Trends in Cell Biology

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Review

Sublethal engagement of apoptotic pathways in residual cancer

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Cytotoxic chemo-, radio-, and targeted therapies frequently elicit apoptotic cancer cell death. Mitochondrial outer membrane permeabilization (MOMP) is a critical, regulated step in this apoptotic pathway. The residual cancer cells that survive treatment serve as the seeds of eventual relapse and are often functionally characterized by their transient tolerance of multiple therapeutic treatments. New studies suggest that, in these cells, a sublethal degree of MOMP, reflective of incomplete apoptotic commitment, is widely observed. Here, we review recent evidence that this sublethal MOMP drives the aggressive features of residual cancer cells while templating a host of unique vulnerabilities, highlighting how failed apoptosis may counterintuitively enable new therapeutic strategies to target residual disease (RD).

Highlights

Mitochondrial outer membrane permeabilization (MOMP) is no longer considered an exclusively lethal event. Incomplete MOMP and minority MOMP are biologically important exceptions to the all-or-nothing viewpoint. These examples of sublethal MOMP provide insight into the true complexity of apoptosis regulation.

Sublethal MOMP generates the drug-tolerant persister (DTP) phenotype through both caspase-independent and caspase-dependent signaling pathways. Cytochrome *c* is the primary factor triggering these cellular changes by activating the integrated stress response (ISR) and the caspase cascade.

Sublethal MOMP offers therapeutic opportunities in the DTP state. Direct targeting of the regulators of the sublethal MOMP, ISR, or DNA damage response pathways may prevent cells from entering the persister state. Downstream of sublethal MOMP, acquired cellular dependencies, such as ferroptosis, emerge and can be therapeutically targeted.

Sublethal apoptotic cell death contributes to cancer therapy resistance

Cancer therapeutics including chemotherapy [1–3], radiotherapy [4], and targeted therapy [5,6] often utilize apoptosis as an effector pathway to eliminate tumor cells. Although these cytotoxic agents result in impressive initial clinical responses, the emergence of therapy resistance is a significant complication that prevents cancer patients from achieving cures from their disease [7]. Therapy resistance is known to occur through diverse genetic and non-genetic mechanisms, which include the selection of pre-existing genetic mutations in the population [8], tumor microenvironment (TME) interactions [9], and cell state plasticity [10]. Recently it was discovered that cells can survive partial engagement of apoptotic cell death [11–15] and that sublethal apoptosis is a novel mechanism of resistance to cytotoxic therapy [16,17]. In this review we define the molecular mechanisms of sublethal apoptosis and discuss how it directly and indirectly contributes to a therapy-resistant state. We then highlight the therapeutic opportunities that sublethal apoptosis creates, which may inform mechanism-based treatment strategies to overcome drug resistance.

Understanding sublethal MOMP

An introduction to MOMP

Apoptosis is a fundamental biological process that eliminates undesirable or aberrant cells through a dedicated cell death mechanism [18]. In normal physiology, apoptotic cell death maintains tissue homeostasis and development [19,20]. Various disease states, including cancer, are known to modify the ability of cells to die [21,22]. Apoptosis occurs through two primary mechanisms: the mitochondrial (or intrinsic) pathway and the death receptor (or extrinsic) pathway [23]. Intrinsic apoptosis is triggered by diverse cellular stressors, including reactive oxygen species (ROS), growth factor deprivation, DNA damage, microtubule disruption, endoplasmic reticulum (ER) stress, and others, resulting in **MOMP** (see Glossary) [22]. Another pathway of apoptosis occurs due to the ligation of the so-called death receptors (e.g., Fas/CD95, TRAIL receptors, TNFR1) of the TNF receptor superfamily. In some vertebrate cells, this extrinsic apoptotic pathway also requires MOMP for cell death to occur [24].

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MOMP is caused and tightly regulated by the BCL-2 protein family, which are identified by the presence of one to four BCL-2 homology (BH) domains [25]. The BCL-2 family includes pro- and antiapoptotic proteins. When activated, the BCL-2 effector proteins (e.g., BAX, BAK) form pores or openings in the outer mitochondrial membrane (OMM), resulting in MOMP. The antiapoptotic BCL-2 proteins (e.g., BCL-2, BCL-xL, MCL-1) prevent this effector-mediated MOMP.

A third class Is the BH3-only proteins (so named because they contain only this domain), which function to regulate the other two classes, either by inhibiting the antiapoptotic proteins ('sensitizers') or by directly activating the effectors ('activators').

Antiapoptotic BCL-2 family proteins contain all four BH domains and exert their antagonistic effects through the sequestration of proapoptotic activators and the direct binding of active BAX and BAK to prevent pore formation in the OMM [26].

On MOMP, proteins of the mitochondrial intermembrane space (IMS), are released to the cytosol. These include cytochrome *c*, which triggers the activation of caspase proteases responsible for the features of apoptosis [27]. Even without caspase activation, extensive MOMP can doom a cell to die due to a general failure of mitochondrial function [28].

Cellular determinants of MOMP

The relative cellular activities of proapoptotic versus antiapoptotic BCL-2 family proteins determine the baseline susceptibility of a cell to the triggering of MOMP following exposure to apoptotic stimuli [29]. Cells are classified across a continuous spectrum of 'apoptotic priming' based on their propensity to successfully engage MOMP on treatment with apoptotic stimuli [30]. For example, cells that rapidly release cytochrome *c* from mitochondria on treatment with apoptotic stimuli are considered highly 'primed' for apoptosis. The degree to which a cell is primed for MOMP is correlated with clinical responsiveness to cytotoxic therapies across cancer types [1–4,31]. An emerging body of research has revealed that apoptotic priming is dictated by both cellular features (mitochondrial size and shape, oncogenic mutations, cell lineage of origin, differentiation status, and metabolic state) and imposed conditions, such as prior exposure to drug therapies and TME interactions, which together determine the cell's propensity to undergo MOMP in response to treatment [32–34].

