Review

The role of PML in the control of apoptotic cell fate: a new key player at ER–mitochondria sites

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The development of malignant tumors results from deregulated proliferation or an inability of cells to undergo apoptotic cell death. Experimental works of the past decade have highlighted the importance of calcium (Ca^{2+}) in the regulation of apoptosis. Several studies indicate that the Ca^{2+} content of the endoplasmic reticulum (ER) determines the cell's sensitivity to apoptotic stress and perturbation of ER Ca^{2+} homeostasis appears to be a key component in the development of several pathological situations. Sensitivity to apoptosis depends on the ability of cells to transfer Ca^{2+} from the ER to the mitochondria. The physical platform for the interplay between the ER and mitochondria is a domain of the ER called the mitochondria-associated membranes (MAMs). The disruption of these contact sites has profound consequences for cellular function, such as imbalances of intracellular Ca^{2+} signaling, cellular stress, and disrupted apoptosis progression. The promyelocytic leukemia (PML) protein has been previously recognized as a critical and essential regulator of multiple apoptotic response. Nevertheless, how PML would exert such broad and fundamental role in apoptosis remained for long time a mystery. In this review, we will discuss how recent results demonstrate that the elusive mechanism whereby the PML tumor suppressor exerts its essential role in apoptosis triggered by Ca^{2+} -dependent stimuli can be attributed to its unexpected and fundamental role at MAMs in the control of the functional cross-talk between ER and mitochondria.

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The Promyelocytic Leukemia Protein

The promyelocytic leukemia (PML) protein is a tumor suppressor gene originally identified at the break point of the t(15;17) chromosomal translocation of acute promyelocytic leukemia (APL), a distinct subtype of acute myeloid leukemia. As a consequence of this translocation, PML fuses to the retinoic acid (RA) receptor alpha ($\text{RAR}\alpha$) gene. Two fusion genes are generated encoding PML- $\text{RAR}\alpha$ and $\text{RAR}\alpha$ -PML fusion proteins, which coexist in the leukemic cells, blocking hematopoietic differentiation (for a review, see Pandolfi,¹ Salomoni and Pandolfi.² PML has, therefore, become the object of intense research on the basis of this premise. Since then, PML has been shown to regulate diverse cellular functions, such as transcriptional regulation, DNA-damage response, sumoylation process, cellular senescence, neoangiogenesis, and, of relevance to this review, apoptosis.^{3,4}

PML belongs to a large family of proteins harboring a tripartite structure that contains a zinc-finger called the RING motif (R) located N-terminally followed by two additional zinc-fingers motifs (B-boxes; B) and an α -helical coiled-coil domain

(CC), collectively referred to as the RBCC domain. The RBCC domain mediates protein–protein interactions and is responsible for PML multimerization and the formation of macromolecular complexes. The C-terminal region of PML is less structured and varies between PML isoforms. Alternative splicing of C-terminal exons is responsible for the existence of at least seven PML isoforms characterized by different C-terminal regions and functional specificity.^{4,5}

PML is typically concentrated in subnuclear macromolecular structures termed PML-nuclear bodies (PML-NBs), of which PML is the essential component. PML-NBs have a diameter of 0.2–1 μm and the shape of a doughnut. PML-NBs are multi-protein dynamic structures that undergo significant changes in number, size, and position, particularly in response to cellular stress.^{4,6} They critically depend on PML to be correctly assembled.⁷

PML functionally interacts with a large number of proteins within PML-NBs.^{4,6} Some are in direct physical contact with PML, while others are not.^{4,6} PML SUMOylation and non-covalent binding of PML to SUMOylated PML through the

