translation of various mRNAs. So-called "strong mRNAs" (e.g., β-actin) are minimally affected by alterations in eIF4F complex formation, whereas "weak mRNAs" strongly depend on eIF4F availability [47]. There are strong evidence that Mcl-1 mRNA exemplifies "weak mRNA" [48–50]. The 5′UTR of Mcl-1 mRNA presumably possesses a substantial secondary structure, which is one of the possible reasons for the "weakness" of Mcl-1 mRNA [50]. Of note, in numerous studies, mTORC1 was reported to positively regulate Mcl-1 synthesis through regulation of CDT [48], while AMP-activated protein kinase (AMPK) led to the opposite effect [49, 51]. Thus, the nutrient and energy sensors of the cell, mTORC1 and AMPK, respectively, are important regulators of the synthesis of short-lived Mcl-1.

Phosphorylation of Mcl-1

Different types of posttranslational modifications ensure a rapid response to cellular needs. Phosphorylation is the most abundant type of posttranslational modification, which tightly regulates the activity, localization, protein–protein interactions, and the stability of individual proteins [52, 53].

Table 1 Transcriptional regulation of Mcl-1.

Transcription factor	Putative binding motif[a]	Transcriptional inducer; signaling pathway	Examples of biological effects	Refs.
Transcriptional activation				
HIF-1	GCGTGCGG −895/−888 bp	Hypoxia	Protection of hepatoma cells against apoptosis (in vitro)	[41]
		Helicobacter pylori induced ROS	Disease pathogenesis of *Helicobacter pylori* in gastric epithelium (in vitro, patient isolates)	[123]
SRF + Elk-1 (act coopera tively)	for SRF: CCTTTTATGG −96/−87 bp for Elk-1: CCGGAAGC −118/−111 bp	Phorbol ester TPA; MEK/ERK pathway EGF; MEK/ERK signaling pathway	Hematopoietic cell differentiation (in vitro) EGF-mediated cell survival in breast cancer cells (in vitro); correlation between activated Elk-1 and Mcl-1 protein level in breast cancer patients was found	[38, 124]
Elk-4	TCCGGAAGCT −119/−110 bp		Protection against apoptosis in GBM (in vitro); correlation between Elk-4 and Mcl-1 mRNAs in GBM patients was found	[125]
ATF4	TTACGTAA −252/−245 bp	ATF4 branch of the UPR	Mcl-1 upregulation in response to UPR in multiple myeloma (in vitro)	[126]
CREB	TTACGTAA −252/−245 bp	PI3K/Akt	Akt-dependent transcription of Mcl-1	[127]
STAT3	TTATGGGAA −92/−84 bp	IL-6 or IFN-α; JAK/STAT	STAT3-dependent transcription of Mcl-1 (in vitro);	[35, 128]
		B-RAF V600E mutation; autocrine IL-6 signaling	Mcl-1 upregulation by B-RAF^{V600E} in melanoma cells (in vitro)	[129]
Ets-1	CCGGAAGG −221/−214 bp	ER stress; IRE1α/XBP-1 and PI3K/Akt	Mcl-1 upregulation by ER stress in melanoma (in vitro)	[130]
RelA (p65 NF-κB subunit)	GGGGTCTTCC +35/+44 bp	TRAIL; MEK/ERK/IκBα degradation	Protection against TRAIL-induced apoptosis (in vitro)	[131]
c-Myc	CACGTG −588/−583 bp	HDAC inhibitors	Control of sensitivity to HDAC inhibitors (in vitro)	[132]
ERα	TGACC −3603/−3599 bp	Estrogen; ERα + Sp1 transcription factor	Estrogen-mediated transcription of Mcl-1 in breast cancer cells (in vitro)	[133]
Transcriptional repression				
E2F1		Flavopiridol treatment	Transcriptional suppression of Mcl-1 in response to stress stimuli (in vitro)	[134]

TPA 12-O-tetradecanoylphorbol 13-acetate, *ATF4* activating transcription factor 4, *CREB* cAMP response element-binding protein, *Ets-1* E26 transformation-specific-1, *ER* endoplasmic reticulum, *EGF* epidermal growth factor, *ERα* estrogen receptor α, *Elk-1,4* ETS domain-containing tyrosine kinase 1,4, *ERK* extracellular signal-regulated kinase, *GBM* glioblastoma multiforme, *HDAC* histone deacetylase, *HIF-1* hypoxia-inducible factor 1, *IL-6* interleukin 6, *IFN-α* interferon-alpha, *JAK* Janus kinase, *NF-κB* nuclear factor kappa-B, *IκBα* NF-κB inhibitor alpha, *PI3K* phosphoinositide 3-kinases, *ROS* reactive oxygen species, *IRE1α* inositol-requiring enzyme 1 alpha, *SRF* serum response factor, *STAT3* signal transducer and activator of transcription protein 3, *Sp1* specificity protein 1, *TRAIL* TNFα-related apoptosis-inducing ligand, *UPR* unfolded protein response

[a]All sequences correspond to accession AF147742; in this table, nucleotide −1 corresponds to nucleotide 3893 in AF147742 (according to Akgul et al. [135])

Table 2 Regulation of Mcl-1 expression by lncRNAs.