Mechanisms of sublethal MOMP

MOMP was initially thought to be an all-or-nothing event [35]. Early studies found that if a cell reached its apoptotic threshold, MOMP would be 'widespread and irreversible', releasing IMS contents from every mitochondrion rapidly and completely. It was thus believed that MOMP was often a lethal event. Nonetheless, biologically significant exceptions to the all-or-nothing viewpoint were discovered. Specific post-mitotic cell types, including oligodendrocytes, cardiomyocytes, and neurons, can survive MOMP through intrinsic resistance mechanisms such as heightened ability to retain mitochondrial membrane potential following cytochrome *c* release [36,37]. Caspase-3 and downstream CAD activity were even found to be required for myoblast differentiation, which suggests a potential developmental role for **sublethal MOMP** [38,39].

It was discovered that if only some mitochondria in a cell undergo MOMP, the effect can be sublethal [12,14,15]. Two forms of sublethal MOMP are incomplete MOMP and minority MOMP, defined by the extent of MOMP (Figure 1). Incomplete MOMP is the process in which most mitochondria undergo MOMP following exposure to an apoptotic stimulus but the cell retains a fraction of undisturbed mitochondria. If caspase activity is inhibited, cells can sometimes survive

Glossary

Drug-tolerant persister (DTP): a population of cells that survive cytotoxic stress events through non-mutational mechanisms and are thought to contribute to the clinical phenomenon of MRD.
Integrated stress response (ISR): a signaling pathway that is activated following cellular stress events that include viral replication, heme deprivation, ER stress, and nutrient depletion. ATF4 is one of the major transcription factors translated following ISR activation and promotes the expression of a stress-response gene program to protect the cell.
Minimal residual disease (MRD): the minority fraction of cancer cells that survive in the body during and after a patient achieves clinical remission.
Mitochondrial outer membrane permeabilization (MOMP): a defining step in the intrinsic apoptosis pathway that is characterized by pores forming in the OMM allowing the diffusion of mitochondrial IMS proteins, including cytochrome *c*, into the cytosol, which triggers caspase activation and protein substrate cleavage.
Sublethal MOMP: engagement of MOMP in a manner that is not fatal to the cell.

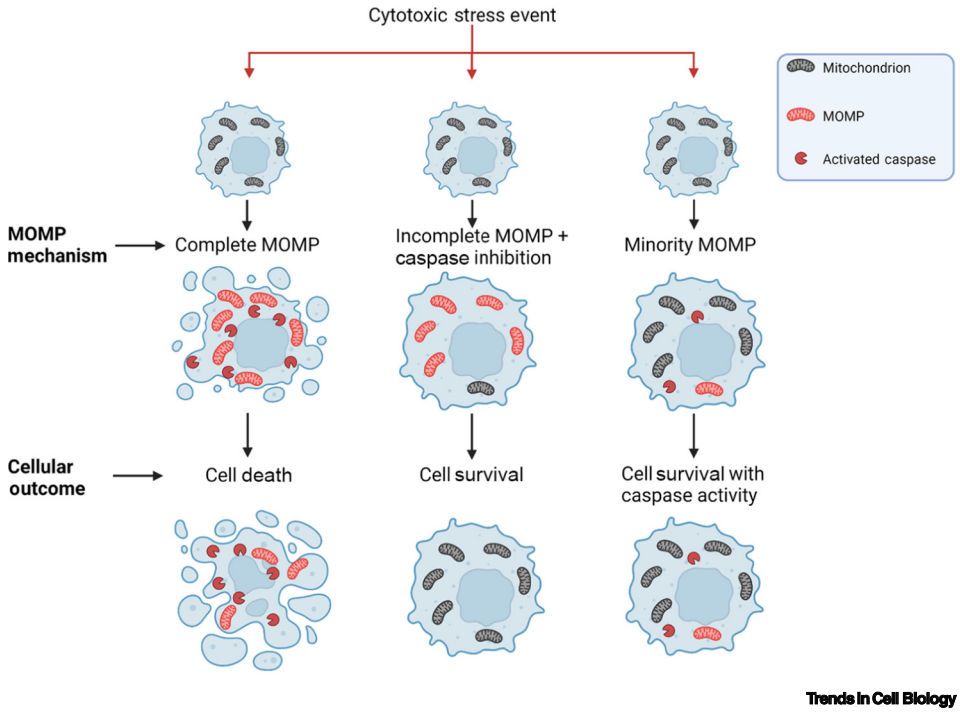

Figure 1. Molecular mechanisms of sublethal mitochondrial outer membrane permeabilization (MOMP). Extrinsic or intrinsic cytotoxic stressors to a cell can cause various mechanisms of MOMP. Complete MOMP occurs when there is widespread and irreversible MOMP across the mitochondrial network. In the case of complete MOMP, the cell demonstrates the hallmark features of apoptosis, including caspase activation, membrane blebbing, and nuclear fragmentation before death. Incomplete MOMP is defined by many, but not all, mitochondria in MOMP following a cytotoxic event and is promoted by caspase inhibition. The classical markers of apoptosis are absent in incomplete MOMP as caspases are necessary for their activation. Minority MOMP is when a small fraction of the mitochondria activate MOMP following a cytotoxic insult. A sublethal amount of caspase activity is found in minority MOMP. Incomplete MOMP and minority MOMP are not lethal to the cell and are classified as sublethal MOMP mechanisms.

incomplete MOMP through repopulation with residual, unprimed mitochondria, especially in cells that retain GAPDH expression [14,15]. Minority MOMP, by contrast, occurs when only a minority fraction of a cell's mitochondria undergo MOMP following exposure to an apoptotic stimulus [12]. Minority MOMP leads to the release of cytochrome *c* and other apoptotic IMS factors, but in quantities small enough to not necessarily be fatal to the cell. Sublethal release of cytochrome *c* following minority MOMP was found to activate caspases and caspase-dependent DNases (CADs), resulting in DNA damage and genomic instability [12,13]. Similarly, sublethal caspase activation in response to death receptor signaling has been shown to induce CAD-dependent DNA damage and genomic instability [40,41].

