

REVIEW

Regulation of apoptosis by PML and the PML-NBs

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The promyelocytic leukemia protein (PML) is a tumor suppressor identified in acute PML and implicated in the pathogenesis of a variety of tumors. PML is essential for the proper assembly of a nuclear macromolecular structure called the PML nuclear body (PML-NB). PML and PML-NBs are functionally promiscuous and have been associated with the regulation of several cellular functions. Above all these is the control of apoptosis, a function of PML whose physiological relevance is emphasized by *in vivo* studies that demonstrate that mice and cells lacking *Pml* are resistant to a vast variety of apoptotic stimuli. The function of PML in regulating apoptosis is not confined to a linear pathway; rather, PML works within a regulatory network that finely tunes various apoptotic pathways, depending on the cellular context and the apoptotic stimulus. Here, we will summarize earlier and recent advances on the molecular mechanisms by which PML regulates apoptosis and the implication of these findings for cancer pathogenesis.

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Introduction

The promyelocytic leukemia tumor suppressor gene (*PML*), also called *MYL*, *RNF71*, *PP8675* and *TRI19*, was cloned in 1991 at the breakpoint of the t(15;17) chromosomal translocation of acute PML (APL), a subtype of acute myeloid leukemia characterized by a block of differentiation at the promyelocytic stage (de Thé *et al.*, 1991; Kakizuka *et al.*, 1991; Pandolfi *et al.*, 1991). In t(15;17), the *PML* gene is juxtaposed to the retinoic acid (RA) receptor- α (*RAR* α) gene giving rise to the fusion protein PML–RAR α (Melnick and Licht, 1999).

Over the past 15 years, *in vitro* and *in vivo* studies have shown that PML–RAR α acts as an oncogene not only by inhibiting RAR α transcriptional function, but also by physically associating and interfering with PML (Piazza *et al.*, 2001). For example, PML–RAR α causes leukemia with APL features in transgenic mice, whereas the dominant negative RAR α mutants, which do not interfere with PML functions, do not (Piazza *et al.*, 2001). In addition, a decrease in *Pml* dosage significantly accelerates leukemogenesis in PML–RAR α transgenic mice (Piazza *et al.*, 2001). At the molecular level, PML–RAR α is thought to inhibit PML by interfering with its typical localization to nuclear macromolecular structures called PML nuclear bodies (PML-NBs). As a consequence, PML-NBs disaggregate in cells expressing PML–RAR α , such as APL blasts, and PML acquires a nuclear microspeckled localization (Melnick and Licht, 1999).

The PML-NBs are functionally promiscuous organelles that have been suggested to regulate a variety of cellular functions. A question that remains still unanswered after years of intense research is which are the functions of PML with physiological relevance. What is certain is that PML possesses functions of a tumor suppressor: PML is a growth suppressor when over-expressed in various cancer cell lines (Liu *et al.*, 1995; Mu *et al.*, 1997; Le *et al.*, 1998), and loss of *Pml* in knockout (KO) mice increases susceptibility to chemical-induced carcinogenesis (Wang *et al.*, 1998) and to spontaneous tumorigenesis (Trotman *et al.*, 2006). In addition, PML expression decreases in human tumors (Gurrieri *et al.*, 2004a) and in some cases low levels of PML correlate with poor disease outcome (Chang *et al.*, 2007).

Analysis of *Pml* null mice has demonstrated that PML exerts a tumor suppressive role at least in part by regulating a number of proapoptotic responses in response to stress and DNA damage (Figure 1) (Wang *et al.*, 1998).

Here, we will review and discuss some of the known molecular mechanisms by which PML controls programmed cell death, with a particular emphasis on the aspects of PML biology that have received more attention recently.

The PML protein and PML-NBs

PML belongs to a large family of proteins harboring a distinctive zinc-finger domain termed the RING

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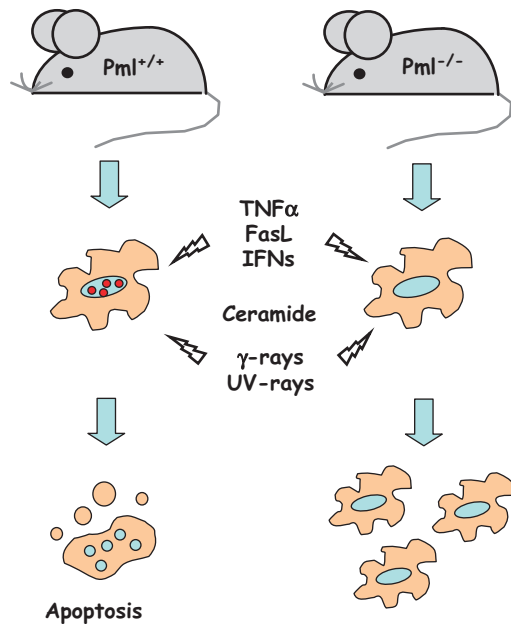


Figure 1 PML is essential for multiple apoptotic pathways. Analysis of mice and cells lacking *Pml* has demonstrated that PML is involved in various apoptotic pathways. Hematopoietic cells and embryonic fibroblasts from *Pml* null mice are resistant to a series of p53-dependent and p53-independent proapoptotic stimuli, including DNA-damaging agents, ceramide and engagement of death receptors. PML, promyelocytic leukemia.

domain, followed by two additional zinc fingers called B-boxes and an α -helical coiled-coil motif, which are collectively referred to as the RBCC domain. The RBCC domain mediates protein–protein interactions and is responsible for PML homomultimerization and nucleation of the PML-NBs (Jensen *et al.*, 2001; Bernardi and Pandolfi, 2007). Also, PML RBCC domain mediates heterodimerization with PML–RAR α (Jensen *et al.*, 2001).