LncRNA	Influence on Mcl-1	Mechanism of action	Possible disease link	Refs.
		Targeting miRNA, which negatively regulates Mcl-1; type of regulation		
MALAT1	Positive	miR-363-3p; sponging	Gallbladder carcinoma	[136]
		miR-101-3p; sponging	Lung adenocarcinoma	[137]
		miR-29a/b-1; increased H3K27me3 modification at the promoter region of miR-29a/b-1	Multiple myeloma	[138]
ANRIL	Positive	miR-127; sponging	Ischemic stroke	[139]
circHIPK3	Positive	miR-193a-3p; sponging	Prostate cancer	[140]
H19	Positive	miR-29b-3p; sponging	Multiple myeloma	[141]
HULC	Positive	miR-124; sponging	Atherosclerosis	[142]
LINC00152	Positive	miR-193a-3p; sponging	Gastric cancer	[143]
		miR-125b; sponging	Ovarian cancer	[144]
MYOSLID	Positive	miR-29c-3p; sponging	Gastric cancer	[145]
PMS2L2	Positive	miR-203; sponging	Osteoarthritis	[146]
SNHG12	Positive	miR-320a; downregulation	Osteosarcoma	[147]
		Other mechanisms		
circOMA1	Positive	Sponges miR-145-5p, which targets mRNA of TPT1. The latter was shown to increase the stability of Mcl-1 protein [19]	Nonfunctioning pituitary adenoma	[148]
PVT1	Positive	Increases Mcl-1 mRNA stability by an unknown mechanism	Renal cell carcinoma	[149]
Linc-ITGB1	Negative	Unknown	Clear cell renal cell carcinoma	[150]

Mcl-1 possesses many potential phosphorylation sites due to the presence of the large regulatory region with two "weak" and two "strong" PEST motifs [11]. Phosphorylation of Mcl-1 is a degradation predictor, although it may also stabilize Mcl-1 and/or control its antiapoptotic activity (i.e., modulate interactions with proapoptotic Bcl-2 family members). Moreover, phosphorylation of one and the same residue can lead to different consequences depending on the phosphorylation status of the other residues.

Phosphorylation of Thr163 is a good example of this intricate regulation. Extracellular signal-regulated kinase (ERK)-mediated phosphorylation of Thr163 (probably in conjunction with phosphorylation of Thr92) results in the increased stability and antiapoptotic activity of Mcl-1 [54, 55]. However, phosphorylation of Thr163 together with Thr92, Ser121, and Ser159 (and, possibly, Ser155) targets Mcl-1 for ubiquitination and subsequent proteasomal degradation [56–59]. In numerous studies, glycogen synthase kinase 3 (GSK-3) was suggested to be a crucial kinase responsible for Ser121, Ser155, Ser159, and Thr163 phosphorylation [56–58]. Triple Mcl-1 mutant S155A + S159A + T163A demonstrates enhanced stability that rescues breast cancer cell line MCF-7 from constitutively active GSK-3 [58]. Even substitution of single residue Ser159 with alanine increased Mcl-1 stability upon GSK-3 activation [57]. In addition, GSK-3-mediated phosphorylation seems to define Mcl-1 antiapoptotic activity independently of proteasomal degradation. In a recent study, histone deacetylase (HDAC) inhibitors did not cause Mcl-1 degradation, despite GSK-3β-dependent phosphorylation of Mcl-1. Nevertheless, GSK-3β activity led to apoptosis, which could be due to the decreased affinity of phosphorylated Mcl-1 to its proapoptotic partners [60]. Intriguingly, during mitotic arrest other kinases [p38 MAPK, JNK1, and casein kinase II (CKII)] are involved in phosphorylation of Mcl-1 at degradation-associated residues, whereas GSK-3 is dispensable in this process in arrested cells. Hence, different kinases can phosphorylate the same residues of Mcl-1 in a context-dependent manner [59].

Thr92 phosphorylation may also lead to different outcomes. As noted above, ERK-mediated phosphorylation at Thr92 and Thr163 was shown to stabilize Mcl-1 [55]. Alternatively, Thr92 phosphorylation serves as a key step in the degradation of Mcl-1 during mitotic arrest. The latter modification is conducted by cyclin-dependent kinase 1 (CDK1) complexed with cyclin B after treatment with microtubule damaging agents (e.g., nocodazole and taxol). The T92A mutation enhances Mcl-1 stability and rendered cells more resistant to apoptosis during prolonged mitotic arrest [61]. Phosphorylation of Thr92 by CDK1 blocks the association of Mcl-1 with protein phosphatase 2A (PP2A) and precedes phosphorylation of Ser121, Ser159, and Thr163. Hence, it was proposed that phosphorylation of Thr92 by CDK1 primes Mcl-1 for subsequent

phosphorylation and degradation by reducing PP2A activity towards Mcl-1 [59].

Intriguingly, recent research has challenged previous data concerning the phosphorylation and stability of Mcl-1 during mitotic arrest. In HeLa cells, the "9A mutant" of Mcl-1, bearing substitutions to alanine in nine phosphorylation sites (i.e., in all known phosphorylation sites, except Ser155), was analyzed. This mutant retained antiapoptotic activity, underwent phosphorylation under mitotic arrest, and degraded as fast as wild-type Mcl-1. Although Ser155 could be responsible for Mcl-1 phosphorylation, neither the priming role of Thr92 nor phosphorylation at multiple sites were required for degradation of Mcl-1 [62]. These results show that our understanding of Mcl-1 regulation via phosphorylation is still incomplete.

