


Perturbing mitosis for anti-cancer therapy: is cell death the only answer?

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Abstract

Interfering with mitosis for cancer treatment is an old concept that has proven highly successful in the clinics. Microtubule poisons are used to treat patients with different types of blood or solid cancer since more than 20 years, but how these drugs achieve clinical response is still unclear. Arresting cells in mitosis can promote their demise, at least in a petri dish. Yet, at the molecular level, this type of cell death is poorly defined and cancer cells often find ways to escape. The signaling pathways activated can lead to mitotic slippage, cell death, or senescence. Therefore, any attempt to unravel the mechanistic action of microtubule poisons will have to investigate aspects of cell cycle control, cell death initiation in mitosis and after slippage, at single-cell resolution. Here, we discuss possible mechanisms and signaling pathways controlling cell death in mitosis or after escape from mitotic arrest, as well as secondary consequences of mitotic errors, particularly sterile inflammation, and finally address the question how clinical efficacy of anti-mitotic drugs may come about and could be improved.

Keywords apoptosis; BCL2 family; mitotic arrest; p53; slippage

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See the Glossary for abbreviations used in this article.

Introduction

Anti-mitotic drugs, including vinca alkaloids or taxanes and derivatives that target microtubules, are used successfully in the clinics to treat multiple types of cancer but negative side effects, such as neurotoxicity and frequent drug resistance, limit their therapeutic use [1]. This has led to the development of second-generation compounds, often referred to as “mitotic blockers”, that aim to interfere with proper spindle formation, chromosome segregation, and/or mitotic exit, for example, by targeting microtubule motor proteins, including kinesins KIF11 or CENP-E, polo-like kinase (PLK)1, or Aurora A kinase, as well as the APC/C E3-ligase

complex, respectively. However, these targeted drugs had overall limited success in clinical trials so far and proved often less effective as before-mentioned microtubule-damaging agents [2]. Along similar lines, another class of compounds, referred to as “mitotic drivers”, triggering premature mitotic exit by targeting key mitotic kinases such as MPS1 or Aurora B, forcing chromosome missegregation or mitotic slippage with cytokinesis failure, respectively, are under development for cancer treatment [2,3]. All these compounds can trigger cell death, the preferred outcome in clinical use [4,5], but also numerical as well as structural chromosomal instability (CIN) and/or tetraploidy-mediated aneuploidy in surviving cells, hallmarks of cancer evolution, and drivers of drug resistance [6].

What all these compounds, spindle poisons, mitotic blockers, and drivers, do have in common is that they can trigger a p53 response in non-transformed cells, as well as in tumors carrying wild-type p53. This response is still poorly understood mechanistically, but clearly distinct from the one induced by classical triggers of DNA damage, such as ionizing radiation or conventional chemotherapy [7,8]. In addition, many (if not all) of these drugs can elicit apoptotic cell death, at least in tissue culture [9–11]. This type of cell death may or may not engage p53, and the basis of cell death paradigms triggered upon erroneous mitosis is considered to be very heterogeneous and molecularly ill-defined, hence often summarized as “mitotic catastrophe” [5,12]. The feature of promoting CIN and aneuploidy upon drug treatment appears as a double-edged sword: On the one hand, aneuploidy itself will have a negative impact on the cellular fitness, but on the other hand, it harbors the inherent problem that therapy exploiting these features will be selecting for complex karyotypes that promote cell survival and aneuploidy tolerance. This is drastically facilitated by p53 loss, leading to more aggressive disease, posing a major challenge for safe clinical application [6,13,14].

Moreover, recent data actually suggest that doses of anti-mitotic drugs needed to induce apoptosis of cancer cells *in vitro* are likely not achieved *in vivo* and, hence, do not efficiently arrest cells in mitosis. For example, paclitaxel rather causes multipolar cell divisions ultimately limiting cancer cell fitness [15]. Ultimately, the clinical efficacy of these drugs may actually also rely on the induction of inflammatory responses in cells experiencing DNA damage upon mitotic errors, either before or upon their, potentially immunogenic, death [15–17]. As the type of cell death induced by these and other

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Glossary

APC/C	anaphase-promoting complex/cyclosome
BAD	BCL2-associated agonist of cell death
BAK1	BCL2 antagonist 1
BAX	BCL2-associated X
BCL2	B-cell CLL/lymphoma 2
BCLX	BCL2-like 1
BH3	BCL2 homology domain 3
BID	BH3-interacting domain death agonist
BIM	BCL2-interacting mediator of cell death
BMF	BCL2-modifying factor
BUB1	budding uninhibited by benzimidazoles 1
CDC20	cell division cycle 20
CDK1	cyclin-dependent kinase 1
CENP-E	centromere protein E
cGAS	cyclic GMP-AMP synthase
CIN	chromosomal instability
IRF3	interferon regulatory factor 3
KIF11	kinesin family member 11
MAD1/2	mitotic arrest deficient 1/2
MCC	mitotic checkpoint complex
MCL1	myeloid cell leukemia sequence 1
MDM2	mouse double minute 2, human homolog of
MOMP	mitochondrial outer membrane permeabilization
MPS1	monopolar spindle 1 kinase
NF-κB	nuclear factor kappa B
PIDD1	p53-induced protein with a death domain 1
PLK1	polo-like kinase 1
PTM	post-translational modification
PUMA	p53-upregulated mediator of apoptosis
SAC	spindle assembly checkpoint
STING	stimulator of interferon genes
TNF	tumor necrosis factor

anti-cancer compounds clearly impacts on anti-cancer immunity, sterile inflammation elicited by anti-mitotic drugs most likely constitutes a highly underappreciated and underexplored feature contributing substantially to their clinical efficacy [17,18].

With this review, we are aiming to highlight most recent developments identifying the molecular machinery that is engaged to execute cell death upon stalled mitosis or upon mitotic exit, with a focus on the BCL2 protein family and their regulation in and out of mitosis. This overview will be complemented with a shorter summary on recent studies identifying novel players leading to p53 activation upon extended mitotic arrest, or in response to supernumerary centrosomes, that have been highlighted recently nicely elsewhere [19,20]. Ultimately, we would also like to end with more thoughts on the cross talk between the mitotic machinery with the one controlling cell death and potential links to sterile inflammation as the latter clearly impacts on the clinical efficacy of drugs aiming to target cancer cells by manipulating mitotic fidelity.

Molecular control of mitotic arrest

Mitotic arrest is implemented by the spindle assembly checkpoint (SAC) machinery which enables and monitors proper segregation of sister chromatids to daughter cells during mitosis. Chromosome segregation requires the mitotic spindle apparatus, whose tubulin fibers originate from two microtubule-organizing centers that, in metazoans, are composed by centrosomes, positioned at opposing

sides of the cell during mitosis. Tubulin fibers attach to special proteinaceous structures, called kinetochores, that assemble on centromeric chromosome regions. The SAC acts as a surveillance mechanism to ensure that the onset of anaphase involving the removal of sister chromatid cohesion and separation only occurs when all chromosomes are attached to the mitotic spindle in the correct configuration, that is known as bi-orientation (Fig 1) [21]. As long as a single chromosome is not bi-oriented, the key effector of the SAC, the so-called mitotic checkpoint complex (MCC), keeps the cells arrested in prometaphase. This is achieved by the inhibition of the large E3 ubiquitin ligase complex called the anaphase-promoting complex or cyclosome (APC/C). The APC/C, once relieved by the inhibition of the SAC, ubiquitinates two key substrates, namely cyclin B and securin, for proteasomal degradation allowing separase to cleave cohesin for chromosome segregation and anaphase progression (Fig 1). The APC/C requires a particular cofactor, CDC20, for degradation of cyclin B and securin in mitosis. This is in turn the target of the control exerted by the SAC on the APC/C [21].

On kinetochores lacking microtubule attachment, the actions of Aurora B kinase lead to the recruitment of monopolar spindle (MPS) 1 kinase to the kinetochore [22]. MPS1 kinase localization at kinetochores relies on binding to members of the outer kinetochore NDC80 complex [23] and is mutually exclusive with microtubule binding [24,25]. MPS1 activity is in turn responsible for the recruitment of several other key checkpoint components such as the MAD1:MAD2, BUB3:BUB1, and BUB3:BUBR1 complexes [21]. How unattached kinetochores that become decorated by checkpoint components can act as catalytic platform for the generation of a

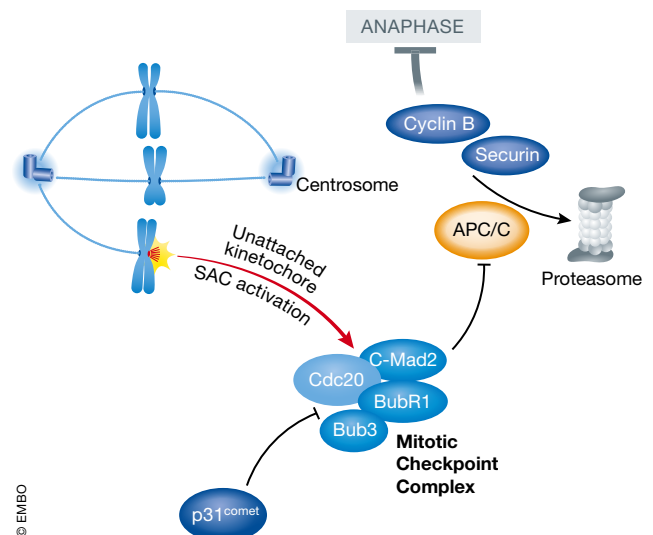


Figure 1. Anti-mitotic drugs activate the spindle assembly checkpoint (SAC).