The C-terminal region of PML is less structured and varies between PML isoforms. Alternative splicing of C-terminal exons determines the existence of at least seven PML isoforms characterized by different C-terminal regions (Jensen *et al.*, 2001; Bernardi and Pandolfi, 2007). Some, if not all, of the isoform-specific C-terminal regions of PML interact with and regulate specific protein partners. For example, p53, c-Myc and Pu1 have been reported to interact with PML isoform IV (Fogal *et al.*, 2000; Bischof *et al.*, 2002; Buschbeck *et al.*, 2006; Yoshida *et al.*, 2007), whereas the acute myeloid leukemia-1 protein binds to PML isoform I (Nguyen *et al.*, 2005). On the whole, however, and as discussed later, the biological relevance of isoform specificity in the context of PML functions has yet to be established due to the lack of information about tissue-specific expression of different isoforms and lack of cell or animal models characterized by the loss of expression of specific isoforms.

In addition to alternative splicing of C-terminal exons, alternative usage of three exons located in the middle of the gene (exons 4, 5 and 6) can also occur,

causing a further increase in the total number of PML protein isoforms (Jensen *et al.*, 2001; Bernardi and Pandolfi, 2007; McNally *et al.*, 2008). Because exon 6 contains a nuclear localization signal, all PML isoforms with different C-terminal regions may in theory come in a cytoplasmic flavor. As discussed later, cytoplasmic forms of PML have been recently implicated in the control of apoptosis. However, once more, lack of a detailed analysis of isoform expression in distinct cellular contexts prevents a better understanding of the physiological significance of these findings.

The PML-NBs

Soon after the *PML* gene was cloned, studies on the expression and localization of the PML protein revealed that PML localizes to distinctive nuclear speckles that defined a new macromolecular structure (Daniel *et al.*, 1993). These new structures have been called different names over the years, until researchers informally agreed to call them simply PML-NBs.

PML nuclear bodies have a diameter of approximately 0.2–1.0 micrometer and the shape of a doughnut, as revealed by electron microscopy studies. They are composed of proteins that form a ring surrounding an empty space. Nucleic acids, such as DNA and RNA, can be found at the periphery of the ring (Boisvert *et al.*, 2000; Delliare and Bazett-Jones, 2004). Besides PML, many heterogeneous proteins localize to the PML-NBs, although, unlike PML, most of these proteins are not constitutive residents of the PML-NBs but rather localize there transiently and after specific stimuli.

Studies in KO animals have shown that PML is essential for the formation of the PML-NBs, because in primary cells lacking *Pml*, constitutive or transient PML-NBs components acquire aberrant nuclear localization and no longer accumulate in nuclear dots (Zhong *et al.*, 2000b). The functional integrity of the PML-NBs is also lost in cells expressing PML–RAR α , such as leukemic blasts from APL patients, because PML–RAR α interacts with endogenous PML and causes delocalization of PML into small nuclear speckles and dispersal of the components of PML-NBs (Melnick and Licht, 1999).

Regulation of PML and PML-NBs by stress conditions

PML-NBs are detected in most cell types in the developing embryo and the adult organism, with some differences in the levels of expression. For example, in the squamous mucosa, basal cells express more PML than superficial, differentiated cells (Cho *et al.*, 1998).

More noticeably, however, PML expression is modulated under stress conditions such as inflammation, oncogenic transformation and proapoptotic stimuli (Bernardi and Pandolfi, 2007). Most of these conditions cause an increase in the number and size of PML-NBs along with the increased expression of PML. PML induction by stress stimuli is often caused by transcriptional upregulation, such as in the case of PML upregulation by interferons (Stadler *et al.*, 1995) and

oncogenic stimuli like Ras^{V12} overexpression (Ferbeyre *et al.*, 2000; de Stanchina *et al.*, 2004).

Besides increasing the levels of PML, many stress stimuli and in particular conditions that induce apoptosis trigger a dynamic spatial and biochemical reorganization of PML-NBs. For example, ultraviolet (UV) irradiation causes the dispersal of PML-NBs into smaller bodies where the c-Jun transcription factor becomes recruited (Seker *et al.*, 2003; Salomoni *et al.*, 2005), whereas γ -irradiation causes an increase in the number of PML-NBs and the recruitment of various factors involved in DNA repair and in checkpoint signaling pathways (Carbone *et al.*, 2002; Dellaire *et al.*, 2006).

As discussed in the following paragraphs, these changes in shape, location and composition of PML-NBs have been coupled to the ability of PML to regulate checkpoint signaling pathways and induction of apoptosis.