Mitochondrial dynamics regulate the extent of MOMP by modulating the distribution of antiapoptotic BCL-2 proteins across the mitochondria [42]. Mitochondrial fusion promotes complete MOMP by providing a homogeneous distribution of apoptotic effector BCL-2 proteins across the mitochondrial network. By contrast, mitochondrial fission promotes sublethal MOMP by producing fragmented mitochondria with uneven BCL-2 protein representation [42].

Anastasis ('rising to life' [11,43]) is a phenomenon in which cells survive and even reverse the effects of caspase activation, including apoptotic blebbing and phosphatidylserine exposure, following the removal of some cytotoxic stressor [44,45]. It is defined by demonstrable, sublethal

caspase activation, and therefore the term can be applied to at least some cases of sublethal MOMP. However, as caspase activation can occur independent of MOMP, it follows that anastasis can occur independent of sublethal MOMP.

Although this review focuses primarily on sublethal engagement of apoptosis, other modes of regulated cell death (e.g., necroptosis, pyroptosis, ferroptosis) might also occur at nonlethal levels [46]. The phenotypic ramifications of nonlethal activation of these alternative modes of cell death in cancer are an area of biology requiring further scientific investigation.

Sublethal MOMP is observed in drug-tolerant persister (DTP) cells

An introduction to DTPs

Minimal RD (MRD) describes the reservoir of tumor cells that fail to undergo drug-induced cell death in a patient with clinical remission. These cells represent the seeds of eventual relapse [47]. While MRD can occur due to the selection of a pre-existing, genetically resistant population of cells, sequencing studies have shown that non-genetic mechanisms of tumor cell survival in MRD occur [48–51]. Cell-autonomous non-genetic survival involves the entry of tumor cells into a **DTP** state, named based on a related phenomenon in antibiotic-tolerant (persister) bacterial cells [52,53].

The DTP state provides a non-mutational mechanism for cells to survive normally lethal events and is associated with diverse epigenetic, transcriptional, and metabolic reprogramming processes [48]. Characteristics of DTPs include reduced replication, hypermutability, altered cellular metabolism, multidrug resistance, and epithelial-to-mesenchymal transition (EMT), and these characteristics are found in DTPs across cancers lineages and drug classes [48]. All of the cardinal features of DTPs are reversible once drug treatment is withdrawn. Barcoding experiments have supported a stochastic model of DTP formation, in which any given cell in a tumor population may have an equal potential to enter the DTP state [49–51], but the stochastic model of DTP formation may be context specific, as pre-existing populations have been identified to give rise to dormant drug-tolerant states [54]. The mechanisms underlying the formation of DTPs remain to be fully elucidated and may be specific to both drug class and cancer lineage [54]. Recent work revealed that persisters that re-enter the cell cycle and begin to proliferate are derived from a distinct lineage compared with non-cycling persister populations [51]. Cycling persisters have a unique metabolic profile that includes increased antioxidant defense genes and a metabolic switch to fatty acid oxidation [51]. Induction of the DTP state following chemotherapy treatment resulted in a transcriptional program resembling the survival program diapause, which is a reversible state of suspended embryological development following a stress event [49,50]. Another transient feature of DTPs is their increased vulnerability to agents that inhibit the lipid peroxidase GPX4, resulting in cell death by ferroptosis [48,55,56]. DTP cells downregulate the reducing cofactors NADPH and glutathione (GSH), which is to likely to contribute to this vulnerability [55].

From this perspective, pharmacological targeting of DTP cells may provide therapeutic opportunities to prevent relapse by eliminating the pool of MRD tumor cells [57], underscoring the vital importance of understanding the biological drivers and vulnerabilities of the DTP state.

Identification of sublethal MOMP in DTPs

The fact that DTP cells derived from different tumor lineages and drug treatments exhibit a remarkable convergence of cardinal phenotypic properties suggests that these properties may have a common origin. Given that the vast majority of cancer therapies lead to engagement of the apoptotic pathway, and that DTP cells are defined by their transient resistance to drug-induced cell death, it is reasonable to imagine that sublethal MOMP could be a conserved feature

of these cells. Two separate reports recently observed sublethal MOMP in DTP cancer cells and provided evidence that it drives key phenotypic properties and therapeutic vulnerabilities of these cells [16,17]. In the first, lung cancer cells treated with a combination of BH3 mimetic drugs targeting BCL-2, BCL-xL, and MCL-1 (using the BCL-2 and BCL-xL inhibitor ABT-737 plus the MCL-1 inhibitor S63845) were found to induce a persister phenotype as defined by reduced cell cycling, reversible chemoresistance, sensitization to ferroptosis, an EMT gene signature, and increased metastatic potential [16]. The persister phenotype in these cells depends on BCL-2 effector proteins, as cells lacking the BCL-2 effector proteins did not demonstrate persister properties following BH3-mimetic treatment [16]. Single-cell RNA-seq analysis of these cells revealed activation of the **integrated stress response (ISR)** as a reversible feature of the persister state [16]. Sublethal MOMP was found to enable the release of cytochrome *c* into the cytosol, where it directly binds to and activates heme-regulated inhibitor (HRI) kinase to initiate the downstream translation of activating transcription factor 4 (ATF4) (Figure 2). Cytochrome *c*, HRI, and ATF4 were all necessary for the DTP phenotype in this setting, and this signaling axis was independent of caspase activation [16]. Finally, engagement of the ISR was found to be sufficient to produce the persister phenotype independent of sublethal MOMP. In the second report, cellular models of non-small cell lung cancer (NSCLC), melanoma, acute myeloid leukemia