Another residue, Ser64, was also phosphorylated during the G2/M phase of the cell cycle [61, 63]. Such modification had no apparent effect on Mcl-1 half-life, as was revealed using phosphonegative (S64A) and phosphomimic (S64E) mutants. However, the S64E mutant of Mcl-1 demonstrated the enhanced affinity to Bak, Bim, and Noxa [63]. Noxa was reported to promote Mcl-1 phosphorylation at Ser64 and Thr70 by checkpoint kinase 2 (Chk2), which subsequently led to the proteasomal degradation of Mcl-1 [64].

In total, at least the following 10 residues of Mcl-1 may undergo phosphorylation: Ser64 [63], Thr68 [62], Thr70 [64], Thr92 [55], Ser121 [65], Ser155 [58], Thr156 [62], Ser159 [57], Ser162 [62], and Thr163 [65]. Nevertheless, most of these sites are not characterized well enough to create a holistic picture of the regulation of Mcl-1 by phosphorylation.

Ubiquitination and degradation of Mcl-1

The ability of Mcl-1 to protect cells from apoptosis is controlled by modulation of the Mcl-1 level, rather than by changing its activity. The proteasomal machinery is crucial for the continuous turnover of Mcl-1 and its degradation in response to different stimuli [13, 66]. As is well acknowledged, K48-linked polyubiquitin chains bound to lysine residues of target proteins serve as signals for proteasomal degradation [67]. Intriguingly, Mcl-1 is able to undergo proteasomal cleavage even in a cell-free system, and the Mcl-$1^{K\rightarrow R}$ mutant (in which all lysines were mutated to arginines) could be degraded as fast as the wild-type protein [68]. Apparently, as a partially intrinsically disordered protein, Mcl-1 undergoes proteasomal degradation by the 20S proteasomes independently of ubiquitin tagging [69]. Nevertheless, numerous studies have demonstrated the crucial role of ubiquitination in the regulation of Mcl-1 turnover (Fig. 1).

Multiple ubiquitin ligases and deubiquitinases orchestrate ubiquitination and the subsequent proteasomal degradation of Mcl-1. Mule (Mcl-1 ubiquitin ligase E3, also known as ARF-BP1) was the first identified Mcl-1 ubiquitin ligase. It contains the BH3-domain, which interacts with the BH3-binding groove of Mcl-1, but not with the grooves of Bcl-2 or Bcl-xL [70, 71]. Mule, thereby, can also compete for the binding of BH3-only proteins to Mcl-1, or vice versa. In particular, the BH3-motif of Bim is able to displace Mule from interacting with Mcl-1, resulting in increased Mcl-1 levels [71]. Instead, Noxa favors the interaction between Mcl-1 and Mule, while abrogating binding with deubiquitinase USP9X (see below) [72]. Mule, therefore, appears to be involved in Noxa-mediated degradation of Mcl-1. Consistently, despite amplified Noxa expression, Mule-deficient primary mouse B cells demonstrate impaired degradation of Mcl-1 after etoposide treatment [73]. Mule may act as a tumor suppressor, while the increased Mcl-1 level in Mule-deficient tumors was shown to protect cells from apoptosis [74].

Membrane-associated RING-CH protein 5 (MARCH5), which localizes to the outer membrane of the mitochondria (OMM) and maintains mitochondrial homeostasis, represents another possible ubiquitin ligase for Mcl-1. Knockdown of MARCH5 reduces ubiquitination and degradation of Mcl-1 [75, 76]. However, it is not yet clear whether MARCH5 directly ubiquitinates Mcl-1. Surprisingly, despite the increased Mcl-1 levels, MARCH5 knockdown sensitized different cancer cell lines to ABT-737 (BH3-mimetic to Bcl-2 and Bcl-xL) treatment. MARCH5 promotes the degradation of Mcl-1 in a Noxa-dependent manner. In MARCH5-silenced cancer cells, knockdown of Noxa abolished both the accumulation of Mcl-1 and the sensitization to ABT-737 [75]. Thus, upon MARCH5 knockdown, the effect of Noxa stabilization might exceed that of Mcl-1.

The ubiquitin ligase Parkin directly ubiquitinates Mcl-1. Normally, the PTEN-induced kinase 1 (PINK1)/Parkin pathway promotes the turnover of moderately depolarized mitochondria, without inducing apoptosis. However, pronounced mitochondrial depolarization [which could take place in response to valinomycin treatment or prolonged exposure to carbonyl cyanide m-chlorophenyl hydrazone (CCCP)] results in Parkin-dependent ubiquitination of Mcl-1, its degradation, and concomitant apoptosis [77, 78]. Thereby, Mcl-1 may serve as a mediator between mitochondrial depolarization and apoptosis.