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Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ANT, adenine nucleotide translocator; Ca^{2+} , calcium; cypD, cyclophilin D; ER, endoplasmic reticulum; IP3, inositol 1,4,5-trisphosphate; IP3Rs, IP3-sensitive Ca^{2+} release channels; MAMs, mitochondria-associated membranes; MEFs, mouse embryonic fibroblasts; PML, promyelocytic leukemia; PML-NBs, PML-nuclear bodies; nuPML, PML chimera targeted to the nucleus; erPML, PML chimera targeted to the outer surface of the ER; RA, retinoic acid; $\text{RAR}\alpha$, retinoic acid receptor alpha; VDAC, voltage-dependent anion channel

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SUMO-binding motif constitutes the nucleation event for subsequent recruitment of SUMOylated proteins and/or proteins containing SUMO-binding motifs to the PML NBs.⁷

In the APL blasts, PML-RAR α associates physically with PML and causes its delocalization into microspeckled nuclear structures with consequent disruption of the PML-NBs.⁸ Loss of PML in a mouse model of APL causes a dramatic acceleration of leukemia and increased incidence of the disease, indicating the importance of the functional disruption of PML and PML-NBs for disease progression.^{1,9} Moreover, PML has been more recently shown to modulate the subcellular localization of the tumor suppressor PTEN.¹⁰ As a consequence, both in APL blasts and in PML-loss conditions, PTEN is excluded from the nucleus,¹¹ in turn suggesting that PTEN delocalization and loss of its nuclear function may have an important role in the pathogenesis of this and possibly other forms of leukaemia. Treatment with drugs commonly used for APL,¹¹ such as all-trans RA or arsenic trioxide, restores normal nuclear PTEN localization.¹⁰

Pml null mice and cells are protected from multiple and diverse apoptotic stimuli (Figure 1).¹² A possible explanation for why *Pml* null cells are resistant to many apoptotic stimuli can be ascribed to the fact that PML can act as a pleiotropic factor in the functional regulation of several pro- and anti-apoptotic factors.¹³

Indeed, PML is functioning as part of a complex tumor-suppressive network. For instance, it is well established that PML is an important factor in the regulation of both p53-dependent and -independent apoptotic pathways.^{13,14} PML activates p53 by several means: by recruiting p53 to PML-NBs by promoting its acetylation and phosphorylation,^{15–18} and by binding and inhibiting Mdm2, the main negative regulator of p53,^{19–21} as well as by promoting p53 de-ubiquitination by the ubiquitin protease HAUSP.^{22,23}

Moreover, PML can act as a suppressor of other major oncogenic pathways, such as the PI3K/Akt pathway, through its ability to interact with the protein phosphatase PP2A and inhibit the nuclear function of Akt, thus leading to suppression of its prosurvival and promitogenic functions.²⁴ In *Pten*^{+/-} animals, reduction of the *Pml* gene dosage results in transition

to invasive carcinoma, which is accompanied by increased Akt phosphorylation,²⁴ suggesting a genetic interaction between the two pathways. Finally, as aforementioned, PML regulates the function of the PTEN, which is the main suppressor of the PI3K pathway. This occurs through inhibition of PTEN de-ubiquitination by HAUSP and its nuclear retention.^{11,25,26} In conclusion, PML-NBs emerge as signaling coordination centers toward the regulation, availability, post-translational modification, and activation of multiple and diverse proteins implicated in apoptotic pathways.

However, no unified mechanism appeared to explain the global resistance of *Pml* null cells to apoptosis. Moreover, all these observations failed to clarify whether or not PML might also be involved directly in the execution of the apoptotic response and if the extra-nuclear fraction of PML could have a role in this respect. Indeed, in addition to its nuclear localization, some PML isoforms are found to accumulate into the cytosolic fraction,⁵ as well as adjacent to mitochondria,²⁷ and to be critical in the regulation of TGF- β signaling and anti-viral responses.^{28,29}

More recently, fractionation analysis by ultracentrifugation, immunogold labeling, and immunofluorescence in mouse embryonic fibroblasts (MEFs) revealed that PML associates to the surface of endoplasmic reticulum (ER) and in the proximity of the mitochondrial membrane at the ER/mitochondria contact sites (MAMs; mitochondria-associated membranes) (Figure 2), suggesting that PML might have additional as yet unidentified functions independent from the PML-NB.³⁰

Importantly, this unexpected intracellular localization of PML has been observed also *in vivo* through fractionation analysis of mouse liver cells (Giorgi, Pinton, and Pandolfi unpublished observations). Further fractionation experiments and immunohistochemical analysis that takes advantage of PML-specific antibodies will address whether the localization of PML at MAM is tissue specific or rather ubiquitous.