Unattached kinetochores trigger the activation of the SAC, leading to inhibition of prometaphase to anaphase transition and mitosis by blocking the activity of the APC/C E3 ligase complex. The mitotic checkpoint complex (MCC) thereby inhibits CDC20 from aiding substrate recognition by the APC/C (e.g., cyclin B or securin, degraded for mitotic exit), thereby enhancing mitotic arrest. MCC function can be antagonized by p31^{comet} that can drive mitotic exit but seemingly also exerts alternative anti-apoptotic functions in cells arrested in mitosis.

diffusible inhibitor effectively targeting the APC/C throughout the whole cell has been a matter of intense investigations during the past 15 years. Central to our understanding of this phenomenon is the so-called MAD2 template model [26], relying on the observation that the SAC component MAD2 has two natively folded states, open O-MAD2 and closed C-MAD2 [27]. O-MAD2 is the unoccupied state where MAD2 may receive one of two binding partners, MAD1 or CDC20. The closed conformation is adopted when one of those two proteins is bound by MAD2 [28]. Critically, the protein can form asymmetric homodimers [29] in which a kinetochore associated and MAD1-bound C-MAD2 can recruit a second moiety of MAD2 in the open conformation [30,31]. The MAD1:C-MAD2 template can catalyze the conformational change of the O-MAD2 moiety, thereby facilitating the formation of a C-MAD2:CDC20 complex, that is then able to associate with BUB3/BUBR1, leading to a fully functional APC/C inhibitor, the MCC [32,33].

Intriguingly, while sequestration of CDC20 by the MCC plays a role in inhibiting the APC/C, the MCC-CDC20 complex can actually bind to the APC/C [34]. There are three main modes of actions currently proposed to explain how the MCC-CDC20 inhibits the action of the APC/C: (i) BubR1 was shown to act as pseudosubstrate for the APC/C without being degraded [35]; (ii) the MCC-CDC20 binds to the APC/C in a way that CDC20 is delocalized from its proper binding site and this then prevents the APC/C from recognizing its substrates [36]; and (iii) the APC/C-bound MCC can bind to free CDC20, negatively influencing its ability to activate the APC/C [37]. Regardless of the mechanistic details, CDC20, a co-activator of the APC/C when devoid of SAC proteins, becomes an APC/C inhibitor when bound to SAC proteins within the MCC.

After proper bipolar attachment of spindle microtubules to the kinetochores of all sister chromatids is established, the SAC is turned off so that the cell can progress in anaphase. There are several mechanisms that concur to effectively shut down the SAC: (i) The MAD1-C-MAD2 complex is stripped away from the kinetochores in a dynein-dependent fashion, therefore terminating the generation of new MCC complexes [38]; (ii) microtubule binding to the NDC80 complex dissociates MPS1 kinase from kinetochores, thereby inhibiting critical phosphorylation events that are required for the recruitment of SAC components to the kinetochore [24,25]; (iii) an important brake of the SAC is p31^{comet} which binds to the dimerization motif of C-MAD2 [39,40], which appears to play a major role in the disassembly of the MCC [41]. While p31^{comet} acts even during SAC signaling to regulate checkpoint homeostasis [42], the regulation of p31^{comet} activity when the SAC has to be silenced remains less clear; and (iv) finally, the actions of the phosphatases PP1 [43,44] and PP2A [45] at the kinetochore are required as well to efficiently terminate the SAC.

Anti-mitotic drugs and their potential biological effects

Anti-mitotic drugs such as vinca alkaloids and taxanes interfere with microtubule dynamics and therefore prevent the mitotic spindle from attaching amphitelicly to kinetochores [46]. This chronically activates the SAC, blocking the cells from progressing through mitosis. The EG5 kinesin inhibitors on the other hand prevent the segregation of the two centrosomes leading to a monopolar spindle and chronic SAC activation [47]. However, two aspects have to be kept

in mind to explain how cells can escape mitotic arrest before undergoing cell death. First, even when the SAC is maximally active, cyclin B is slowly degraded [48]. Once cyclin B levels drop beneath a critical threshold, the cell can exit mitosis, here, often into a polyploid interphase. This is referred to as mitotic slippage or mitotic checkpoint adaption [48]. The second issue is that while one unattached kinetochore is indeed sufficient to activate the SAC, the SAC does not work in a bimodal fashion but can exert varying signaling strength [49]. Hence, different mitotic inhibitors can activate the SAC to a different extent [49]. It therefore follows that intrinsic factors such as the levels of MAD2 [49] or p31^{comet} [50] influence the strength of the SAC and, by extension, the speed and rate of mitotic slippage without fully abolishing the actions of the SAC.

Intriguingly, different cell types studied for the long-term consequences of mitotic arrest by live-cell imaging often show a strikingly different behavior, that is, mitotic cell death vs. slippage and mitotic exit, preventing death in mitosis. Seminal work by the Taylor laboratory has led them to propose the competing network hypothesis that accounts for the differential behavior of cells in this setting [9,12]. In short, the idea behind the model is that not only the molecular machinery controlling cell division but also the apoptotic machinery is activated in mitosis and that mitotic arrest is intrinsically pro-apoptotic. Hence, when a cell gets trapped in mitosis, an apoptotic activity builds up, ultimately leading to activation of proteases of the caspase family and cell death, while on the other hand, mitotic exit allows escape from death, at least in the next G1-phase. As such, the gradual degradation of cyclin B by APC/C activity sets the time to mitotic exit that can differ between cell types but also between individual cells of the same cell line in a single experiment, generating often heterogeneous response patterns *in vitro*. When cells experience a drop of cyclin B levels below a critical threshold before mitochondrial integrity is perturbed, cells exit mitosis and reset the apoptotic clock back to baseline [9,12,51]. Not surprisingly, a series of cell death proteins are defined substrates of mitotic kinases and become heavily modified in mitosis but are then quickly de-phosphorylated again upon mitotic exit (see below). In support of the competing network model, overexpression of anti-apoptotic proteins, such as B-cell CLL/lymphoma 2 (BCL2), can prevent death of mitotically arrested cells long enough for cyclin B levels to drop below a threshold for slippage. Similarly, deletion or inhibition of key apoptotic effectors, including the BCL2 family members BAX/BAK1, or chemical inhibition of caspases induces similar effects [5,9,11,52,53]. On the other hand, preventing mitotic exit, for example, by depletion/deletion of CDC20, pharmacologic inhibition of the APC/C, or overexpression of (non-degradable) cyclin B, and arresting cells in M-phase with high efficiency ultimately result in apoptosis [9,53–56]. Extended mitotic arrest is also associated with increased autophagy rates that even seems to contribute to death to some degree and a metabolic switch to enhanced glycolysis for survival, but eventually, all cells die. This death is characterized by necrosis-like morphology when cells are unable to trigger apoptosis or exit mitosis in due time [53].

p53 activation in response to mitotic errors

Interestingly, delaying mitotic exit beyond a given threshold in time (about 90 min in RPE1 cells [8]) can promote a p53 response in

mitosis that is “carried over” into the next G1-phase [8]. This response involves p53-binding protein 1 (53BP1) and the de-ubiquitinating enzyme USP28 that counteracts MDM2, thereby promoting stabilization of p53 promoting cell cycle arrest in the next G1-phase [57–59]. In addition to extended mitotic duration itself, chromosome congression and segregation defects can also result in p53 stabilization [60,61]. Yet, whether the quantitative measurement of mitotic timing and sensing the fidelity of chromosome segregation are mechanistically coupled or independent pathways remains to be clarified. Irrespective of the activating mechanism, whether the p53 response to mitotic abnormalities initiates already in mitosis and contributes to cell death induced by microtubule-damaging agents remains unclear at present. Given the postulated absence of transcription in mitotically arrested cells, this appears unlikely. However, this dogma has been challenged lately [62]. Moreover, albeit not generally accepted, non-canonical direct cell death-promoting functions of p53 have been reported [63]. Certainly, this p53 activity can impact on cell fate in the next G1-phase when transcription is reinitiated, as several BCL2 family proteins, such as BAX, PUMA, or NOXA, are confirmed p53 targets [63]. It remains possible that these genes are bookmarked by p53 already in mitosis for low-level transcription and swift expression upon mitotic exit [62], defining post-mitotic cell fate (Fig 2).

Remarkably, however, in some settings, discussed in more detail below, increasing apoptotic thresholds in cells arrested in mitosis only delays cell death without resulting in increased mitotic slippage, suggesting that both thresholds are affected simultaneously. Moreover, caspase activation has been noted to repeatedly occur at low level in mitotic cells, leading to the activation of caspase-activate DNAase and DNA damage that needs to be corrected after mitotic exit as otherwise fostering genomic instability [7]. Finally, it has to be pointed out here that key apoptotic effectors, such as caspase-3 or caspase-7, have been repeatedly linked to impaired

proliferation or migration of several cell types in the blood, for example, by disabling p21 [64] or p27, by proteolysis [65]. Hence, not all caspase activation noted in mitosis must necessarily lead to apoptosis or genomic instability, but may ultimately contribute to proliferation or migration control by targeted proteolysis of selected substrates. Along similar lines, overexpression of BCL2 or loss of pro-apoptotic effectors, such as BIM, has been linked to impaired proliferation of lymphocytes upon mitogen stimulation [66]. Whether this phenomenon is due to impaired caspase-3 activation during M-phase downstream of mitochondrial outer membrane permeabilization (MOMP) in a fraction of mitochondria [7,67] is entirely unclear, as these initial observations rather indicate a problem with G1/S transition [68,69]. Yet, these problems may originate in the previous M-phase.

The fate of cells that actually escape mitotic arrest in the presence of microtubule poisons or targeted drugs by slippage is actually less well understood. Clearly, such cells are usually characterized by frequently failing cytokinesis and the presence of micronuclei that are a particular danger to genomic stability when the captured chromosomes or chromosome fragments re-integrated after replication into the main genome. This can happen randomly in a shotgun-like approach referred to as chromothripsis [70]. In order to avoid this, the p53 network usually becomes activated which ultimately can trigger the death of such micronuclei-bearing cells or promote their senescence [71]. Yet, the proficiency of this checkpoint seems to be variable and even cells carrying functional p53 may re-enter the cell cycle, depending on the mitogenic load and their histone methylation status.