Several other ubiquitin ligases of Mcl-1 ($SCF^{\beta\text{-}TrCP}$, SCF^{FBW7}, TRIM17) promote Mcl-1 ubiquitination in a phosphorylation-dependent manner. SCF (Skp1, Cul1, F-box-protein) is a multicomponent E3 ubiquitin ligase complex, which contains interchangeable F-box proteins for substrate recognition. Three different F-box proteins, FBW7 [56, 59], β-TrCP [58], and FBXO4 [79], were reported to mediate ubiquitination of Mcl-1 by SCF. Mcl-1 contains

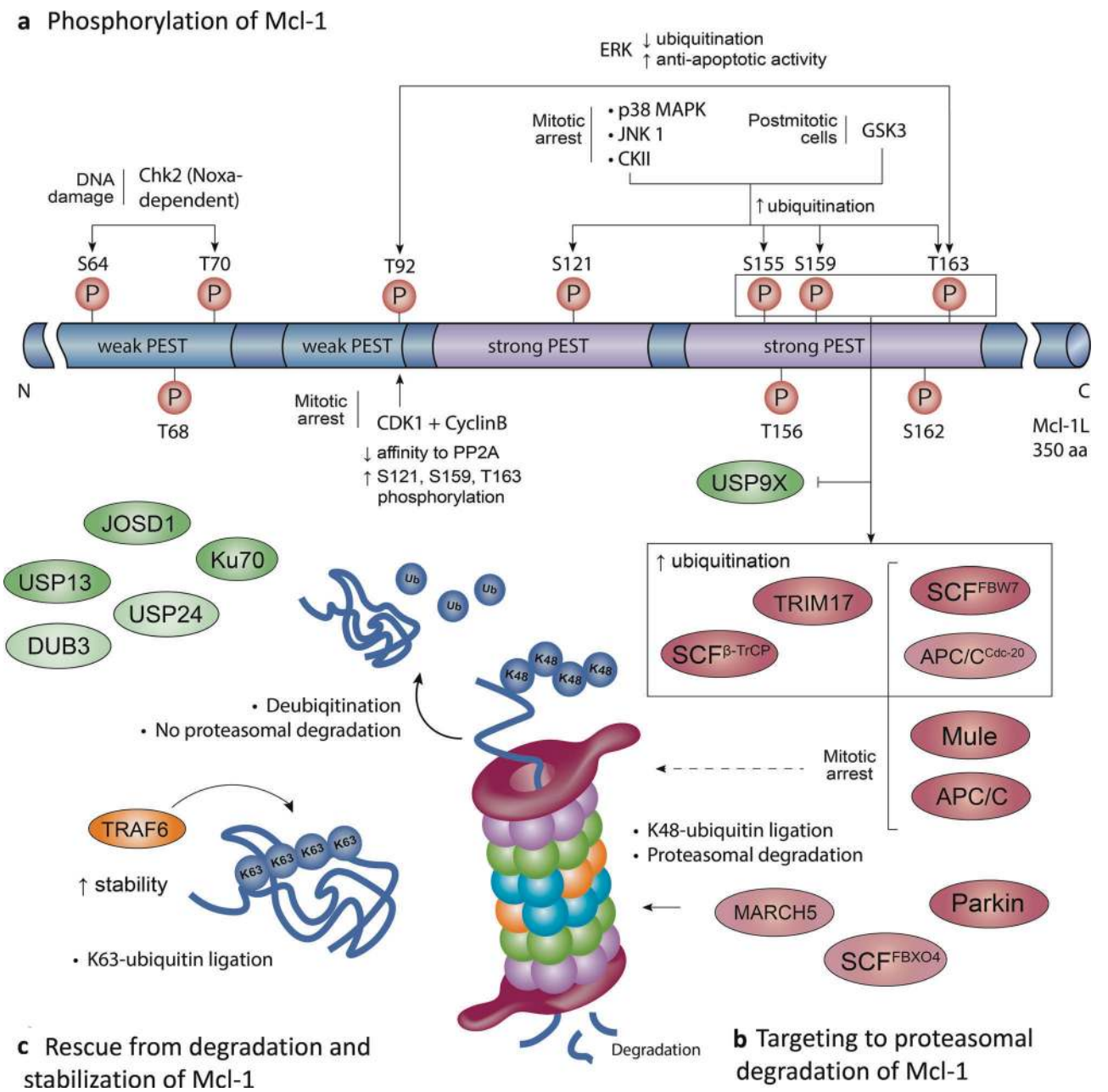

Fig. 1 Phosphorylation, ubiquitination, and deubiquitination of Mcl-1. a A distinctive feature of Mcl-1 is the presence of two weak and two strong PEST motifs in its N-terminal region. PEST motifs are typical for proteins with relatively short half-lives, and they contain many sites of phosphorylation. Depending on the modified sites, Mcl-1 phosphorylation can lead to different effects, such as changes in Mcl-1 affinity to the binding partner, stabilization, or destabilization. For instance, phosphorylation of multiple residues in Mcl-1 degron motifs 116–125 and 154–163 targets the protein for ubiquitination and subsequent proteasomal degradation. **b** Several ubiquitin ligases and deubiquitinating enzymes were described as regulators of Mcl-1 degradation. Ubiquitin ligases that form K48-linked ubiquitin chains target Mcl-1 for proteasomal degradation. Some of them ($SCF^{β\text{-}TrCP}$, SCF^{FBW7}, TRIM17, and APC/C^{Cdc20}) ubiquitinate Mcl-1 in a phosphorylation-dependent manner. SCF^{FBW7}, APC/C, and Mule were proposed to play roles during mitotic arrest. However, a recent study suggests that APC/C is a key ubiquitin ligase of Mcl-1 during mitotic arrest, while SCF^{FBW7} and Mule are not (see Fig. 2). **c** The ubiquitin ligase TRAF6 mediates K63-linked polyubiquitination and thus stabilizes Mcl-1. Deubiquitinating enzymes USP9X, USP13, USP24, DUB3, JOSD1, and Ku70 "reverse" Mcl-1 K48-linked ubiquitination and prevent its proteasomal degradation. To our knowledge, there is no data (using an in vitro enzyme activity assay) of the direct regulation of Mcl-1 by deubiquitinating peptidases USP24 and DUB3 and ubiquitin ligases MARCH5, APC/C^{Cdc20}, and FBXO4 (depicted in pale colors), which allows for the possibility of indirect regulation of Mcl-1 by these enzymes. S serine, T threonine, Ub ubiquitin, K48 ubiquitination with lysine 48 linked ubiquitin, K63 ubiquitination with lysine 63 linked ubiquitin, Chk2 checkpoint kinase 2, ERK extracellular signal-regulated kinase, p38 MAPK p38 mitogen-activated protein kinase, JNK1 c-Jun N-terminal kinase 1, CKII casein kinase II, GSK3 glycogen synthase kinase 3, CDK1 cyclin-dependent kinase 1, PP2A protein phosphatase 2A, APC anaphase-promoting complex, $APC/C^{Cdc\text{-}20}$ APC complexed with the cell-division cycle protein 20, Mule Mcl-1 ubiquitin ligase E3, MARCH5 membrane-associated RING-CH protein 5.