The ER–Mitochondria Contact Sites as a Hot Spot Signaling Unit

Mitochondrial and ER networks are fundamental for the maintenance of cellular homeostasis and for the determination of cell fate under stress conditions.³¹ Structural and functional studies revealed that as much as 20% of the mitochondrial surface is in direct contact with the ER, underscoring the dynamic and highly regulated communication between the ER and mitochondria.³²

These specific zones of close contact between ER and mitochondria have been termed MAMs by Jean Vance who characterized for the first time, from a biochemical point of view, the intimate relationship between the two compartments.³³

Experiments in living cells with ER and mitochondria differentially labeled by mutants of GFP have subsequently demonstrated the existence of a physical interaction between the two organelles and highlighted the functional importance of these contact sites.³²

The physiological function of the close apposition between ER and mitochondria is related to bioenergetics and cell survival.

Figure 1 PML is critical in multiple apoptotic pathways. The absence of PML inhibits cell death induced by various apoptotic stimuli. PML is critical for both transcription-dependent (e.g., p53-mediated responses) responses as well as transcription-independent early apoptotic responses

Figure 2 Intracellular localization of PML. The PML protein accumulates in the nucleus where it forms PML-nuclear bodies (PML-NBs), as well as at endoplasmic reticulum (ER) in the contact sites with mitochondria (MAMs). In both sites, PML interacts with several proteins (indicated in figure as IP: interacting proteins) generating multi-protein complexes of large molecular size

MAMs contain multiple phospholipid- and glycosphingolipid-synthesizing enzymes, including long-chain fatty acid-CoA ligase type 4 (FACL4) and phosphatidylserine synthase-1 (PSS-1), and support direct transfer of lipids between the ER and mitochondria.^{34,35}

In addition to the function in phospholipid homeostasis, MAMs have been implicated in the metabolism of cholesterol^{36,37} and its metabolites and in the trafficking of sphingolipids.³⁸

MAMs have also an important role in ER-mitochondrial calcium (Ca^{2+}) transfer. In support of this notion, western blot analysis of MAMs fraction after subcellular fractionation showed a selective enrichment of signaling elements,³⁹ such as the inositol 1,4,5-trisphosphate (IP3)-sensitive Ca^{2+} release channels (IP3Rs),⁴⁰ the most important molecular component of Ca^{2+} handling machinery, identifying these

zones as 'hotspots' of Ca^{2+} transfer from the ER to the mitochondria (Figures 3 and 4).

Interestingly, on the mitochondria side, this specialized domain is enriched in components of the permeability transition pore.^{41,42} This large-conductance channel (which has attracted a large interest given its potential role in pathophysiological conditions) includes the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), cyclophilin D, and other proteins (including pro-apoptotic members of the Bcl-2 family such as BAX/BAK). Both VDAC and ANT were shown by us and other groups to be enriched in the MAMs fraction (Figure 4a).³⁹

The continuous flow of Ca^{2+} between these two organelles regulates processes ranging from ER chaperone-assisted folding of newly synthesized proteins to the regulation of mitochondria-localized dehydrogenases involved in

Figure 3 PML localization at ER/MAM is critical for calcium-dependent apoptotic cell death. Re-expression of an erPML chimera does rescue the sensitivity to cell death of PML null cells. In this case, the ER Ca^{2+} release induced by various apoptotic stimuli causes a mitochondrial Ca^{2+} overload and in turn the damage of the mitochondrial structure with release of apoptotic factors. On the contrary, the re-introduction of a nuPML chimera is able to rescue the PML-NBs formation, but not the sensitivity to apoptosis. In this case, the absence of PML at the ER/MAMs sites still impairs ER- Ca^{2+} release and in turn mitochondrial damage and the apoptotic response