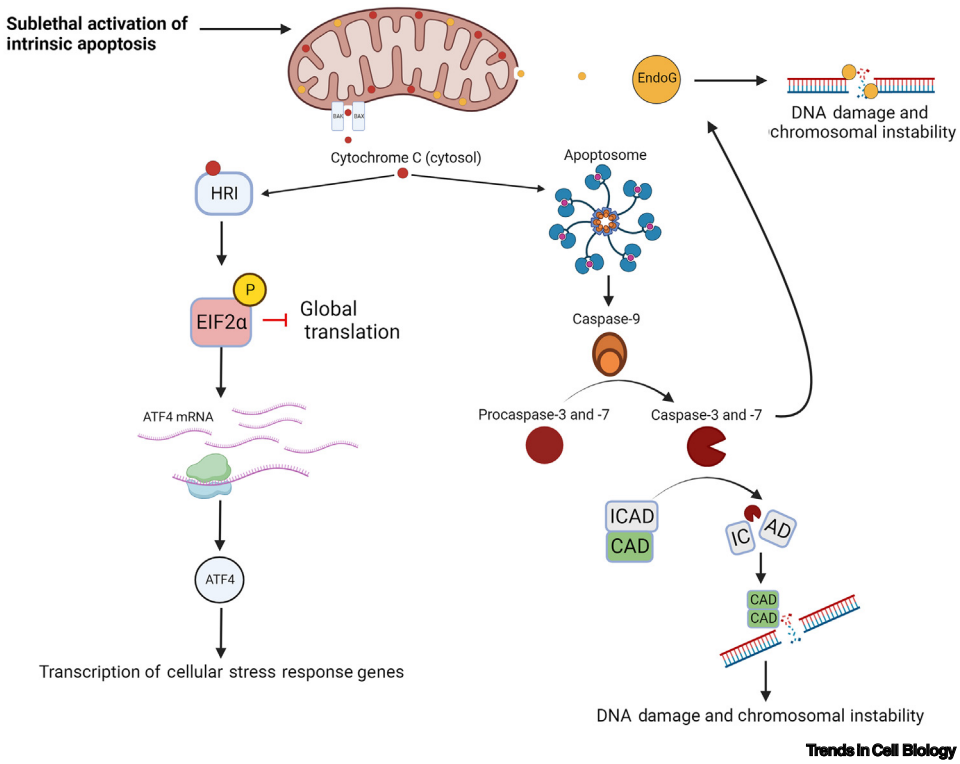

Figure 2. Sublethal mitochondrial outer membrane permeabilization (MOMP) promotes integrated stress response signaling and DNA damage. The release of cytochrome *c* from the intermembrane space (IMS) through BAX and BAK into the cytoplasm triggers the caspase-independent pathway of heme-regulated inhibitor (HRI)–EIF2α–activating transcription factor 4 (ATF4) and the caspase cascade. Direct binding of cytochrome *c* to HRI triggers its activation and downstream phosphorylation of EIF2α. Phospho-EIF2α inhibits global translation and stimulates ATF4 translation. ATF4 initiates transcription in a set of genes involved in the stress response. Activated apoptotic caspases facilitate the release of endonuclease G (EndoG) from the IMS. Inhibitor of caspase-activated DNase (CAD) (ICAD) is cleaved by caspases, which allows the activation of CAD through homodimerization. CAD and EndoG are DNases that introduce DNA breaks in genomic DNA.

(AML), and pancreatic ductal adenocarcinoma (PDAC) surviving treatment with their cognate oncogene-targeted therapies were found to exhibit DNA damage marked by phosphorylation of ataxia-telangiectasia mutated protein (p-ATM) and gamma H2A histone family member X (γ-H2AX) [17]. This therapy-induced DNA damage depended on activation of the proapoptotic factors BIM (an activator BH3-only protein) and BAX. Specifically, BAX-dependent sublethal MOMP led to the release of cytochrome *c*, activating downstream effector caspases and CAD-mediated damage. DTPs generated from targeted therapy treatment were eradicated by genetic or pharmacological inhibition of ATM. This finding is likely to reflect the requirement for DTP cells to resolve toxic double-strand breaks (DSBs) caused by sublethal MOMP and CAD activation (Figure 2) [17]. Interestingly, the results of these studies were corroborated by another recent report identifying an ATF4 dependency and increased rates of DNA damage in drug-tolerant melanoma cells [58].

Together, these findings illustrate the critical role of sublethal MOMP in shaping the formation and vulnerabilities of DTP cells. While evidence suggests that DTP formation may not always require sublethal MOMP [54], we speculate that it may be a common driver of this state in response to diverse drug treatments provided the treatment engages apoptosis [1–6].

These findings also prompt consideration of the broader ISR and its relationship to sublethal MOMP. The ISR is an adaptive signaling pathway activated in response to diverse cellular stressors. Extrinsic stressors include viral infection, heme deprivation, and nutrient depletion, activating any of three kinases (PKR, HRI, and GCN2, respectively). ER stress results from an accumulation of unfolded proteins in an intrinsic source of ISR activation via the kinase PERK [59]. In cancer, specific oncogenic pathways are known to hijack the ISR for tumor-promoting effects [59]. Activation of caspases may occur with or without MOMP and can also contribute to ISR signaling through proteolytic activation of PKR [60]. Therefore, while sublethal MOMP engages the ISR and promotes caspase activation and DNA damage, there are other ways these features can be engaged in stressed cells. Nevertheless, as many cancer therapies engage the mitochondrial pathway of apoptosis [1–6], it follows that sublethal MOMP-driven ISR activation may be a general feature of DTP generation and, therefore, a unifying concept underlying the persister phenomenon.