two degron motifs for interaction with FBW7 (residues 116–125 and 154–163) and one degron motif for binding to β-TrCP (residues 157–162) [59]. Phosphorylation of Ser121, Ser159, and Thr163 (e.g., by GSK-3) in the corresponding degrons of Mcl-1 is an essential step for the interaction with $SCF^{\beta\text{-TrCP}}$ and SCF^{FBW7}. Consequently, FBW7 and β-TrCP are important participants of GSK-3-mediated degradation of Mcl-1 [56, 58], and they can act in a redundant manner [80]. A recent study demonstrated that FBW7 may act in a context-dependent manner since the downregulation of FBW7 in cholangiocarcinoma cells affects Mcl-1 degradation in cisplatin-treated cells, but not in untreated cells [81]. Ubiquitin ligase TRIM17 was shown to ubiquitinate Mcl-1 in primary mouse cerebellar granule neurons after phosphorylation by GSK-3, and this resulted in neuronal apoptosis [82]. Recently, SCF^{FBXO4} was proposed to ubiquitinate Mcl-1. However, it is not clear whether FBXO4 serves as an adapter for phosphorylated Mcl-1, and the precise mechanisms of FBXO4-mediated Mcl-1 ubiquitination also remain to be elucidated [79].

A separate issue is the degradation of Mcl-1 during prolonged mitotic arrest, which occurs in response to chromosome segregation defects, e.g., after the treatment with microtubule poisons. Phosphorylation-dependent Mcl-1 degradation seems to be a decisive factor in the separation of mitotic arrest and apoptosis [61]. Previously, APC/C^{Cdc20} (APC/C complexed with substrate recognition adapter Cdc20) [61], Mule [83], and SCF^{FBW7} were proposed as ubiquitin ligase systems responsible for ubiquitination of Mcl-1 during mitotic arrest [59]. At the same time, in another report these ubiquitin ligases were dispensable for Mcl-1 degradation in arrested cells [84]. This paradox could arise from the false-positive results in the initial experiments: decreased degradation of Mcl-1 after silencing Mule, FBW7, and Cdc20 might be detected in cells that slipped out of mitosis and not in arrested ones. Nevertheless, recently, using live-cell imaging, APC/C (independently of its activator Cdc20) was claimed to direct Mcl-1 degradation during mitotic arrest [85]. Live-cell imaging might be used in future studies to address possible roles of other ubiquitin ligases in the degradation of Mcl-1 during mitotic arrest in various cell lines. An understanding of such mechanisms could be useful for improving strategies to eliminate cancer cells with antimitotic drugs [85, 86] (Fig. 2).

Several ubiquitin ligases tag their substrates with K63-linked polyubiquitin chains, which serve as nondegradative signals in various intracellular processes. TRAF6 is a well-known K63 ubiquitin ligase, which, among other substrates, ubiquitinates Mcl-1 [87, 88]. Such modification stabilizes Mcl-1 by preventing its interaction with the 20S-proteasome (allegedly, owing to the steric hindrance). Intriguingly, four C-terminal lysine residues of Mcl-1, but not those in the N-terminal regulatory region, were subjected to K63 ubiquitination by TRAF6 [87].