How p53 becomes activated in tetraploid G1 cells was subject of intense investigations. Recent evidence from our own group suggests that caspase-2 is needed for this response and, by cleaving the E3 ligase, MDM2, usually degrading p53, at a conserved site, is stabilizing the transcription factor. Of note, this process is actually

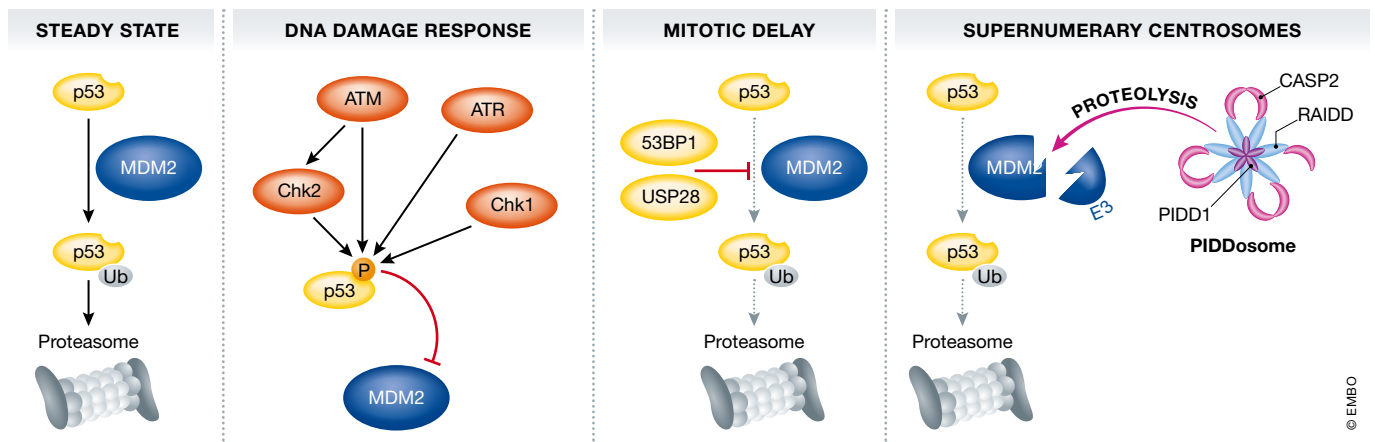


Figure 2. Multiple independent pathways can lead to p53 stabilization.

In steady state, cytoplasmic p53 levels are kept low by continuous MDM2-mediated ubiquitination and proteasomal degradation. Within the canonical DNA damage response, p53/MDM2 interactions are neutralized by phosphorylation of p53, abrogating MDM2 binding, that are executed by DDR kinases ATM, ATR, CHK2, and CHK1, depending on the type of DNA damage encountered. Cells spending an extended period of time in mitosis are also able to activate p53 for subsequent cell cycle arrest in the next G1-phase. There, MDM2 activity is antagonized by the activity of a de-ubiquitinating enzyme, USP28, all held together by the p53-binding protein 53BP1. Finally, upon cytokinesis failure, extra centrosomes present in such cells activate the PIDDosome multiprotein complex, comprised out of the p53-induced protein with a death domain (PIDD)1, a linker protein called RAIDD and a protease of the caspase family, that is, caspase-2. Upon activation in the PIDDosome, caspase-2 can process MDM2, removes its E3 ligase domain, and thereby promotes p53 stabilization.

driven by the extra centrosome in such cells and requires assembly of the PIDDosome multiprotein complex that drives caspase-2 activation [20,72]. In consequence, a unique p53 response is induced that is distinct from the one elicited by canonical DNA damage or extended mitotic arrest, as mentioned above [19]. It can be assumed that such cells again build up an apoptotic activity that, in part, may depend on proteins modified in and carried over from the previous M-phase [9,11]. What ultimately pushes the cell toward induction of apoptosis vs. cell cycle arrest and senescence under such conditions remains to be defined in full. Clearly, loss of p53, seen in many cancers, will ultimately preclude the activation of a set of cell death and cell cycle arrest regulators, facilitating the escape of such micronucleated tetraploid G1 cells, promoting the selection of more complex karyotypes culminating in aneuploidy tolerance, a major drive of tumor evolution (Fig 2).

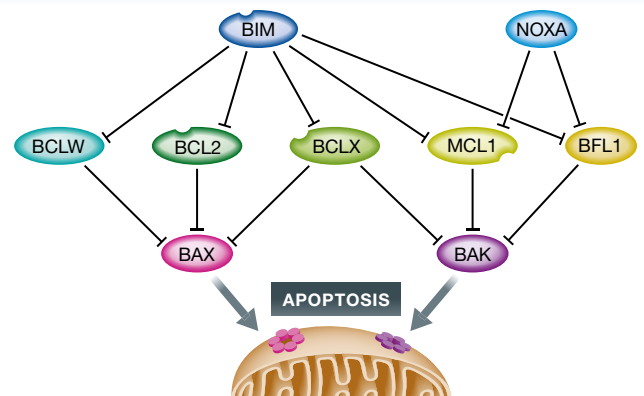
Molecular control of mitotic and post-mitotic cell death

As pointed out above, the death of mitotically arrested cells is controlled predominantly by the BCL2 family that comprises a set of anti-apoptotic molecules, including BCL2, BCLXL, MCL1, BCLW, and BFL1 (also known as A1), their antagonists of the “BH3-only” subgroup, such as BIM, BID, PUMA, NOXA, BAD, and BMF, as well as the central regulators of MOMP, BAX, and BAK1 (reviewed in [73,74]). Their activation can be fine-tuned by some BH3-only proteins directly, most notably BIM and BID, that facilitate insertion into and dimerization of BAX or BAK1 in the mitochondrial outer membrane (MOM). BAX or BAK1 homodimers ultimately oligomerize into higher order structures that permeate the mitochondrial outer membrane (MOM) forming a toroidal pore [75,76], allowing release of apoptogenic factors, such as cytochrome c, from the inner membrane space that control caspase activation for apoptotic cell death [74] (Fig 3A).

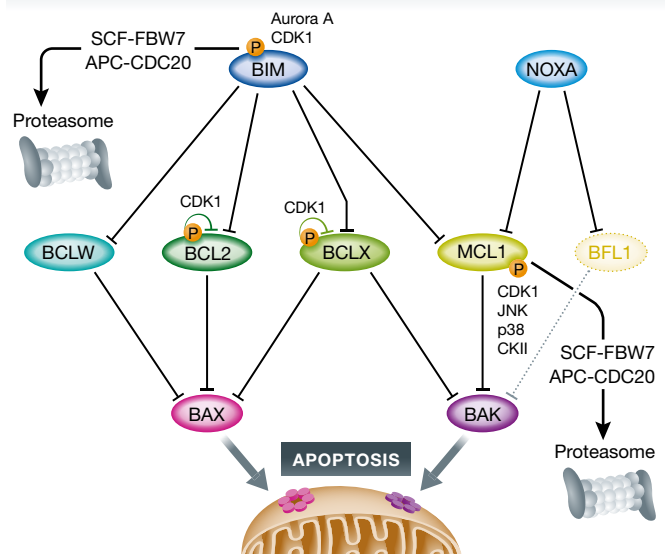
BCL2 family proteins have been studied early on in mitotic cell death and have been recognized as *bona fide* substrates of stress-induced and mitotic kinases (see below). Yet, early findings were often highly contradictory, leaving behind a blurry picture related to the functional relevance of these modifications (reviewed in [77]). From the helicopter view, it seems all clear. Deletion of BAX and BAK1 blocks mitotic cell death (Fig 3B) and promotes mitotic slippage, albeit delayed cell division accompanied by chromosome segregation errors can also be observed [52]. As apoptosis is

abrogated in the absence of BAX and BAK1, either event should be followed by cell cycle arrest and senescence in the next tetraploid G1-phase, at least in primary cells [78,79]. In the absence of p53, or a forced reduction in arrest proficiency by INK4a or p21 RNAi, the outcome becomes less clear and cells can continue to cycle and

A STEADY STATE



B MITOTIC ARREST



C PROLONGED MITOSIS | SUPERNUMERARY CENTROSOMES

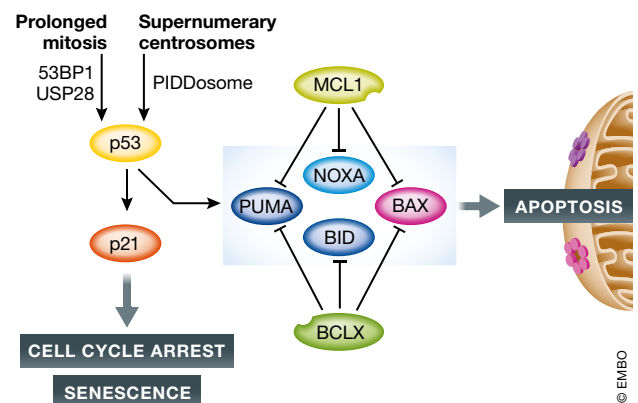


Figure 3. BCL2 family proteins implicated in the control of mitotic cell death or cell death after mitotic slippage.

(A) In healthy cells, a homeostatic equilibrium between cell death initiating BH3-only proteins (blue), anti-apoptotic BCL2 family proteins (green), and cell death executioners (pink/purple) is maintained. (B) Upon perturbation of this equilibrium, for example, by the action of anti-mitotic drugs and prolonged arrest in mitosis, a series of events, including phosphorylation on and proteasomal degradation of pro-survival BCL2 proteins, shifts the balance, favoring BAX/BAK1 activation. (C) Mitotic slippage or SAC adaptation can allow escape from mitotic cell death, yet newly initiated or carried over signaling cues impact on cell fate of such “post-mitotic” cells. This can culminate in the induction of cell death, again potentially involving p53 plus a set of BCL2 family proteins that may have become active or changed in quantity in the preceding and prolonged M-phase.

become aneuploidy [72,78]. Clearly, many such slipped tetraploid G1 cells do eventually die [10,11].

In contrast to mitotic death, the molecular basis of this subsequent death in G1-phase (Fig 3C) is poorly investigated and hard to dissect experimentally from death signals that may have been already initiated in the previous extended M-phase, yet kill the cell only after mitotic exit. A possible means to address this is to compare the cell death machinery recruited by mitotic blockers with that of mitotic drivers used in combination with selective inhibitors of the BCL2 family. Such an analysis has been done and supports the idea that certain BCL2 proteins are critical for survival upon mitotic exit, in particular MCL1 and BCLX, at least in colorectal cancer cell lines [10,11]. Moreover, anti-apoptotic BCL2 proteins were reported to be heavily modified and inhibited by post-translational modifications (Table 1) or promoted for degradation by the proteasome, lowering the apoptotic threshold [77]. Based on the minimalistic, *C. elegans*-derived model of BCL2-regulated apoptosis, supported in part also by mammalian cell line studies, inhibiting or removing all BCL2-like pro-survival proteins in a given cell can suffice to promote BAX activation and apoptosis [80]. This, however, was raising the question whether BH3-only proteins are actually needed to promote or fine-tune apoptosis under conditions of extended mitotic arrest. An important note on the side here is that the trigger used to arrest cells in mitosis, for example, microtubule-damaging agents or targeted anti-mitotic drugs, does not seem to grossly affect the molecular mode of cell death execution upon extended mitotic arrest.