Sublethal MOMP shapes the DTP state

As previously described, DTP cells across cancer lineages contain the shared qualities of slowed cell cycling, induction of genes associated with EMT, multidrug tolerance, sensitization to ferroptosis, and increased mutability [48]. In the following section, we highlight the possible role of sublethal MOMP in fostering these phenotypic traits (Figure 3).

Sublethal MOMP inhibits cellular proliferation

Sublethal MOMP-enabled release of cytochrome *c* directly activates HRI-ATF4 to slow cell cycling, associated with inhibition of the mammalian target of rapamycin (mTOR) growth signaling pathway. PC9 persister cells exhibit a downregulated mTOR gene signature dependent on BCL-2 effectors and ATF4 [16]. HRI/ATF4 signaling has previously been linked to mTOR inhibition in various settings. One group identified that oligomycin treatment increased Sestrin2 and Redd1, known repressors of the mTOR signaling complex, in a HRI/ATF4-dependent manner [61]. The inhibition of mTOR by ATF4 through Sestrin2 was also identified in cancer cells treated with multiple ER stress agents [62,63]. Serum starvation and glucose depletion similarly led to ATF4-dependent activation of Redd1 and Sestrin2, respectively, which inhibited mTOR in these contexts [64,65]. Finally, HRI/ATF4 signaling has also been shown to inhibit mTOR signaling through growth factor receptor-bound protein 10 (Grb10) activation in erythroid progenitor

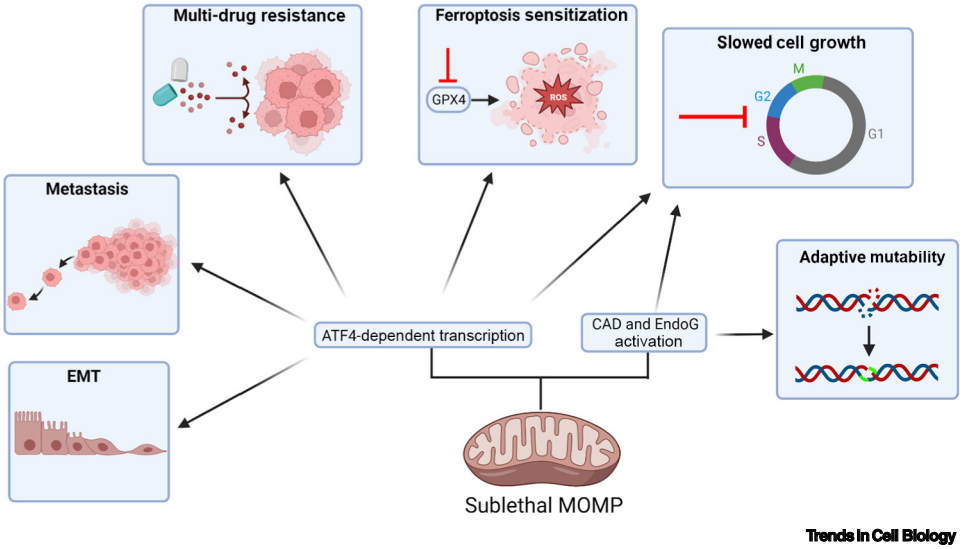

Figure 3. Contributions of sublethal mitochondrial outer membrane permeabilization (MOMP) to the drug-tolerant persister (DTP) state. Sublethal MOMP promotes the phenotypic characteristics of the persister state through activating transcription factor 4 (ATF4)- and DNase-dependent signaling events. ATF4 signaling triggers epithelial-to-mesenchymal transition (EMT), metastasis, multidrug resistance (MDR), ferroptosis sensitization, and slowed cellular proliferation in persister cells. Caspase-activated DNase (CAD) and endonuclease G (EndoG) may inhibit cellular proliferation and adaptive mutability by introducing DNA damage and genomic instability in the persister cells.

cells [66]. Altogether, the activation of ATF4 by sublethal MOMP is a potent mechanism to negatively regulate cell proliferation by inhibiting mTOR.

Increased DNA damage may provide a secondary mechanism to slow cellular growth under conditions of sublethal MOMP. CAD activity in cells experiencing sublethal MOMP is known to activate DNA DSBs that result in genomic instability and the DNA damage response (DDR) [12,13,17,67]. Breast cancer and sarcoma cell lines treated with mitotic inhibitors stimulated sublethal MOMP, which led to cell-cycle arrest through CAD-dependent DNA damage, activation of p53, and induction of p21 [68,69]. BH3-mimetic-treated renal cell carcinoma cells also exited the cell cycle following sublethal MOMP-dependent DNA damage [70]. Cell cycling is known to stall during the repair of DSBs until DNA repair effector proteins appropriately fix the lesion [71] and cells may permanently exit the cell cycle during DSB repair through the p53-induced expression of p21 [72]. Although the heterogeneity of cell-fate outcomes following DSBs is not fully elucidated, it was found that the amplitude of ATM- and Rad3-related (ATR)-dependent checkpoint signaling is associated with cell-cycle exit [73]. The DNA damage created in cells undergoing sublethal MOMP alters their ability to complete the cell cycle successfully and may contribute to slowed cellular proliferation in the persister state [68,69].