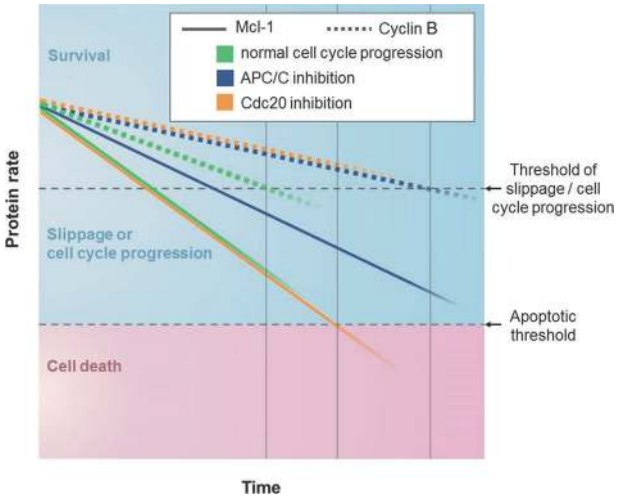

Fig. 2 Degradation of Mcl-1 upon treatment with antimitotic drugs. Mcl-1 is one of the key switches between prolonged arrest in mitosis and cell death. The level of Mcl-1 changes as the cell cycle progresses, peaking in G2 and declining in mitosis. Prolonged mitotic arrest eventually leads to a drop in Mcl-1 levels below the threshold of apoptosis induction. Degradation of Mcl-1 is responsible for the cell death induced by microtubule poisons [61]. However, which machinery controls proteasomal degradation of Mcl-1 during mitotic arrest is still debatable. Previously, APC/C^{Cdc20}, Mule, and SCF^{FBW7} were reported to target Mcl-1 for proteasomal degradation in arrested cells [59, 61, 83]. It is noteworthy, though, that microtubule poisons lead to the degradation of cyclin B, resulting in mitotic slippage. Hence, data concerning the roles of various ubiquitin ligases may reflect events in cells after mitotic slippage, but not in arrested ones. Recently, Allan et al. revised their previous data about the role of APC/C^{Cdc20} in the degradation of Mcl-1 during mitotic arrest (see the figure). Instead, APC/C (independently of its activator Cdc20) was shown to be a key determinant of this process [85]. According to the proposed model, low activity of APC/C provides a slow decrease in the level of Mcl-1 (blue line), which ultimately reaches the threshold of apoptosis induction. This coincides with a decrease in the level of cyclin B (dashed blue line) and, if the latter prevails, cells slip out of mitosis before apoptosis induction. Earlier, it was shown that targeting Cdc20 leads to an increase in the ratio of apoptotic/slipped cells in comparison with the action of microtubule poisons [86]. The model proposed by Allan et al. demonstrates the possible mechanism of this phenomenon. Microtubule poisons lead to the inhibition of APC/C, which affects the degradation of both Mcl-1 and cyclin B. However, while cyclin B requires Cdc20 for degradation, Mcl-1 does not. Consequently, targeting Cdc20 slows down the degradation of cyclin B (dashed orange line), but not the degradation of Mcl-1 (orange line), thus favoring apoptosis [85]. Meanwhile, during normal mitosis, the degradation of cyclin B (dashed green line) ensures cell cycle progression before the level of Mcl-1 (green line) drops below the threshold of apoptosis induction. Please note, the curves for Mcl-1 and cyclin B levels are not proportional to each other, so the level of Mcl-1 should not be directly compared with the level of cyclin B. In general, degradation of Mcl-1 during mitotic arrest depends on APC/C, while degradation of cyclin B depends on both APC/C and Cdc20. These data show the molecular basis of various outcomes after the treatment of cells with antimitotic drugs and the decisive role of Mcl-1 in determining cell fate.

As the name implies, deubiquitinases reverse the process of ubiquitination, inhibiting proteasomal degradation of their substrates. Yet, USP9X, USP13, USP24, JOSD1, DUB3, and Ku70 were found to promote deubiquitination of Mcl-1. USP9X deubiquitinates Mcl-1, depending on the phosphorylation state of the degradation-associated residues Ser155, Ser159, and Thr163. The substitution of these residues with alanine favors interactions between USP9X and Mcl-1, whereas a phosphomimic (S155E + S159E + T163E) mutant of Mcl-1 demonstrates a decreased affinity towards USP9X. Consistently, GSK-3 inhibition prevents stress-induced dissociation of USP9X from Mcl-1 [89]. Furthermore, Noxa was shown to disrupt USP9X/Mcl-1 interactions, resulting in ubiquitin-dependent degradation of Mcl-1 [72].

Similarly, deubiquitinase USP24 can interact with Mcl-1, while the knockdown of USP24 resulted in decreased Mcl-1 levels. No in vitro assays for direct deubiquitination of Mcl-1 by USP24 were performed [90]. Next, it was reported that USP13 was a novel deubiquitinase for Mcl-1. Apparently, in several cancer cell lines, Mcl-1 stability depends on USP13 rather than USP9X [91]. Two recent studies revealed DUB3 and JOSD1 as deubiquitinases of Mcl-1 [31, 32]. Overexpression of DUB3 resulted in an increase in the level of Mcl-1, while DUB3 knockdown led to increased ubiquitination of Mcl-1. Although no in vitro assay for direct deubiquitination of Mcl-1 was demonstrated, the interaction between DUB3 and the N-terminus of Mcl-1 suggests that DUB3 might act as a direct deubiquitinase of Mcl-1. It was also shown that among the three N-terminal lysines of Mcl-1 – K5, K40, and K136 – DUB3, as well as USP9X, promoted deubiquitination at K40 [31]. Similarly to DUB3, JOSD1 interacted with the N-terminus of Mcl-1, and it was demonstrated that JOSD1 directly cleaved the K48-linked polyubiquitin chains bound to Mcl-1 [32].