A number of more recent studies came to similar conclusions regarding the initiation phase of mitotic cell death, that is, a subgroup of BH3-only proteins is involved in activating BAX and BAK1 in mitotically arrested cells. The Taylor group conducted a genome-wide RNAi screen defining candidates that promote cell death in mitosis [11]. Next to a group of genes controlling mitotic entry, including cyclin B, CDK1, and EMI1, they also noted a selection against MYC. In follow-up analyses, they found evidence that the ability of MYC to increase levels of certain BH3-only proteins, BIM, BID, and NOXA, was critical to determine the fate of mitotically arrested cells and that MYC-driven repression of BCLX favored apoptosis in mitosis (Fig 3B). Consistently, chemical inhibition of BCLX sensitized cells with low MYC levels toward mitotic as well as post-mitotic cell death in different epithelial cancer cells [11]. The sensitizing effect of BCLX inhibition for mitotic cell death has been noted before by the Mitchison group using RNAi and a broad-spectrum BCL2 inhibitor, ABT-263, that also blocks BCLX and BCLW [81]. RNAi against BCL2 or BCLW had minor sensitizing effects in some cells (e.g., U2OS), but showed no effect in others (e.g., A549). BCLW is an understudied BCL2 pro-survival homologue that is found frequently expressed in epithelial cancer cells and that may act redundantly with BCLX. Silencing by RNAi was shown to accelerate mitotic cell death also in HeLa cells [82], a phenomenon also seen by Shi and colleagues [81].

Of note, in another RNAi-based screening approach for regulators of mitotic checkpoint adaptation and slippage, the Yu laboratory concluded a critical role for NOXA in cell death, but the authors did not find a significant contribution of BIM [50], that has been implicated in this process before by others [83,84]. We conducted an RNAi-based mini-screen in HeLa cells to address the question whether and which BH3-only proteins may be involved in mitotic

cell death and identified BIM and NOXA as potential modulators of BAX and BAK1 activation in this setting [52]. Together with our analysis of post-translational modifications on BCL2, BCLX, and MCL1, as well as MCL1 protein turnover, we propose here an adopted M-death model to explain the role of the BCL2 family network in deleting cells arrested for extended periods of time in mitosis (Fig 3B). Our model finds clear support by the findings of the Taylor, Mitchison, and Yu laboratories (discussed in detail below). We propose that CDK1-mediated phosphorylation of BCL2 and BCLX potentially serves to lower the threshold for apoptosis induction to a certain degree by changing relative binding affinities while degradation of MCL1 acts as the actual molecular timer. MCL1 degradation is driven by NOXA (see below) that liberates BIM from sequestration by MCL1 to take out BCLX (and potentially BCLW; not tested by us), promoting death in mitosis. The contribution of BID to this process may be cell type-dependent [85] or become critical only upon mitotic exit [86], as may be the contribution of other BH3-only proteins not scoring in any of the RNAi screens conducted but able to act in a redundant manner with BIM, most notably PUMA [87] or BMF [88]. Finally, similar to BCLX, MCL1 levels appear to be particularly important for cells which survive mitotic arrest by slippage as its depletion significantly increases cell death in such cells [54,86]. Similarly, cells with low-level expression of MCL1, such as DLD1 colon cancer cells, or cells where MCL1 levels were reduced by RNAi are highly sensitive to BCLX inhibitors when combined with mitotic drivers, such as the Aurora B inhibitor ZM447439 [10,86].

What remains obscure is that most studies actually failed to detect a significant contribution of BCL2 for the survival of M-arrested cells, as neither RNAi-mediated ablation nor chemical inhibition using ABT-199 sensitized epithelial cancer cells efficiently to death in mitosis [81,82,89] while overexpression of BCL2 clearly protects [9,52,90]. One possible explanation may be that BCL2 binds poorly to BAK1, and this may be the preferred executor of mitotic cell death, as it will be liberated and activated upon MCL1 degradation, restrained then only via BCLX in this setting. This may also explain the sensitizing effects of WEHI-539, selectively targeting BCLX, toward anti-mitotic drugs. On the other hand, ABT-199 is most effective in leukemia which is more often BCL2-dependent, such as B-cell-derived chronic lymphocytic leukemia (CLL) [91] and some types of acute myeloid leukemias [92]. Sensitizing effects of RNAi-mediated ablation or ABT-199 treatment have been reported in K-562 and Jurkat leukemia cell lines treated with vinca alkaloids [93]. Hence, the relative dependence on BCLX vs. BCL2 for survival in mitotic arrest seems to be cell type-dependent and co-defined by the set of BH3-only antagonists expressed or activated under these conditions.

Alternative modes of cell death are well established. So far, the evidence that death receptors and its ligands, such as FAS/FASL, TNFR1/TNF, or TRAILR/TRAIL, all members of tumor necrosis factor receptor/ligand superfamily that depend on caspase-8 to mediate apoptosis or promote cell survival do play a role here is limited. However, non-apoptotic functions of caspase-8, its antagonist, c-FLIP, and their adapter, FADD, have been reported to regulate proliferation rates upon mitogenic stimulation of splenocytes and in hepatocytes [94,95]. If anything, these receptor/ligand pairs may be engaged to induce or contribute to inflammatory signaling under conditions of mitotic arrest or upon slippage in tetraploid

Table 1. Post-translational modifications of BCL2-family members

Protein	Residue modified	Kinase/PPase	Reported effects	Function	Reference No.
Anti-apoptotic proteins					
BCL2	Undefined	Undefined	Phosphorylation noted during mitosis and taxol-induced growth arrest; not involved in protein degradation	Unknown/not tested	[101]
	T69, S70, S87	JNK, ASK1	Observed during cell cycle progression to inactivate BCL2 at G2/M at S70; monitoring the fidelity of chromosome segregation	Pro-apoptotic	[102]
	All	PP1 phosphatase	Observed during slippage; suggested to reactivate the anti-apoptotic function of BCL2	Pro-survival	[107]
	T56	CDK1/cyclin B	Phosphorylated during mitosis; found in nuclear structures in prophase; at later mitotic stages, P-BCL2 localizes on mitotic chromosomes; relied on use of one particular antibody	Unknown/not tested	[178]
	Undefined	CDK1/cyclin B	During mitosis transient and incomplete phosphorylation of BCL2; sustained activity of CDK1 during mitotic arrest leads to increase in phosphorylation at multiple S/T sites; inactivation of anti-apoptotic function; prevents association with BAX/BAK	Pro-apoptotic	[104,113,179]
	S70	CDK1/cyclin B	Higher affinity to bind BAK1 and BIM in mitosis in cell-free conditions; enhanced protection against apoptosis induced by chemotherapeutic drugs	Pro-survival	[93]
	T56, S70	CDK1/cyclin B	Prometaphase arrest-dependent; protein still localized in mitochondrial fraction irrespective of their phosphorylation; abrogates anti-apoptotic function	Pro-apoptotic	[103]
BCLX	S62	JNK	Taxol triggers phosphorylation during G2/M; increased apoptosis	Pro-apoptotic	[180]
	S62	CDK1/cyclin B	Phosphorylation after vinblastine treatment; phosphorylation weakens interaction with BAX; phospho-defective BCLX mutant retained ability to bind	Pro-apoptotic	[134]
	S62	CDK1/cyclin B	During mitosis transient and incomplete phosphorylation of BCLX; sustained activity of CDK1 during mitotic arrest leads to increase in phosphorylation at multiple S/T sites; inactivation of anti-apoptotic function; prevents association with BAX/BAK	Pro-apoptotic	[113]
	S62, S49	PLK3	S62-modified BCLX may interact with APC/C during mitotic checkpoint; S49 modified version seen during S- and G2-phase but disappears in early mitosis—reappears during telophase; destabilizes G2-arrest and slows cytokinesis; accumulates at centrosomes in G2 after DNA damage; retains anti-apoptotic effect	Unknown/not tested	[114]
	S62	PLK1/JNK2	Phosphorylated during normal cell cycle but accumulates in G2 after DNA damage; during that arrest phosphorylation at S62 promotes BCLX accumulation in nucleoli -> meets CDK1 and traps CDK1 to avoid timely entry into mitosis	Unknown/not tested	[181]
	S62	CDK1/cyclin B	Loss of BCLX function is the major driver of mitotic death; phosphorylation and degradation of MCL1 and phosphorylation of BCLX are both critical	Pro-apoptotic	[179]
	Undefined	CDK1/cyclin B	CDK1/cyclin B is responsible for the strong phosphorylation of BCLX and BCL2 upon prometaphase arrest; only phosphorylation of BCLX activates intrinsic apoptosis	Pro-apoptotic	[108]