Sublethal MOMP contributes to EMT and metastasis

The ISR can stimulate EMT and promote metastases [74]. Phosphorylation of eIF2α via PERK was shown to be constitutively active in cells undergoing EMT and PERK-induced ATF4 expression drove the metastatic potential in EMT cells, as inhibition of this axis decreased the cells' ability to form *in vitro* tumorspheres, migrate, and establish *in vivo* lung tumors following tail-vein injection [75]. Microarray data from breast, colon, gastric, and lung cancer patients showed a strong correlation between EMT genes and ATF4-targeted genes [75]. *CREB3L1*, a target gene of ATF4, was identified as a driver of the ATF4-dependent increased metastatic potential in EMT cells [76].

The Initiation of EMT in persister cells is thought to contribute to tumor invasiveness, metastasis, chemoresistance, and immune escape [48,77,78]. BH3-mimetic-induced PC9 persister cells exhibit an enriched EMT gene signature and increased metastatic potential in a manner dependent on sublethal MOMP. Over time, this EMT signature reverted to parental levels [16]. Moreover, the activation of ATF4 by sublethal MOMP was the driving force for the induction of EMT genes and their enhanced metastatic capacity [16]. This work implicates the ISR, resulting from sublethal MOMP, as a driver of EMT and metastasis in persister cells. Besides the ISR, sublethal MOMP can increase melanoma invasiveness and metastatic capacity through JNK–AP1 signaling in a caspase-independent manner [79].

Sublethal MOMP promotes chemoresistance

As cells enter the persister state following drug treatment, they resist the primary therapy and other drug classes [48]. *EGFR*-mutant PC9 persister cells generated following epidermal growth factor receptor (EGFR) inhibitor treatment were found to be insensitive to cisplatin chemotherapy, suggesting that the resistance mechanism is not drug-class specific [53]. The activation of the ISR by sublethal MOMP was found to promote multidrug resistance in persister cells [16]. BH3-mimetic-induced *EGFR*-mutant PC9 persister cells, for example, were shown to be reversibly resistant to the primary BH3-mimetic therapy and an EGFR inhibitor, a microtubule stabilizer, and platinum-based chemotherapy. Loss of ATF4 in PC9 persister cells restored sensitization to targeted and conventional chemotherapies.

ATF4 and the ISR can promote or inhibit drug-treatment resistance depending on the specific context. In multiple myeloma, resistance to the proteasome inhibitor bortezomib was mediated through transcriptional upregulation of MCL-1 by ATF4 [80]. PERK inhibition and promotion of eIF2α activity through ER stress sensitizes *BCL-ABL* chronic myeloid leukemia cells to ABL inhibition with imatinib and *BRAF*-mutant melanoma cells to BRAF inhibition with PLX-4720, respectively [81,82]. Activation of the ISR was cytoprotective in pancreatic cancer cells following gemcitabine treatment [83]. Poor prognosis and resistance to radiotherapy in triple-negative breast cancer were associated with the activation of ATF4 [84]. Finally, ATF4 was required to resist the chemotherapeutic agent 5-fluorouracil in colorectal cancer cells [85].

Alternatively, ATF4 may sensitize cancer to specific therapies, including BCL-2 inhibition in AML [86]. It was also shown that targeting mitochondrial translation with the bacterial 30S ribosome inhibitor tigecycline is a vulnerability in the colorectal cancer cell line DLD-1 in a manner that is dependent on the activation of ISR [87]. The modulation of therapy responsiveness by ATF4 thus may depend on the treatment and cancer cell lineage.

Sublethal MOMP and ferroptosis sensitization

Ferroptosis is a programmed cell death pathway genetically and morphologically distinct from apoptosis, necroptosis, and autophagy. It is characterized by depletion of GSH and NADPH and impaired GPX4 activity, leading to dysfunctional lipid peroxidase metabolism and a toxic accumulation of iron-dependent ROS [56]. As discussed earlier, DTPs acquire a dependence on the lipid peroxidase GPX4. The cytochrome *c*–HRI–ATF4 axis, discussed earlier, was observed to downregulate genes involved in GSH metabolism and increase ferroptosis sensitivity in PC9 persister cells [16]. A second study showed that ABT-737 treatment decreased GSH levels in a caspase-independent manner, which may be explained by the above signaling axis [88]. ATF4 has previously been shown to induce the expression of ChaC GSH-specific gamma-glutamylcyclotransferase 1 (CHAC1), which degrades GSH and sensitizes triple-negative breast cancer to ferroptosis [89]. Accordingly, ATF4-dependent CHAC1 upregulation was similarly observed in PC9 persister cells compared with parental controls [16]. It is worth

noting that ATF4 has been reported to play a dual role in ferroptosis regulation [56]. ATF4 can protect cells against ferroptosis by upregulating heat shock 70-kDa protein 5 (HSPA5) and SLC7A11 expression [90–93]. The conflicting roles of ATF4 in ferroptosis regulation may depend on its specific activation conditions. Current data suggest that, in cells in the persister state, it appears to act primarily to sensitize to this form of cell death.

Sublethal MOMP and adaptive mutability

Apoptosis leads to the activation of numerous nucleases to facilitate the degradation of DNA and RNA in the cell [94]. A hallmark feature of apoptosis is nuclear DNA fragmentation, performed primarily by the dsDNA nuclease CAD. In a healthy cell, CAD activity is restricted through heterodimerization with the inhibitor of CAD (ICAD) protein. In apoptosis, ICAD is proteolytically cleaved by caspases. Degradation of ICAD frees CAD, allowing it to homodimerize and activate its nuclease ability [67]. In cancer cells, CAD was recently discovered to induce genome-wide DNA breaks secondary to genotoxic therapy in a caspase-independent manner, which inhibited premature mitotic progression and promoted cell survival, suggesting an additional mechanism of genomic instability independent of sublethal MOMP [95]. A second DNase that participates in nuclear DNA fragmentation is endonuclease G (EndoG). EndoG can be released from the mitochondria to translocate to the nucleus during apoptosis. Apoptotic caspases are required for the successful release of EndoG from the mitochondria but are not required for its nuclease activity [96,97].