Somewhat unexpectedly, the DNA repair protein Ku70 appears to stabilize Mcl-1 through deubiquitination [92]. The deubiquitinating activity of Ku70 has been insufficiently explored, and, so far, Bax and Mcl-1 are the only known substrates for deubiquitination by Ku70 [92, 93]. It could be proposed that Ku70 regulates the interplay between the DNA damage response and apoptosis through the regulation of Mcl-1 or controls its nonapoptotic functions. Indeed, further studies are necessary to address these possibilities. Nevertheless, it is clear that ubiquitination and proteasomal degradation represent some of the most important mechanisms for the modulation of Mcl-1 levels.

Mcl-1 downregulation during cell death execution

Elimination of prosurvival factors is required for the efficient execution of cell death. During apoptosis, Mcl-1 is cleaved by executioner caspases at D127 and D157 [94] and Granzyme B at D117 and, to a lesser extent, at D127 and D157 [95]. Although the resulting C-terminal fragments of Mcl-1 are still able to form antiapoptotic BH3-binding groove, their binding profiles seem to be altered [94, 95]. There is some uncertainty about how Mcl-1 cleavage affects its functions. While several studies have demonstrated proapoptotic activity of Mcl-1 cleavage fragments [96, 97], others have failed to do so [94, 98]. The functions of Mcl-1 cleavage products seem to be context-dependent, and this issue requires further elucidation. Another mechanism of Mcl-1 downregulation during apoptosis is global mRNA decay by exonuclease DIS3 mitotic control homolog-like 2 (DIS3L2), which leads to the arrest of protein synthesis and a subsequent drop in Mcl-1 levels [99]. Taken together, these mechanisms ensure the elimination of Mcl-1 during execution of apoptotic cell death. This circumstance should be considered when interpreting the decrease in Mcl-1 levels, e.g., by western blot analysis, in response to different apoptosis-inducing agents since the downregulation of Mcl-1 could represent both a cause and/or a consequence of apoptosis.

Dysregulation of Mcl-1

Overexpression of Mcl-1 confers high oncogenic potential due to the decreased susceptibility to apoptotic stimuli. The corresponding dysregulation may occur in two common ways. First, *MCL1* is the proposed amplification target gene, and cancer cells with amplifications in the *MCL1* genomic locus depend on Mcl-1 for survival [100]. In addition, specific short sequence insertions in the *MCL1* promoter were shown to correlate with the increased expression of Mcl-1 and a worse prognosis in chronic lymphocytic leukemia patients [101]. Similar to the *BCL2* proto-oncogene, *MCL1* is located at a chromosomal fragile site [102]. However, unlike *BCL2*, *MCL1* rarely undergoes chromosomal translocations [102]. Point mutations in *MCL1* are also uncommon events, with no specific mutational hotspots identified [103]. Overall, genomic dysregulation of *MCL1* mainly involves gene amplifications, but not translocations or point mutations.

Second, alterations in multiple signaling pathways and the regulatory mechanisms mentioned earlier may affect Mcl-1 expression. For example, in acute lymphoblastic leukemia cell lines, both stabilization (through phosphorylation of Thr92 and Thr163) and increased antiapoptotic activity (through phosphorylation of Ser64) of Mcl-1 contribute to acquired resistance to ABT-737 treatment [104]. Another regulator of Mcl-1, the serine/threonine kinase GSK-3, plays dual roles in cancer [105]. Nevertheless, GSK-3 activation could be useful in overcoming the Mcl-1-mediated resistance to apoptosis. Since Akt negatively

regulates GSK-3 [57], targeting Akt leads to GSK-3-dependent Mcl-1 degradation [80]. Next, reduced FBW7 activity results in the stabilization of Mcl-1 in cancer cell lines of different origins [106, 107]. Interestingly, *FBW7*-mutant cancer cells exhibit resistance to ABT-737 and docetaxel, while being highly sensitive to HDAC inhibitors [108]. Hence, epigenetic regulation might represent a promising strategy for the treatment of tumors that overexpress Mcl-1. Of note, some types of histone modifications were shown to regulate Mcl-1 expression in cancer cells. In osteosarcoma cells, binding of histone H3 trimethylated at lys27 (H3K27me3), a mark of transcriptional repression, to the *MCL1* gene locus was directly correlated with sensitivity to cisplatin [109]. Finally, monoubiquitination of H2A orchestrated by deubiquitinase BAP1 and ubiquitin ligase RNF2 was found to silence both Mcl-1 and Bcl-2 expression [110].

There are numerous other examples illustrating how cancer cells can abuse various molecular mechanisms in order to upregulate Mcl-1. Meanwhile, one of the most important issues is whether the mechanisms of Mcl-1 regulation listed above have clinical relevance. There are several studies that have demonstrated a correlation between Mcl-1 regulators and prognosis and/or Mcl-1 levels in patients. As such, in pancreatic cancer patients, a decrease in FBW7 expression correlates with Mcl-1 accumulation and a poor prognosis [111]. A correlation between USP9X and Mcl-1 expression was found in several cancers, including follicular lymphoma and colon adenocarcinoma. Moreover, in patients with multiple myeloma, an increase in USP9X mRNA was associated with a poor prognosis [89]. Immunohistochemistry analysis revealed a strong correlation between USP13 and Mcl-1 levels in ovarian cancer tissues [91]. Similar results were shown for the recently identified deubiquitinases of Mcl-1, JOSD1 and DUB3. In addition to the correlation between JOSD1 or DUB3 and Mcl-1 levels, all three proteins were correlated with a poor outcome in ovarian cancer patients [31, 32].