ATP-producing Krebs cycle reactions, and the activation of Ca^{2+} -dependent enzymes that execute cell death programs.⁴³ Hence alterations in the ER/mitochondrial coupling and in Ca^{2+} homeostatic mechanisms provides a powerful molecular basis for the activation of apoptosis.³¹

ER/Mitochondrial Coupling in Ca^{2+} Signal and Apoptosis

The ER is the main Ca^{2+} store of the mammalian cell, mitochondria are the powerhouse of the cell and they need Ca^{2+} signal to carry out their function. If we consider that Ca^{2+} ion is one of the most important mediators of life and death (and that mitochondria are main effectors of these processes), then the dynamic cross-talk between ER and mitochondria in the regulation of Ca^{2+} signal becomes a key step in physiology and pathology⁴⁴ (Figure 4).

On this basis, it has been argued that the switch from a life to a death signal occurs when normal Ca^{2+} distribution between the ER and the mitochondria is distorted leading to a breakdown of mitochondrial functions.⁴⁵ During normal signaling, there is a continuous flow of Ca^{2+} between these two organelles where a small bolus of Ca^{2+} is periodically released to the cytoplasm to be then re-sequestered with a proportion passing through the mitochondria.⁴⁶

In contrast, perturbation of ER/mitochondrial coupling provide a massive and/or a prolonged mitochondrial Ca^{2+} overload that in turn activates apoptosis.^{44,47} Indeed, a wide number of apoptotic stimuli, such as ceramide, arachidonic acid, and oxidative stress induced by H_2O_2 or menadione, trigger both a progressive release of Ca^{2+} from the ER and an activation of the capacitative Ca^{2+} influx. This sustained ER

Ca^{2+} release, in turn, induced a mitochondrial Ca^{2+} overload with a consequent release of mitochondria proteins involved in the apoptotic process, such as cytochrome *c*, AIF, and Smac/Diablo.^{44,48}

In this respect, conditions that reduce the ER Ca^{2+} storage and thus Ca^{2+} released from ER to mitochondria decrease the probability of Ca^{2+} -dependent apoptosis;⁴⁹ on the other hand, an increase in the Ca^{2+} released has the opposite effect.

Interestingly, over-expression of the anti-apoptotic protein Bcl-2 decreases the steady-state Ca^{2+} content of the ER, resulting in a reduced amount of agonist-releasable Ca^{2+} and in a diminution of cytosolic and mitochondrial Ca^{2+} response.^{48,50} Thus, by diminishing ER Ca^{2+} levels, Bcl-2 is able to protect from Ca^{2+} -dependent apoptotic stimuli.⁵¹ Moreover, knocking out the pro-apoptotics Bax and Bak leads to a dramatic reduction of the steady-state Ca^{2+} concentration in the ER, rendering the knock-out cells more resistant to apoptosis.⁵² On the contrary, after over-expression of Bax, the ER Ca^{2+} content increases and the cells are more susceptible to apoptosis treatment.⁵³ Therefore, manipulating the level of ER Ca^{2+} , adjusting the load of Ca^{2+} imposed upon mitochondria, it is possible to regulate apoptosis.