Sublethal MOMP is a potent inducer of DNA damage and genomic instability. Stimulation of sublethal MOMP in an osteosarcoma cell line, U2OS, and a cervical cancer cell line, HeLa, with both BH3-mimetic treatment and sublethal expression of the activated BH3-only protein tBID promoted markers of DNA damage, including phosphorylation of γ-H2AX. These cells exhibited increased genomic instability, as evidenced by increased micronuclei and gene amplifications [12]. NSCLC, AML, and PDAC cell lines treated with small-molecule inhibitors targeting oncogenic driver pathways stimulated sublethal MOMP-dependent DNA damage and activated ATM [17]. In both contexts, the observed DNA damage depended on caspases and CAD. Sublethal activation of caspase-3 following low-dose radiotherapy triggered DNA damage and increased the rate of chromosome aberrations and translocations in MCF10A and mouse bone marrow cells, respectively. The DNA damage in this context was caspase and EndoG dependent [13].

Persister cells arising from bacteria and cancer populations display increased rates of genome-wide mutagenesis [98–100]. Persister cells are proposed to downregulate mismatch repair (MMR) and homologous recombination (HR) enzymes, resulting in a less efficient DNA damage repair system. Persisters resulting from targeted therapy of cancer cells show altered expression of high- to low-fidelity DNA polymerases and express APOBEC3A and APOBEC3B proteins to further contribute to a hypermutable state [101,102]. It is poorly understood how persister cells shift to low-fidelity DNA repair pathways, but mTOR [103] and transforming growth factor beta (TGF-β) [104] have been discovered to modulate DNA repair in specific cancer contexts. However, it suggests that persisters are a genetically malleable reservoir from which acquired resistance may be cultivated.

Sublethal MOMP generates targetable vulnerabilities in DTPs

Sublethal MOMP alters the fitness landscape of persister cells and creates susceptibility networks that can be rationally targeted. A sublethal MOMP gene signature was identified from differentially expressed genes (DEGs) in BH3-mimetic-treated wild-type cells versus $ATF4^{-/-}$ and $BAX^{-/-}BAK^{-/-}$ cells. The 'apoptosis persister signature' was found to be significantly elevated

in RD patient samples compared with treatment-naïve (TN) or progressive disease (PD) samples, suggesting that sublethal MOMP biology is indeed active in RD and may be therapeutically targeted [16]. Direct targeting of pathways that control the persister phenotype may prevent the survival system from being engaged and enhance killing by the primary drug insult. Alternatively, indirect targeting of the persister state through its downstream dependency hubs may facilitate synergistic partnerships with standard-of-care therapeutics.

Targeting the generation of the persister state

The ISR, leading to the expression of ATF4, represents a potential target of therapeutic intervention to limit DTP generation. In one study, *BRAF*-mutant melanoma cells that survived treatment with a BRAF inhibitor displayed increased expression of ATF4, and silencing of ATF4 reduced the numbers of these persisters [58]. In pancreatic cancer mouse models, the ISR inhibitor ISRIB increased apoptotic markers and decreased tumor growth when paired with the chemotherapeutic agent gemcitabine [83]. Pairing the tyrosine kinase inhibitor imatinib with ISRIB synergistically decreased the engraftment rate of imatinib-resistant chronic myeloid leukemia blasts [105]. Similarly, ISRIB treatment decreased the metastatic ability of pancreatic cancers in humanized mouse models [83]. Further investigation into ISRIB or its more bioactive derivative 2bAc [106] as a tool to eliminate persister cells is warranted to understand whether the ISR is a viable target for the prevention of DTP generation and MRD.

If sublethal MOMP and the activation of HRI is indeed a general mechanism for the induction of DTPs [16], targeting HRI may be a therapeutic strategy to prevent DTP generation. Although the options are limited, pharmacological inhibition of HRI can be achieved with a series of indeno[1,2-c]pyrazoles [107,108]. Downstream transcriptional targets of ATF4 that control specific features of the persister phenotype may provide added targetable opportunities.

Sublethal MOMP releases several IMS proteins into the cytosol that go on to facilitate numerous cellular changes. Each of these downstream signaling events may create novel cellular vulnerabilities. An example of this concept is the dependency on DNA damage repair pathways in persister cells created by CAD activation. NSCLC tumors progressing on EGFR treatment showed elevated phosphorylation of ATM compared with matched, treatment-naïve tumor samples. Furthermore, patients with both an *EGFR* driver mutation and a rare *ATM* loss-of-function mutation showed a significant progression-free survival advantage when treated with first-generation EGFR inhibitors [17]. *In vitro*, it was found that EGFR inhibitors activated sublethal MOMP and induced DNA damage through CAD. Pairing EGFR inhibitors with genetic or pharmacological inactivation of ATM eradicated PC9 persister cells. *In vivo*, NSCLC tumor models demonstrated that the combination treatment with EGFR and ATM inhibitors significantly outperformed either agent alone [17]. Sublethal MOMP thus indirectly creates a heightened dependence on DDR pathways due to its activation of CAD. As caspases are known to target thousands of proteins for degradation, numerous signaling cascades may become activated or inactivated in the sublethal MOMP state, which may contribute to as-yet-undefined dependency networks. Future investigation into sublethal MOMP signaling may reveal additional vulnerabilities of the persister state.