Studying the relationship between Mcl-1 and its regulators might have practical significance for precision medicine approaches. For example, the expression of several ubiquitin ligases/deubiquitinases at mRNA levels could be assessed with convenient RNA-seq techniques to predict Mcl-1-dependence in tumors. However, since posttranslational regulation of Mcl-1 implies changes in Mcl-1 protein levels and not in mRNA abundance, more complicated approaches, such as immunohistochemistry, could be required to assess the increase/decrease in Mcl-1 levels. In this case, dysregulation of ubiquitin ligases/deubiquitinases of Mcl-1 could serve as a predictive biomarker for the use of Mcl-1-targeted therapies. In general, translating our knowledge of the regulation of Mcl-1 into clinical practice is highly relevant.

Indirect inhibition of Mcl-1: to be or not to be?

Previously, numerous attempts have been made to target various regulators of Mcl-1 in order to neutralize its anti-apoptotic activity in cancer cells. Whereas preclinical studies have demonstrated promising results for a variety of agents and approaches, which could potentially downregulate Mcl-1, only a few of them were translated into clinical trials. In particular, the CDK inhibitors alvocidib and dinaciclib were evaluated as potential indirect inhibitors of Mcl-1. However, the conducted trials failed to demonstrate the efficacy of the studied compounds in decreasing Mcl-1 levels. While several reports did not disclose the influence on Mcl-1 [112, 113], one report demonstrated only weak efficacy of alvocidib in the context of downregulation of Mcl-1 [114]. Nevertheless, new clinical trials with CDK inhibitors are being conducted focusing on changes in Mcl-1 levels as a pharmacodynamic effect (NCT04017546, NCT03739554) or Mcl-1-dependency as a biomarker of sensitivity (NCT03298984, NCT02520011). Hopefully, new studies will demonstrate better efficacy of CDK inhibitors as modulators of Mcl-1.

The rationale for the use of CDK inhibitors for targeting Mcl-1 is that these compounds block global mRNA synthesis, which results in a dramatic change in the level of short-lived proteins [115]. There are many other mechanisms through which potential indirect inhibitors of Mcl-1 could act. In theory, each node in the complex net of regulation of Mcl-1 could be targeted in order to downregulate this oncogenic protein. For example, WP1130, which inhibits several deubiquitinases, including USP9X and USP24, induced apoptosis in Mcl-1-dependent myeloma cells [90]. Inhibitors of mTORC1 might decrease Mcl-1 levels through suppression of CDT [48]. Mcl-1 can also be downregulated by calorie restriction [116], which represents a promising approach for cancer therapy [117]. Recent work demonstrates that feeding/fasting cycles in combination with metformin inhibit tumor growth through the downregulation of Mcl-1 in a GSK-3β-dependent manner [118]. In addition, tyrosine kinase inhibitors (TKIs), as exemplified by sorafenib, lead to decreases in Mcl-1 through various mechanisms [119, 120]. Pharmacological agents that upregulate BH3-only proteins targeting Mcl-1 represent another tool for indirect inhibition of this protein [121, 122]. This is especially relevant for transcriptional inducers of Noxa, as this protein demonstrates high selectivity to Mcl-1 over other antiapoptotic proteins, Bcl-2 and Bcl-xL. In general, there are numerous ways of indirect inhibition of Mcl-1.

Meanwhile, recent advances in the development of small molecule inhibitors targeting Mcl-1 have provided us with powerful tools that could be used to block the antiapoptotic activity of Mcl-1. If these compounds could be effective in

clinical settings, should we consider indirect inhibitors of Mcl-1 as potential drugs? There are at least two reasons why this question should be answered positively. First, indirect Mcl-1 targeting could be better for the inhibition of Mcl-1 specifically in cancer cells. Mcl-1 is essential for the survival of some types of normal cells, and BH3-mimetics would result in the inhibition of Mcl-1 both in normal cells and in cancer cells. If, for instance, Mcl-1 is upregulated due to the increased activity of some deubiquitinases, the inhibition of the "reason" (i.e., deubiquitinases, in this example) rather than the "consequence" (i.e., Mcl-1) would diminish Mcl-1 predominantly in cancer but not in normal cells. Secondly, compounds such as CDK inhibitors and TKIs influence multiple cellular pathways, and the decrease in the Mcl-1 level represents one of the possible mechanisms of their action. In case such therapeutic agents could efficiently downregulate Mcl-1, its direct inhibition by BH3-mimetics could be dispensable. Altogether, we speculate that, at least in some cases, indirect inhibitors of Mcl-1 might be a more favorable option for cancer therapy instead of direct antagonists.

Conclusion

Here, we have shed light on the regulatory circuits that modulate the expression and activity of the antiapoptotic protein Mcl-1. As discussed above, Mcl-1 is a short-lived protein that can be regulated through distinct mechanisms, including posttranslational modifications. These features of Mcl-1 have been used in many experimental studies to target Mcl-1 in cancer cells. Yet, translating this knowledge for practical applications is of great importance. We anticipate that further studies will focus on correlative analyses between the expression of Mcl-1 and its regulators in patients, as well as on the significance of various patterns of expression for the prediction of therapy responses. With the development of next generation sequencing technologies, substantial progress should be done in the understanding of mechanisms relevant for the dysregulation of Mcl-1 in patients. This would give new options for precision medicine approaches and improve therapy for cancer patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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