This concept was corroborated by the case of PML that was discovered to modulate Ca^{2+} homeostasis and apoptosis in view of its ability to localize and function at the ER and MAMs, as mentioned above³⁰ (Figure 3). Indeed, in the absence of *Pml*, the ER steady-state values are lower and the decreases of $[\text{Ca}^{2+}]_{\text{ER}}$ after agonist treatment are drastically smaller and lower compared with *Pml*^{+/+} MEFs. In agreement with the observed low $[\text{Ca}^{2+}]_{\text{ER}}$, the $[\text{Ca}^{2+}]$ increases evoked in the cytosol and mitochondria both by ATP stimulation and by

Figure 4 Schematic representation of ER-mitochondria Ca^{2+} cross-talk perturbations in physiological and oncogenic states. **(a)** In normal cells, the PML protein is in protein complexes with the type 3 inositol triphosphate receptor (IP3R-3), the protein kinase Akt and the phosphatase PP2a. This complex is fundamental to regulate the phosphorylation state of IP3R-3, while the Bcl-2-Bax ratio controls the phosphorylation state of the type 1 of IP3R (IP3R-1). In normal conditions, the stimulation of ER Ca^{2+} release through IP3 generating stimuli allows a transient mitochondrial Ca^{2+} uptake and in turn the stimulation of mitochondrial ATP production through the mitochondrial respiratory chain (MRC). In contrast, a Ca^{2+} -dependent pro-apoptotic stimulus induces a much larger Ca^{2+} release from the ER with a dramatic effect on mitochondria, which induces the release of cytochrome c and the opening of the mitochondrial permeability transition pore. This chain of event, combined with the activation of cytoplasmic effectors, is an important trigger of the apoptotic program. **(b)** In cancer cells, where PML is often missing or when the Bcl-2-Bax ratio is abnormal, IP3R-3 and IP3R-1 are, respectively, hyper-phosphorylated as demonstrated on Pml KO, Bax/Bak KO, or Bcl-2 KI cells. Under these conditions, pro-apoptotic stimuli induce a smaller release of Ca^{2+} from ER, with minor repercussions on mitochondrial physiology and intracellular Ca^{2+} -modulated processes

oxidative apoptotic stimulation were significantly smaller in *Pml*^{-/-} than in *Pml*^{+/+} MEFs and cells were unable to die. Furthermore, the expression of a PML chimera that exclusively localizes to the outer surface of the ER (erPML) in *Pml*^{-/-} MEFs restores mitochondrial Ca^{2+} signals to values comparable to those measured in *Pml*^{+/+} MEFs re-establishing also their sensitivity to the apoptosis. On the contrary, a PML targeted exclusively to the nucleus, restores the formation of NB but is not able to rescue Ca^{2+} response and the sensitivity to ER-stress-dependent cell death³⁰ (Figure 3).

Altogether, these data highlight the link between Ca^{2+} homeostasis and the regulation of apoptotic cell death and clearly indicate ER/mitochondria contacts as a critical, although not unique checkpoints.

IP3Rs as Target of Cell Death

The most important molecular component of the Ca^{2+} handling machinery of the ER is represented by the IP3Rs. IP3Rs are ligand-gated channels that serve to discharge Ca^{2+} from ER stores in response to agonist stimulation.^{40,54} After IP3-mediated release of Ca^{2+} from the ER through the IP3 receptor, high- Ca^{2+} microdomains (estimated to be in the range of 50–100 μM) are generated at the tight ER-mitochondrial junctions, activating the low-affinity mitochondrial Ca^{2+} uniporter and resulting in mitochondrial Ca^{2+} uptake³² (Figure 4).

Being directly responsible for mitochondrial Ca^{2+} overload, the release of Ca^{2+} from ER stores by IP3Rs is linked to multiple models of apoptosis.⁵⁵ Indeed, cells deficient of IP3R are resistant to apoptosis^{56–58} (Figure 4).

There are three isoforms of IP3Rs: IP3R-1 to 3. At present, it is not clear whether different IP3 receptor isoforms have an equivalent role in apoptosis, but types 1 and 3 have been described as important in mediating Ca^{2+} -dependent cell death.⁵⁹ Importantly, their function is regulated by post-transcriptional modifications. In particular IP3R phosphorylation appears to be a key common feature for modulation of channel function and, as consequence, apoptotic signaling.^{60–62}

In this respect, the Korsmeyer's group found that Bcl-2 and IP3R-1 physically interact at the ER surface and proposed a model by which Bcl-2 family members regulate IP3R-1 phosphorylation to control the rate of ER Ca^{2+} leak from intracellular stores and, as consequence, the apoptotic response⁶³ (Figure 4).