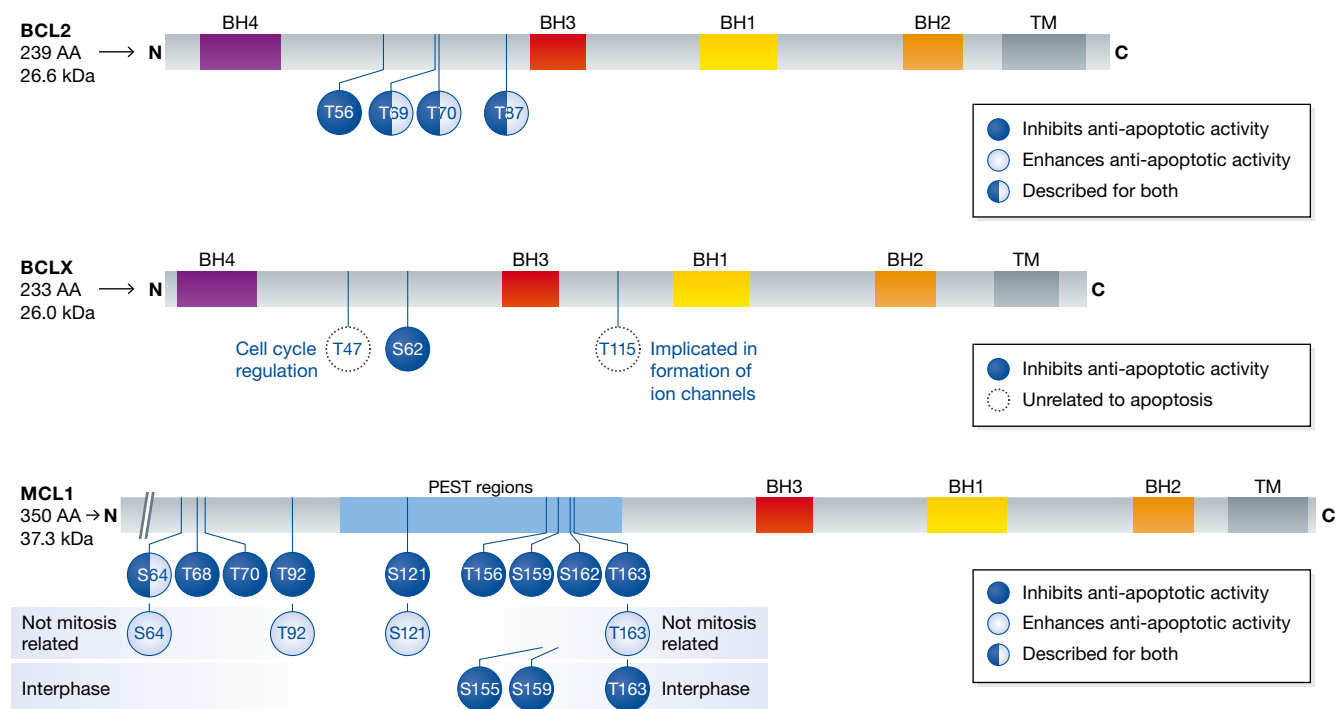
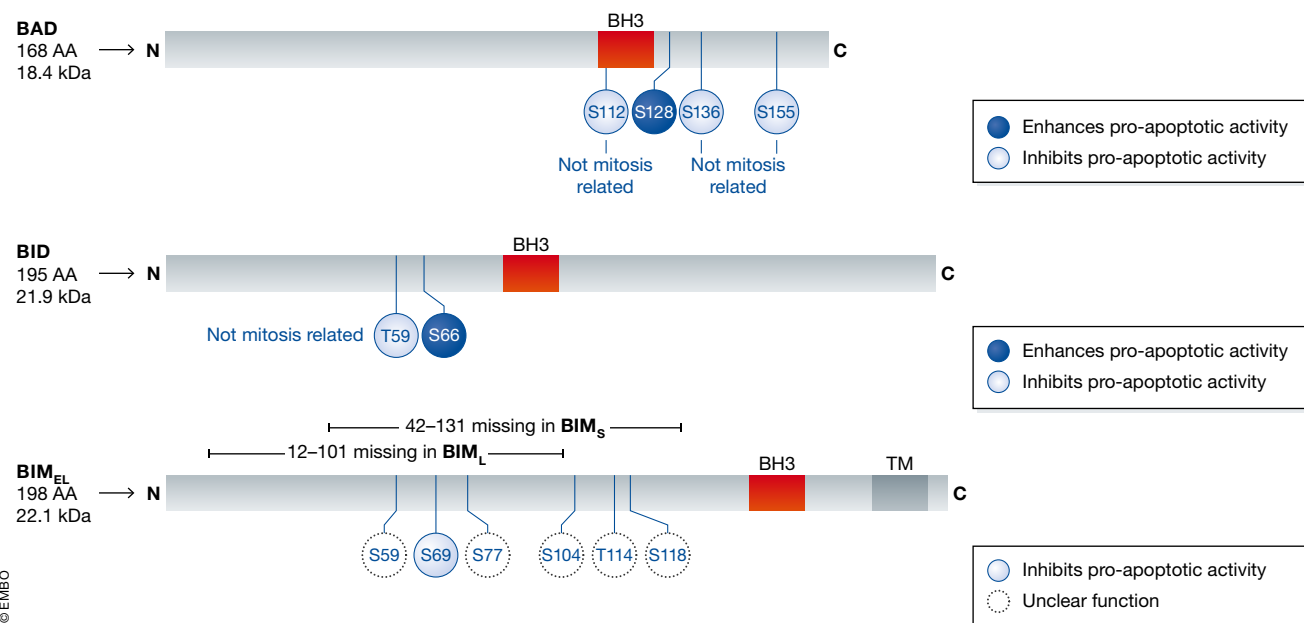
Table 1. (continued)

Protein	Residue modified	Kinase/PPase	Reported effects	Function	Reference No.
	S62	Not defined	Observed during mitotic arrest; decreases affinity of BCLX for BAX -> impaired protection from mitotic cell death	Pro-apoptotic	[89]
MCL1	S64	CDK1/2 or JNK	Observed during mitosis; enhances binding to BAK1, NOXA, and BIM; enhancing pro-survival function; does not influence protein stability	Pro-survival	[182]
	T92 + S64	CDK1/cyclin B	Seen during mitotic arrest; signal for APC/CDC20 to ubiquitinate MCL1 -> proteasomal degradation; S64 also phosphorylated in interphase but mutation of this site does not stabilize MCL1	Pro-apoptotic	[117]
	S64, T92, S121, S159, T163	CDK1/cyclin B, p38, CKII, and JNK	T92 targeted by CDK1/cyclin B -> allows further phosphorylation by other kinases, leading to the recruitment of SCF/FBW7 for MCL1 degradation; phosphorylation on T159 and S163 reduces stability	Unknown/not tested	[118]
	T92	CDK1/cyclin B	Seen during mitosis, induces proteasomal degradation; release of bound BAK for apoptosis	Pro-apoptotic	[121]
	S64, T68, T70, T92, S121, T156, S159, S162, T163	For novel sites: GSK3, CDK1, AMPK	CDK1-mediated phosphorylation at T92 is dispensable for mitotic arrest-induced MCL1 phosphorylation and degradation; mutation of five putative CDK1 sites to alanine cannot prevent degradation of MCL1	Pro-apoptotic	[122]
Pro-apoptotic proteins					
BAX	S184	AKT/PKB	Inhibits apoptosis, sequestration of BAX in the cytoplasm, heterodimerizes with MCL1, BCLX, or BFL1	Pro-survival	[136]
BAK	T108	BMX	Inhibition of autoactivation	Pro-survival	[137]
BIM	S69	ERK1/2	ERK-mediated -> induces ubiquitination and SCF/ β TrCP1-dependent degradation during mitotic arrest	Pro-survival	[139]
	S55, S65, S73, S100, T112, S114		Mutating all sites abrogates phosphorylation of BIM during mitosis; CDK1 but not ERK1/2 or ERK5 phosphorylates BIMEL during mitosis	Unknown/not tested	[143]
	S93, S94, S98	PP2A phosphatase	Reverses phosphorylation by Aurora A, de-phosphorylation stabilizes BIMEL after mitotic exit	Pro-apoptotic	[140]
BID	S66	CDK1/cyclin B	Phosphorylated as cells enter mitosis, phosphorylation lost during transition from meta- to anaphase; sensitizes mitochondria for MOMP during mitotic arrest	Pro-apoptotic	[85]
BAD	S128	Undefined	Enhances the apoptotic activity during mitosis; dominant negative mutant fails to prevent taxol-induced apoptosis	Pro-apoptotic	[151]

G1 cells that may become senescent [71,78,79]. Similarly, necroptosis, a form of cell death engaged downstream of these receptors when caspase-8 is blocked [96], has not been linked to mitotic or post-mitotic cell death so far. Caspase-2, on the other hand, has been implicated in mitotic catastrophe a long time ago [97], as well as in the execution of cell death induced by anti-mitotic drugs [98]. Yet, while it does not significantly contribute to mitotic cell death [72,99], it eventually may be involved in the initiation of post-mitotic cell death via mechanisms that remain to be defined in full. This response might involve the BH3-only protein BID [13,85] that can be processed and activated by caspase-2 [100].

Post-translational modifications of pro-survival BCL2 family proteins and their relevance for mitotic cell death and cell death after slippage

The founding member of the family, BCL2, was described early on to be phosphorylated during mitotic arrest [101]. Multiple phosphorylation sites were identified, including T69, S70 and S87 [102], T56 [103], and possibly also T74 [93], all located in an unstructured loop of the protein between alpha 1 (BH4 domain) and alpha 2 (BH3 domain). Phos-tag gel analysis actually suggests that these phosphorylation events are quantitative as even exogenously overexpressed BCL2 gets fully phosphorylated in M-arrested HeLa cells [52]. The

A ANTI-APOPTOTIC PROTEINS**B PRO-APOPTOTIC PROTEINS****Figure 4. BCL2 family proteins targeted by phosphorylation in mitosis.**

The BCL2 protein family controls cell death upon extended mitotic arrest. Multiple phosphorylation events of pro- and anti-apoptotic family members have been found in mitosis. Not all of them have been functionally characterized, but generally, it is believed that phosphorylation on pro-survival proteins reduces their function, while the same type of modification promotes the death function of pro-apoptotic BH3-only proteins. A detailed list of reported phosphorylation events in and out of mitosis, their potential impact on function, the kinases and phosphatases involved, and the related reference can be extracted from Table 1.

exact role of these phosphorylation events remains controversial however (Fig 4A, Table 1). Through the use of point mutations of the various residues, generating either phospho-defective alanine

mutations or phospho-mimetic glutamate mutations, some studies have concluded that phosphorylation of BCL2 is inhibiting its pro-survival function [52,102,104], while others reported on enhanced

anti-apoptotic function [68,105]. One report actually suggests that a conformational change in the normally unstructured loop region rather than the inserted charge affects BCL2 structure and behavior. Mutagenesis at position S70, usually targeted by CDK1 for phosphorylation, increased the binding affinity of BCL2 for BAK1 and BIM in surface plasmon resonance analyses and increased cell death protection in leukemia cells regardless of whether a negatively charged or aliphatic amino acid was introduced [93].