Targeting the acquired vulnerabilities of the persister state

Heightened sensitivity to ferroptosis is a common feature of DTPs across the cancer lineage. Persister cells were found to exhibit significant depletion of GSH and NADPH and were specifically vulnerable to both genetic and pharmacological inhibition of GPX4 [16,55]. *GPX4* knockout in *BRAF*-mutant melanoma tumors prevented the outgrowth of persister cells following treatment with combination BRAF (dabrafenib) and MEK (trametinib) inhibition *in vivo* [55]. EMT is a feature

of both persister and drug-resistant states and was discovered to promote a vulnerability to ferroptosis. Induction of EMT in epithelial-like cell lines through TGF-β treatment, drug resistance, or ectopic expression of EMT transcription factors was sufficient to increase their dependence on GPX4 *in vitro* and *in vivo* [109]. These findings suggest that ferroptosis is an attractive target in persister cells.

Ferroptosis can be induced through cystine depletion, inhibition of the cystine and glutamine antiporter system Xc^-, or inhibition of GPX4. Currently, the availability of specific and bioavailable GPX4 inhibitors is limited. Additionally, systemic targeting of GPX4 may lead to undesirable off-target effects, including renal failure [110,111], and may be tumor promoting in an immunocompetent host [112]. Investigation of cystine depletion and system Xc^- as therapeutic targets in persister cells has not been evaluated but they may be attractive alternatives to GPX4 inhibition. SLC7A11 is a subunit of the system Xc^- antiporter that is found to be specifically overexpressed in certain cancers, and no developmental defects are observed in SLC7A11$^{-/-}$ mice [111,113]. Imidazole ketone erastin (IKE) was recently discovered to be a selective inhibitor of system Xc^- and is the first system Xc^- inhibitor with favorable *in vivo* pharmacological properties. IKE induced tumor-specific ferroptosis in a diffuse large B cell lymphoma (DLBCL) mouse model and reduced overall tumor burden [114]. Understanding how persister cells will respond to pharmacological ferroptosis induction *in vivo* requires further evaluation.

Concluding remarks

Sublethal cellular stressors activate MOMP and caspases at levels that are nonlethal to the cancer cell but nevertheless alter its biological state and dependencies considerably. Sublethal MOMP was identified in both preclinical and clinical models of RD, where it initiated downstream signaling events that contribute to the phenotypic properties of DTPs. Stimulation of the ISR by cytosolic cytochrome *c* inhibits cellular proliferation, promotes chemoresistance, activates EMT, and sensitizes the cell to ferroptosis. MOMP-dependent DNases and CADs introduce DSBs and increase the genomic instability of DTPs. The widespread molecular alterations initiated by sublethal MOMP come at an exploitable cost to the cell. Sublethal MOMP creates a state with heightened dependence on ferroptosis, ISR, and DDR pathways that can be specifically targeted to achieve cellular eradication. Both persisters and genetically resistant clones comprising residual tumors can activate sublethal MOMP [17]. Thus, the impact of sublethal MOMP on the biology and vulnerabilities of DTP cells may extend to their irreversibly resistant brethren. This suggests that targeting vulnerabilities secondary to sublethal MOMP may be a powerful strategy to overcome multiple mechanisms of persistence and resistance (see Outstanding questions).

Acknowledgments

We are grateful to the many researchers who contributed to our understanding of sublethal MOMP and residual tumor cell biology and apologize that we could not cite all of the relevant research due to space restrictions. This work is supported by Cancer Research UK (DRCNPG-Jun22\100011 to S.W.G.T.), the US National Institutes of Health (R35CA231620 to D.R.G. and R01CA266389, R01CA263593, and U54CA231630 to K.C.W.), the US Department of Defense (W81XWH-21-1-0362 to K.C.W.), the Prostate Cancer Foundation (to K.C.W.), and the Duke Medical Scientist Training Program (T32GM007171 to S.T.K.).

Declaration of interests

K.C.W. is a founder of and consultant and equity holder at Tavros Therapeutics and Celldom and an SAB member and equity holder at Decrypt Biomedicine and Simple Therapeutics, and has performed consulting work for Guidepoint Global, Bantam Pharmaceuticals, and Apple Tree Partners. S.W.G.T. is a consultant for Exosite Therapeutics. D.R.G. is a consultant for Sonata Therapeutics. S.T.K. has no conflicts of interest.

Outstanding questions

Following MOMP, both IMS and mitochondrial matrix contents diffuse into the cytosol. Are there species beyond cytochrome *c* whose release during sublethal MOMP contributes to the DTP phenotype? For example, it is expected that sublethal MOMP may lead to inflammatory signaling through the release of mtDNA or mtRNA [115,116] or nuclear factor kappa B (NF-κB) activation through the downregulation of IAP proteins [117].

Does sublethal MOMP engagement result in the same phenotypic consequences in development and normal physiology as it does when it is activated by cytotoxic therapy?

As caspases are known to cleave hundreds of substrates following their activation, are there additional phenotypic manifestations of sublethal MOMP that depend on caspase activity?

Can sublethal MOMP also have tumor suppressor activity; for instance, through cGAS-STING activation?

Is increased apoptotic priming selected for in cancer cells through its ability to promote sublethal MOMP?

To what extent is sublethal MOMP responsible for MRD and for the properties of the residual cells?

Are there pre-existing transcriptional states that predispose cells to sublethal MOMP and DTP generation, and are such states associated with developmental plasticity?

Is there a general, transcriptional signature associated with cells that have undergone sublethal MOMP?

Does sublethal MOMP-driven DNA damage, and subsequent activation of DNA repair mechanisms, contribute to the DTP state or accelerate the acquisition of drug resistance in persister cells; for example, by increasing mutability?

We understand mitochondrial fusion, ferroptosis, ISR, and DDR pathways as rational therapeutic targets in sublethal MOMP-induced persister cells. Are there additional dependencies associated with sublethal MOMP?

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