Recent data showed that IP3R-3, localized in the MAMs, has a selective role in the induction of apoptosis by preferentially transmitting apoptotic Ca^{2+} signals to mitochondria.^{64,65} Accordingly, siRNA silencing of IP3R-3 blocked apoptosis,⁶⁵ as KO of IP3R-3 significantly decreased agonist-induced mitochondrial Ca^{2+} uptake.⁶⁶

IP3Rs possess consensus sequences for phosphorylation by numerous kinases, including protein kinase B (Akt/PKB).⁶⁰ This is an interesting observation, because in some cancer cells in which Akt is constitutively active (e.g., prostatic carcinoma cells), IP3Rs are hyper-phosphorylated.⁶⁰ In turn, the hyper-phosphorylation of IP3Rs by Akt inhibits ER Ca^{2+} release and reduces significantly cellular sensitivity to Ca^{2+} -mediated pro-apoptotic stimulation.^{62,67}

Interestingly, similarly to Bcl-2 with IP3R-1, the tumor suppressor PML was found to physically interact with IP3R-3 modulating its phosphorylation state. Indeed in MEF *Pml*^{-/-} cells, IP3R-3 is hyper-phosphorylated. This was demonstrated to be mediated by a specific multi-protein complex, localized at ER/MAMs contact sites, including PML, IP3R-3, the protein phosphatase PP2a, and Akt. In particular, PML appeared to be essential for the binding of PP2a to the IP3R-3, hence favoring IP3R-3 de-phosphorylation (Figures 3 and 4).

Thus in the absence of PML, the unopposed action of Akt at ER due to an impaired PP2a activity leads to a hyper-phosphorylation of IP3R-3 and in turn a reduced Ca^{2+} flux from ER to mitochondria rendering cells resistant to apoptotic Ca^{2+} -dependent stimuli³⁰ (Figures 3 and 4b).

The elucidation of the role of IP3R-3 in Ca^{2+} transfer from the ER to mitochondria, of its molecular mechanism and of the regulatory effect of its phosphorylation may reveal a novel unexplored pharmacological target in apoptosis.

Conclusions and Future Directions: Beyond the PML-NB

PML is a critical and essential regulator of multiple apoptotic responses. While the reported role of PML in the modulation of p53 transcription could explain some of its pro-apoptotic functions, it failed to reconcile the fundamental role played by PML in the transcription-independent early apoptotic response.

The identification of PML at the ER-MAMs regions addressed this outstanding question. Indeed, the unexpected extra-nuclear MAMs-associated PML-dependent pathway for the control of Ca^{2+} homeostasis and in turn Ca^{2+} -dependent apoptosis provides a compelling explanation for such a pleiotropic role. PML exerts this role by orchestrating at ER/MAMs sites, as in the NBs, the function of different key proteins involved in cell death processes.

Further experiments will address whether this function is required also *in vivo* for tissue homeostasis, and in which tissues this function is critical. The generation of an inducible erPML transgenic mouse model will also allow to determine whether the PML function at the ER/MAMs sites is fundamental (and sufficient) to trigger Ca^{2+} -dependent apoptosis in tumor models.

Strikingly, the final outcome of a PML functional loss at the cellular level is similar to the one observed in cells over-expressing Bcl-2 or lacking of Bax/Bak (albeit through a completely different molecular mechanism): a reduced mitochondrial Ca^{2+} overload upon pro-apoptotic stimuli that dramatically blunts the apoptotic response.

This in turn highlights a new extra-nuclear PML function critical for regulation of cell survival through the ER-mitochondria Ca^{2+} -dependent cross-talk.

Conflict of interest

The authors declare no conflict of interest.

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