Another possible effect investigated was BCL2 protein stability after its phosphorylation when MCF7 cells were challenged with paclitaxel [106]. Non-phosphorylated BCL2 showed reduced half-life, though we and others did not note degradation of BCL2 during mitotic arrest [52,90]. The kinase most frequently implicated in modifying BCL2 is CDK1 as it was able to phosphorylate BCL2 in cells [93] and the CDK inhibitor roscovitine was able to prevent this [103,105]. After escaping mitotic arrest by slippage BCL2 is rapidly de-phosphorylated by PP1 phosphatase [107]. Even though BCL2 overexpression can protect cancer cells effectively from mitotic death [52,90], combinatorial treatment of anti-mitotic drugs with inhibitors selectively targeting individual BCL2 family proteins suggests that, at least in epithelial cancer cells, BCLX is more critical than BCL2 for survival during and possibly after slipping from extended mitotic arrest [81,89,93,108]. In conclusion, the physiological relevance of BCL2 phosphorylation for cell death initiation in this context remains uncertain. It is tempting to speculate that some of these phosphorylation events on T69, S70, and S87, reported to abrogate also interaction with the autophagy regulator Beclin1 in response to starvation [109,110], may serve to facilitate the activation of cell death by alternative means. Studies by Domenech and colleagues actually suggest that this initially protective and energy-restoring response can contribute to cell death during extended mitotic arrest [53]. Regardless of the ultimate truth, it seems fair to say that phosphorylation of BCL2 in mitosis does not substantially modulate its core survival function or interaction pattern with other BCL2 family proteins, but may act to fine-tune the apoptotic threshold by controlling affinities of established interaction partners.

Like BCL2, BCLX is readily detectable as being phosphorylated in a highly quantitative manner during mitotic arrest and similar to BCL2, remains highly stable [111]. It was shown that the higher molecular weight complexes containing BCLX found in normal cells in cross-linking experiments are readily lost after treatment with microtubule-targeting agents [112]. The only mitotic phosphorylation site characterized in BCLX so far, on S62, again contained in the unstructured loop of the protein between helices alpha 1 and alpha 2, is also targeted by CDK1/cyclin B [113]. The effect of this phosphorylation has been shown to inhibit the anti-apoptotic potential of BCLX [89,113]. Numerous alternative phospho-sites in BCLX have been described, such as phosphorylation on Ser49, targeted by polo-like kinase (PLK)3, but this modification is not detectable prior telophase [114]. Yet, these phospho-sites appear to be critical for cell cycle fidelity, as overexpression of site mutants negatively affects chromosomal stability in diploid fibroblasts [115]. The consequences of BCLX phosphorylation on complex formation with either BAX/BAK1 or BH3-only proteins, however, are poorly understood (Fig 4A, Table 1).

Without doubt, MCL1 seems to be the most crucial survival factor within the BCL2 family, as suggested by multiple studies investigating consequences of its loss by genetic means (reviewed in

[116]). Again, MCL1 has been shown to be subjected to intense phosphorylation events during mitotic arrest (Fig 4A, Table 1), but in stark difference to BCL2 and BCLX, this phosphorylation has been reported as the prerequisite for the degradation of MCL1 in mitosis. The first report on this topic demonstrated phosphorylation on S64 and T92 by CDK1/cyclin B, with mainly T92 leading to the degradation of MCL1 in the proteasome after ubiquitination by the APC/C-CDC20 E3-ligase complex [117]. Later, researchers at Genentech defined the mitotic phosphorylation events on S64, S121, S159, and T163, with only the latter two influencing the stability of MCL1 [118]. The publication identified JNK1, p38, CKII, and CDK1 as kinases involved in phosphorylation and the SCF-FBW7 complex as the executing E3 ubiquitin ligase degrading MCL1 in mitosis and as a key factor determining sensitivity to anti-mitotic drugs in cancer. At the same time, another group found that two of the sites, S159 and T163, can be phosphorylated by GSK3, which targets MCL1 for SCF-FBW7-dependent degradation [119]. However, this was described in asynchronous, cycling cells without any mitotic inhibitors. Consistent with these reports, tumors that have lost FBW7 function were shown to be more resistant to anti-mitotic agents and showed poorer prognosis [118,119].

JNK-driven phosphorylation [120] and CDK1-driven phosphorylation [121] were also found by other groups to be important for the de-stabilization of MCL1 in mitotically arrested cells. The role of the reported phosphorylation sites, however, has been put into questions lately. A recent study interrogated all previously defined mitotic phospho-sites on MCL1 in mitosis, including three newly defined ones coming to a surprising conclusion: The pan phospho-defective alanine mutant version of MCL1, carrying 9 alanine substitutions, was degraded as rapidly as the wild-type version of the protein during mitotic arrest [122]. Additionally, the E3 ubiquitin ligases described to degrade MCL1 in mitosis were equally challenged in a recent study [54] as the authors were unable to stabilize MCL1 with either the APC/C-CDC20 inhibitors apcin and proTAME nor knockdown of SCF-FBW7. Together, these data suggest that MCL1 turnover is executed by different E3 ligases in a highly redundant manner and that phosphorylation events have little impact on MCL1 protein stability in mitosis in most cells. Along these lines, it is worth mentioning that an “all KR” mutant of MCL1 is degraded as effectively as wild-type MCL1 in cells after cycloheximide treatment [123], suggesting either a role for N-terminal ubiquitination, acting as a backup, or alternative routes of protein degradation, for example, directly in the 20S-proteasome or, potentially, in lysosomes.

Independently of E3 ligases and phosphorylation events, it was shown that the BH3-only protein NOXA promotes the degradation of MCL1 in mitosis and thereby defines the sensitivity of epithelial cancer cells to anti-mitotic drugs [52]. Similarly, the Yu laboratory identified NOXA in an RNAi-based screen for factors controlling SAC proficiency, linking it also to MCL1 inhibition and as a target of the pro-survival role of p31comet, controlling SAC duration [50]. It is worth mentioning that NOXA levels accumulate most in G2 and drop again in M and upon mitotic exit in HeLa cells [52]. Yet, in M-arrested cells, NOXA gets co-degraded with MCL1 and its ablation by RNAi leads to increased MCL1 stability and prolonged survival [50,52]. Of note, also a KR mutant version of NOXA appears to be degraded with similar kinetics than wild-type protein [124]. As NOXA and MCL1 are only degraded effectively when complexed with each other [125], all these studies interrogating the individual proteins

may have failed because none of them investigated the half-life of KR versions of both proteins complexed together in one cell, or because of NOXA as an intrinsically unstructured protein, dragging the complex directly into the 20S proteasome.

Surprisingly, under conditions of NOXA RNAi, BIM levels increase and it is tempting to speculate that this is due to sequestration by MCL1 and/or BCLX. Accordingly, simultaneous knockdown of BIM and NOXA prolongs survival of mitotically arrested cells, while inhibition of BCLX promotes death in mitosis [10,11,81]. Clearly, NOXA controls MCL1 stability, and consistent with a timer function of MCL1 in mitosis, miRNAs that affect MCL1 protein levels, such as miR29b and miR101, were shown to modulate sensitivity to microtubule-targeting agents [126,127]. Unexpectedly, however, simultaneous depletion of NOXA and BIM did not only affect time to death upon mitotic arrest but also increased the mitotic duration until slippage [52]. This finding implicates a certain cross talk between the two competing networks.

BCLW, mainly known for its essential role in spermatogenesis [116], has been neglected in most studies investigating mitotic cell death. However, BCLW can play a role in setting the apoptotic threshold in HeLa or U2OS cells arrested in mitosis as siRNA against or knockout of BCLW shortens the duration until mitotic death [81,82]. No post-translational modifications have been reported on BCLW in mitosis so far. It was shown that BCLW is a direct target of miR107 and that this miRNA is downregulated in a taxol-resistant A549 derivative when compared to the parental cell line [128]. Yet, clearly, this effect of miRNA repression may involve de-repression of alternative targets, next to BCLW. Lastly, similar to BCLW, BFL1/A1 is still understudied, when compared to the other pro-survival BCL2 family members mentioned so far. While protective roles from multiple apoptotic stimuli activating mitochondrial cell death were reported when BFL1 is overexpressed, nothing is known about its role in mitotic cell death, nor have PTMs been analyzed in that context. Clearly, BFL1 has a short half-life affected by ubiquitination [129,130]. However, it might be an attractive target in some types of blood cancer, such as diffuse large B-cell lymphoma (DLBCL) or myeloid malignancies as well as melanoma skin cancer, as these tumors appear to frequently use aberrant expression of BFL1 to secure survival [131]. Notably, it was shown that BFL1 knockdown, in contrast to BCL2 inhibition, sensitizes a B lymphoblastoid IM9 tumor cell line, expressing low levels of MCL1, to vincristine [132]. Furthermore, all-trans retinoic acid (ATRA) protects NB4 acute promyelocytic leukemia cells from taxol-mediated cell death by upregulating BFL1 [133] suggesting a role in mitotic cell death in some cell types.

Post-translational modifications of pro-apoptotic BCL2 family proteins and their relevance for mitotic cell death and cell death after slippage

BAX and BAK1 are redundant executors of MOMP, and hence, treatment of cells with mitotic inhibitors eventually culminates in their activation and oligomerization prior cell death [112,134]. Accordingly, BAX and BAK1 deficiency or depletion results in a significant survival benefit during mitotic arrest and promotes a strong slippage phenotype. In some cellular systems, BAK1 seems to be playing a

dominant role [52,134,135], consistent with its preferential interaction with MCL1, when compared to BAX [74]. Little information is out there if BAX or BAK1 are undergoing PTMs in mitosis and if such modifications modulate their activity during mitotic arrest. Clearly, survival kinases such as AKT can phosphorylate BAX on S184 to restrain its pro-death function [136] and phosphorylation on T108 of BAK1, elicited, for example, by the tyrosine kinase BMX, antagonized by PTPN5 phosphatase, has been shown to negatively regulate BAK1 oligomerization activity [137,138]. Whether any of these or other residues are modified in mitosis is unknown. It is also unknown whether BAX/BAK1 DKO cells that escape mitotic arrest by slippage eventually die by non-apoptotic cell death, become genomically unstable or simply undergo senescence.

While BAX/BAK1 is essential for mitochondrial apoptosis despite being functionally redundant (Fig 3A), the large group of pro-apoptotic BH3-only proteins are usually highly redundant and none of them is essential for apoptosis, nor is any combination tested genetically so far (reviewed in [73]). BIM is the most thoroughly investigated BH3-only protein, including during mitotic arrest (Fig 4B, Table 1). What remains undisputed is that BIM is heavily phosphorylated during mitotic arrest. It was shown that the residues S69 [139] as well as S94 and S98 are phosphorylated by Aurora A kinase with the latter two residues leading to ubiquitination and SCF- β TrCP1-dependent degradation during mitotic arrest [140]. Counterbalancing this is the action of the phosphatase PP2A since its inhibitor, okadaic acid, or siRNA against this phosphatase drastically increases the phosphorylation and de-stabilizes BIM [140]. The Aurora A-dependent degradation of BIM by SCF- β TrCP1 is somewhat at odds with a report documenting direct binding of BIM to the APC/CDC20 complex in late mitosis, targeting it for proteasomal degradation [141]. Again, chemical inhibition of the APC or CDC20 RNAi leads to an accumulation of BIM that was identified to carry two destruction (D)-BOX motifs in position 72–87 [142]. The binding between BIM and APC/CDC20 was shown to be counteracted by the phosphorylation of BIM on S83, most likely mediated by PKA. This notion is at odds with the observation that the mutation of six different residues (S55, S65, S73, S100, T112, and S114), considered to be targets of CDK1/cyclin B1 in mitosis, abrogated the mobility shift of mitotic BIM protein seen in SDS-PAGE [143]. Moreover, while there is supportive evidence for a cell death executing role of BIM in cells challenged with mitotic inhibitors [52,84,144], some studies failed to recapitulate these findings [135,139]. These differences may often be due to alternative experimental design or knockdown efficiency in RNAi experiments. Along that lines, the transcription factor FOXO3—controlling *BIM* transcription—was shown to mediate sensitivity to long-term taxol treatment in MCF-7 breast cancer cells [144]. As transcription supposedly stops in mitosis, it remains plausible that BIM levels in post-mitotic G1 cells that may have escaped mitotic arrest are controlled by FOXO3 that may bookmark the gene locus already in M-phase and thereby control overall drug sensitivity. Furthermore, ERK—while probably not responsible for the mitotic phosphorylation of BIM [143]—can confer resistance to low doses of microtubule-targeting agents. ERK inhibition together with low dosage of MTAs caused dramatic BIM-dependent mitotic death [145]. This, however, may be explained by increased baseline levels of BIM, due to ERK inhibition that may sensitize HT1080 cells to taxol treatment in mitosis and potentially other cell death triggers. Together, these studies document that BIM

is setting a threshold for cell death in mitosis and that this threshold is fine-tuned by BIM phosphorylation that can happen before or upon mitotic arrest (Fig 4B, Table 1).

As discussed in part already above, NOXA is a critical player in the execution of mitotic cell death as its depletion by siRNA is able to give a significant survival benefit upon extended mitotic arrest [50] and profoundly impairs the mitotic degradation of MCL1 [52]. This potentially leads to reduced BIM activity, as it accumulates in NOXA RNAi cells. As BIM can interact with comparable affinity with all BCL2 pro-survival homologues, it is most likely then bound by excess MCL1, free BCL2, BCLX, or BCLW, depending on the cell type-dependent makeup. How NOXA protein levels are regulated during cell cycle progression is unclear, as is the question whether it gets post-translationally modified before or during mitotic entry. CDK5-mediated phosphorylation of NOXA has been reported to control its localization and the cellular response to glucose deprivation [146]. Of note, inhibiting glycolysis using inhibitors of PFKFB3 is speeding up mitotic cell death in combination with taxol [53]. Clearly, NOXA levels are highest in G2-phase, before getting co-degraded with MCL1 in mitosis. Together with BIM, NOXA promotes caspase activation and double deficiency or combined targeting by RNAi increases the time to death in mitotically arrested cells [11,52]. Yet, ultimately, these cells do die in mitosis, as the rate of slippage was not significantly affected, suggesting additional redundancies or compensatory activation of other BH3-only proteins. Whether and how de-ubiquitinating enzymes reported to regulate stability of BIM [147], NOXA [148], or MCL1 [149] affect mitotic cell fate remains to be formally tested.

Genetic analysis in mice has demonstrated beyond doubt that BH3-only proteins usually act in a redundant manner [73], and hence, it is not surprising that other members of this subgroup may contribute in one or the other cell type or experimental setting. Of note, the BH3-only protein BID, usually considered to link extrinsic to intrinsic apoptosis pathways upon caspase-8-mediated proteolysis [150], appears to undergo an activating phosphorylation event during mitotic arrest at S66 by a yet to be identified kinase (Fig 4B, Table 1) in the absence of caspase-mediated processing, promoting its mitochondrial localization [85]. Furthermore, depletion of BID could protect cells from the toxic effects of mitotic inhibitors [85] but this effect was not seen by others [52] and hence may be an either cell type-specific effect or affecting cell death after slippage [13,86]. Finally, the BH3-only protein BAD can become phosphorylated during mitotic arrest at S128 in a CDK1- and JNK-dependent manner (Fig 4B, Table 1), but the relevance for mitotic cell death remains unclear [105,151]. Usually, phosphorylation of BAD at other residues, such as S112, S136, or S155, is inhibitory and mediated upon growth factor or cytokine addition via the kinases PKA or PKB/AKT [152]. S128 phosphorylation, however, appears to interfere with these inhibitory phosphorylation events and reduces binding of BAD to 14-3-3 proteins that retain them in the cytoplasm [151]. Little is known about the remaining members of the BH3-only subgroup. One study suggests that the BH3-only protein BMF may promote BIM-mediated breast cancer cell death in response to taxol, yet it is unclear whether this is happening during mitotic arrest or upon slippage [90]. As BMF has a more restricted expression pattern as BIM, its contribution may be cell type-dependent [88]. No meaningful information is currently available for the ER-stress engaged BH3-only protein BIK/NBK

[153] in mitotic cell death and HRK, preferentially found expressed in post-mitotic neurons [154].

While the role of BH3-only proteins in mitotic cell death has been investigated to a reasonable degree, their role in cell death upon slippage is less clear. As indicated above, BID might be relevant for post-mitotic death upon SAC override using the MPS1 kinase inhibitor reversine in p53-deficient cells as a substrate for caspase-2 [13]. Yet, BID cleavage can also be observed in colon cancer cell lines proficient for p53 where slippage has been induced by Aurora B kinase inhibition, rendering them highly dependent on BCLX for survival. These cells are further characterized by an accumulation of NOXA, PUMA, and/or BIK. Knockdown of NOXA by RNAi clearly protected these cells from cell death upon slippage in the presence a BCLX inhibitor, as did knockdown of BAX, while RNAi against the other BH3-only proteins induced was not protective [86].

Linking mitotic errors to inflammation and immunity

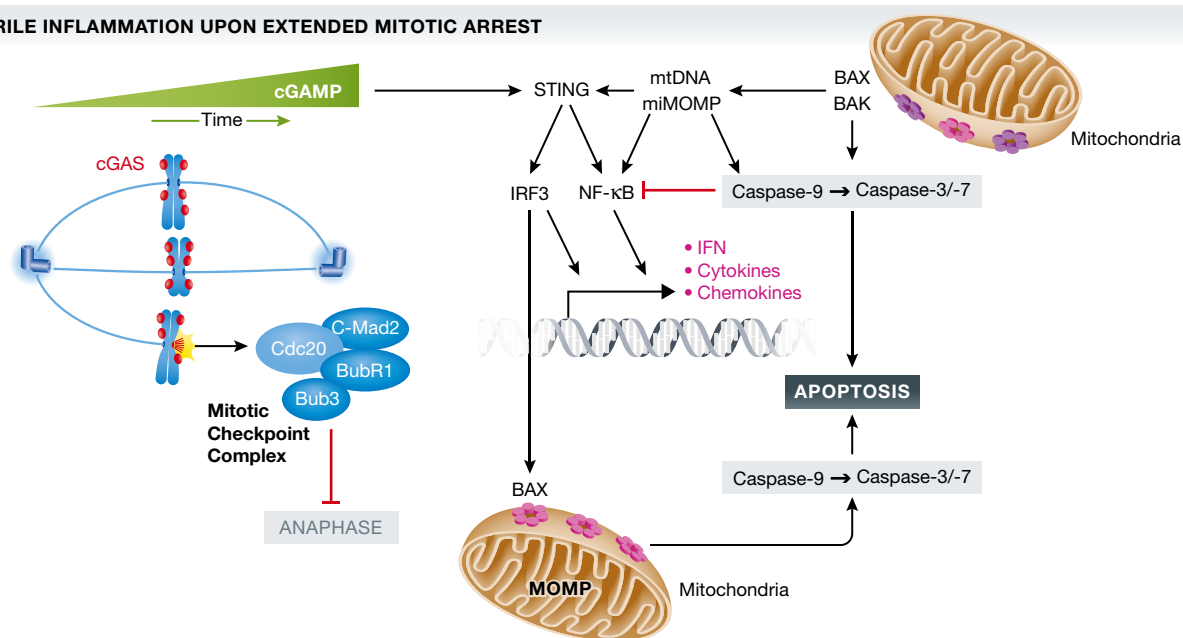
DNA damage can arise in response to chromosome segregation errors in various ways. For example, physical constriction of lagging chromosomes in the cleavage furrow during cytokinesis elicits a canonical DNA damage response in daughter cells inheriting damaged chromosomes or chromosome fragments [155]. Alternatively, DNA damage arises within micronuclei after whole-chromosome missegregation during asynchronous replication and subsequent chromosome fragmentation [70]. It is well-appreciated that DNA damage can cause activation of NF- κ B signaling triggering a subsequent inflammatory response leading to cytokine and chemokine release [156]. These cytokines can alert the immune system that aids the efficacy of anti-cancer therapy. In contrast to pathogen-driven inflammatory cytokine-mediated activation of NF- κ B, this sterile inflammatory response appears to occur with delayed and often bimodal kinetics but is mechanistically poorly understood. Early studies suggest involvement of RIPK1-regulated signal transduction as well as a need for ATM-kinase-mediated phosphorylation of NEMO/IKK γ and PARP1 activity [157–159]. Moreover, PIDD1, discussed above in the context of p53 activation, has been suggested to act as a missing link connecting DNA damage with RIPK1 and NEMO-driven NF- κ B activation [160,161]. Yet, how damaged DNA is actually sensed to trigger this inflammatory pathway remained largely unclear.

Nucleic acids, including double-stranded (ds) DNA of foreign or self-origin, have been recognized as pathogen- or danger-associated molecular patterns (PAMP/DAMP), respectively, and different sensory systems have developed during evolution to alert the immune system in their presence. Toll-like receptor (TLR)9 [162] as well as cytoplasmic cGAS [163] can respond to lysosomal or cytoplasmic dsDNA, respectively, inducing NF- κ B and interferon signaling. Cytoplasmic dsDNA elicits a subsequent interferon response by engaging ER-resident STING via the cGAS-synthesized second messenger, the cyclic dinucleotide cGAMP, to promote TBK1-dependent phosphorylation and activation of the transcription factor IRF3 [163]. Keeping this in mind, one may actually wonder why the cGAS/STING pathway sensing cytosolic DNA is not activated automatically during mitotic cell division when DNA is present in large amounts and unshielded in the cytoplasm. Even more intriguingly, cGAS can actually bind to nucleosome-bound DNA yet

appears less active in this context [preprint: 164]. While this phenomenon may be due to inhibitory signals preventing full activation of cGAS that remain to be discovered in normal mitosis, it has been recognized that cGAS indeed becomes activated in response to DNA-damaging cues, including irradiation, but also after nocodazole treatment, usually arresting cells in mitosis [18,165]. Remarkably, the activation of this pathway required mitotic traverse and was linked to the formation of micronuclei, as a consequence of these insults (Fig 5A). Moreover, even sterile means to increase micronuclei formation, such as genetic alterations in RNase H2, associated with autoimmunity in humans, favoring formation of

chromosome bridges, promoted a cGAS/STING-driven inflammatory response [165,166]. This was nicely corroborated by single-cell RNAseq analysis of cells with or without micronuclei [165]. Importantly, the nuclear envelope forming around micronuclei is notoriously unstable and prone to disassemble due to lamin breakdown, leading to early recruitment of cGAS. This interaction allows synthesis of the second messenger cGAMP and the induction of inflammatory genes in a STING-dependent manner [163]. It is intriguing to note that STING is an ER-resident protein and that breakdown of nuclear membranes surrounding micronuclei allows chromatin to become invaded by underlying ER membranes [167], bringing all

A STERILE INFLAMMATION UPON EXTENDED MITOTIC ARREST



B STERILE INFLAMMATION UPON MITOTIC EXIT

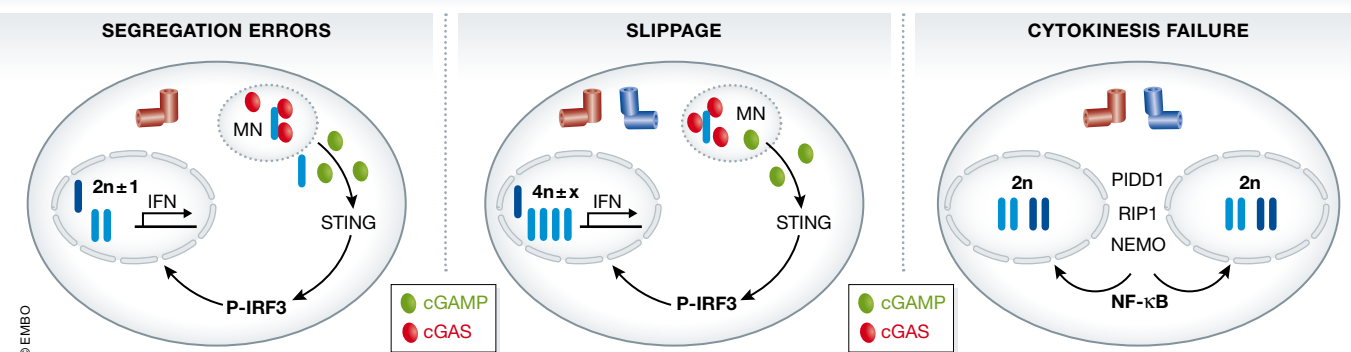


Figure 5. Linking mitotic arrest and slippage to inflammation and immunity.

(A) The dsDNA immune sensor cGAS, found associated with chromatin in mitosis, can synthesize a second messenger cyclic AMP/GMP from ATP and GTP that activates an ER-resident signaling molecule, stimulator of interferon genes (STING). STING can trigger the activation of transcription factors, including the interferon response factor (IRF3) and NF-κB, leading to the production type I interferons, IFN, as well as a set of inflammatory cytokines and chemokines to alert the immune system and neighboring cells. A non-transcriptional cell death activating function of IRF3 at mitochondria may contribute to mitotic cell death directly. Moreover, mitochondrial outer membrane permeabilization in a minority of mitochondria (miMOMP), frequently seen during mitotic arrest, could lead to NF-κB-driven inflammation when caspase activation is impaired while released mitochondrial (mt)DNA could lead to STING-dependent IFN production. (B) Errors in mitosis that lead to micronucleation in the next G1-phase, such as chromosome missegregation or slippage, alert the immune system via recruiting the cGAS/STING signaling pathway, described above. cGAS enters micronuclei upon lamin breakdown, binds to nucleosomal chromatin, and produces the second messenger cGAMP for STING activation and IFN signaling. Cytokinesis failure that does not lead to micronucleation, yet tetraploidy, may engage pro-inflammatory signaling via the activation of the so-called NEMO-PIDDosome complex, leading to NF-κB activation.

components needed for signaling into close proximity. Together, these recent studies show how genomic instability is linked to the immune system and explain why cancer cells that are often prone to micronucleation, for example, due to p53 loss, select against expression of components of this signaling axis, that is, in order to avoid sterile inflammation and immune cell activation (Fig 5A).

What does all this have to do with mitotic cell death? Not much, possibly post-mitotic death at best, or post-mitotic induction of senescence and the associated senescence-associated secretory phenotype (SASP) [168–170]. The latter may ultimately lead to the activation of death receptor signaling. Yet, one recent report on bioRxiv actually provides evidence that upon extended mitotic arrest, cGAS can be detected along mitotic chromosomes and that blocking cGAS/STING signaling delays mitotic death upon taxol treatment when slippage is blocked by APC/C inhibition [preprint: 164]. Intriguingly, cell death appears to involve a non-transcriptional activity of IRF3 that has previously been reported in the context of infection with dsRNA viruses, that is, to be able to directly activate BAX via protein–protein interaction at the mitochondrial outer membrane [171]. Of note, IRF3 contains a structural motif that closely resembles a BH3 domain and that may facilitate BAX oligomerization, in a way similar to certain effector BH3-only proteins [172]. Clearly, pro-death effects of cGAS/STING agonists have been reported meanwhile in many different settings, including T [173]- and B-cell leukemia [174]. Yet, the molecular mode of cell death remains to be fully defined (see Box 1).

Implications for cancer therapy

One is tempted to speculate that drugs targeting mitotic kinases, microtubule motors, or the APC/C may have failed in the clinic so far, because they kill cells in mitosis too effectively. Classical microtubule-targeting agents, on the other hand, are only moderate killers promoting slippage and micronuclei formation around lagging chromosomes, thereby leading to treatment-associated inflammation in the next G1-phase. It remains to be noted once more that clinically achieved doses of microtubule-targeting agents are ineffective in killing cancer cells in mitosis *in vitro*, but promote subsequent multipolar cell divisions, chromosome missegregation, and micronuclei formation [15]. *In vivo*, this may amplify inflammatory response, triggering strong anti-tumor immune responses affecting the efficacy of anti-mitotic drugs, but also the effectiveness of immunotherapy, as shown most recently [18]. While early on during treatment, CIN and subsequent micronucleation may be beneficial for treatment responses because of its pro-inflammatory nature (Fig 5B), aneuploid karyotypes may be selected that eventually allow tumor cell survival and immune escape. Again, such a phenomenon has already been documented in melanoma patients subjected to immunotherapy. In a retrospective analysis of clinical trials, Davoli and colleagues noted an inverse correlation between the degree of aneuploidy, tumor immune cell infiltration, and treatment response of melanoma patients [175]. Consistently, hyperploid cancer cells, prone to become aneuploidy more easily, show an ER-stress response due to proteotoxic stress and are eventually cleared by the immune system [176].

While it has become clear that mitotic cell death is controlled by the BCL2 family and that blocking individual BCL2 pro-survival factors using BH3 mimetics can actually increase the efficacy of anti-mitotic drugs, it remains to be seen whether clinical benefit can be

Box 1: In need of answers

- (i) What is the contribution of cell death to the clinical efficacy of anti-mitotic drugs?
- (ii) Are phosphorylation events detected on different BCL2 proteins during prolonged mitosis relevant for therapy efficacy?
- (iii) Is apoptosis the only type of cell death induced in response to anti-mitotic drugs?
- (iv) Do the cell death modalities and/or mediators differ in mitosis vs. death upon mitotic slippage?
- (v) What determines if p53 activation in response to mitotic errors triggers cell death vs. cell cycle arrest/senescence?
- (vi) Can prolonged mitotic arrest lead to inflammation and what prevents cGAS activation during normal mitosis?
- (vii) Do extra centrosomes represent a *bona fide* danger signal that can drive inflammation?

improved. In light of the above, one could also envision that any strategy that increases mitotic cell death will eventually fail to increase therapy success, as only apoptotic death that is already poorly immunogenic *per se* will be enhanced, while curtailing secondary inflammatory events. As such, one could also postulate that drugs that promote cell death in the next G1-phase in cells that escape mitotic arrest, either as micronucleated tetraploids or as aneuploidy diploids, might be clinically more successful. Therefore, mitotic drivers, such as MPS1 or Aurora B inhibitors that foster segregation errors, and, indirectly, subsequent micronuclei-driven inflammation seem interesting alternatives to be combined with BH3 mimetics and immune checkpoint blockade for more effective anti-cancer therapy. Alternatively, any means that would convert non-inflammatory apoptosis into a more immunogenic type of cell death might do the trick and improve treatment success. This idea finds support in recent observations documenting that inhibiting caspases after MOMP improves the effects of anti-cancer therapeutics by promoting an TNF-driven inflammatory response, activated by proteins released from the mitochondrial inner membrane space, neutralizing inhibitor of apoptosis proteins (IAPs) by promoting their degradation. This leads to NF- κ B-dependent inflammation, M1 macrophage polarization, and T-cell-dependent anti-tumor immunity [177].

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Conflict of interest

The authors declare that they have no conflict of interest.

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