New insights into the regulation of PML stability

Post-translational regulation of PML and, more specifically, the regulation of PML stability, had not been intensely investigated until a few years ago, when it was first reported that PML abundance is regulated by the ubiquitin-proteasome pathway (Scaglioni *et al.*, 2006). PML is phosphorylated on many tyrosine, serine and threonine residues (Chang *et al.*, 1995), some of which have been mapped and shown to be constitutively phosphorylated (Hayakawa and Privalsky, 2004; Scaglioni *et al.*, 2006; Tagata *et al.*, 2008). Phosphorylation of PML controls many aspects of PML function, including interaction with other proteins, sumoylation and ubiquitination (Wei *et al.*, 2003; Hayakawa and Privalsky, 2004; Scaglioni *et al.*, 2006; Tagata *et al.*, 2008). With regard to PML catabolism, it has been reported that PML is polyubiquitinated upon phosphorylation by the serine-threonine kinase CK2, a proto-oncogene frequently overexpressed or aberrantly activated in tumors (Scaglioni *et al.*, 2006). These data helped explain, at least in part, why PML expression is lost in solid tumors (Gurrieri *et al.*, 2004a). Indeed, in lung cancer samples, PML levels inversely correlate with CK2 activity (Scaglioni *et al.*, 2006). However, these studies did not establish the identity of the ubiquitin ligase in charge of PML ubiquitination. This issue has been addressed more recently by two groups who have not only identified a ubiquitin ligase catalysing PML ubiquitination, but also have revealed a novel and unexpected connection between sumoylation and ubiquitination (Lallemand-Breitenbach *et al.*, 2008; Petrie and Zelent, 2008; Tatham *et al.*, 2008).

Sumoylation is a post-translational modification that consists of the covalent linkage of a small protein similar to ubiquitin, SUMO, to a selected substrate (Seeler and Dejean, 2003). Unlike ubiquitination that causes prevalently, although not exclusively, protein catabolism, sumoylation had been so far linked to nucleocytoplasmic transport, regulation of gene expression and subnuclear architecture, and even to the inhibition

of protein degradation, but had never been positively implicated in protein degradation (Petrie and Zelent, 2008). Lallemand-Breitenbach *et al.* (2008) and Tatham *et al.* (2008) have now identified in PML the first example of a protein degraded by the ubiquitin-proteasome pathway in a SUMO-dependent manner (Petrie and Zelent, 2008).

It had long been known that PML is SUMO-modified on three lysines (65, 160 and 490) by SUMO1 as well as SUMO2/3, which unlike SUMO1 form polymeric chains (Fu *et al.*, 2005; Mukhopadhyay *et al.*, 2006). Sumoylation of PML, coupled with the presence of a SUMO-binding domain, had been previously linked to proper assembly of PML-NBs and to the recruitment of proteins such as Daxx and CBP (Ishov *et al.*, 1999; Zhong *et al.*, 2000a; Best *et al.*, 2002; Shen *et al.*, 2006). These new papers surprisingly link the sumoylation of PML to its degradation through recognition by the E3 ubiquitin ligase RNF4/SNURF, a protein containing four SUMO interaction motifs (Lallemand-Breitenbach *et al.*, 2008; Tatham *et al.*, 2008). Interestingly, it is also suggested that both mono- and polysumoylation by SUMO1 and SUMO2, respectively are involved in the degradation of PML. This novel mechanism of degradation was shown to occur upon arsenic trioxide treatment, a condition known to promote PML degradation, but also to some extent at steady-state conditions (Petrie and Zelent, 2008). Although these studies do not address whether RNF4/SNURF is the ubiquitin ligase that mediates PML degradation upon CK2 phosphorylation in addition to sumoylation, they certainly add an interesting twist not only to our understanding of PML catabolism, but also of the sumoylation processes in general.

Nevertheless, in the context of PML degradation matters are complicated by another recent publication that also addresses the issue of PML stability. This work reveals that PML degradation is also mediated by the peptidyl-prolyl *cis-trans* isomerase Pin1, which binds to phosphorylated PML and promotes a conformational change that triggers its degradation (Reineke *et al.*, 2008). Surprisingly however, while PML sumoylation was found to be necessary for RNF4/SNURF-mediated degradation, sumoylation is suggested to inhibit PML degradation induced by Pin1 (Reineke *et al.*, 2008).

This apparent controversy could be resolved assuming that PML sumoylation on different lysines and/or by different SUMO proteins has different and specific meanings, as suggested by Petrie and Zelent (Petrie and Zelent, 2008). In other words, sumoylation on lysine 160 by SUMO2 and SUMO1 would promote PML degradation by RNF4/SNURF, whereas sumoylation on lysine 490 by SUMO1 would inhibit PML degradation by Pin1 (Figure 2) (Lallemand-Breitenbach *et al.*, 2008; Petrie and Zelent, 2008). While obviously more work is required to rigorously address this hypothesis, what is emerging clearly from these recent studies is that PML sumoylation is a tightly controlled process and it seems reasonable to presume that not all PML sumoylation events have the same outcome (Petrie and Zelent, 2008).

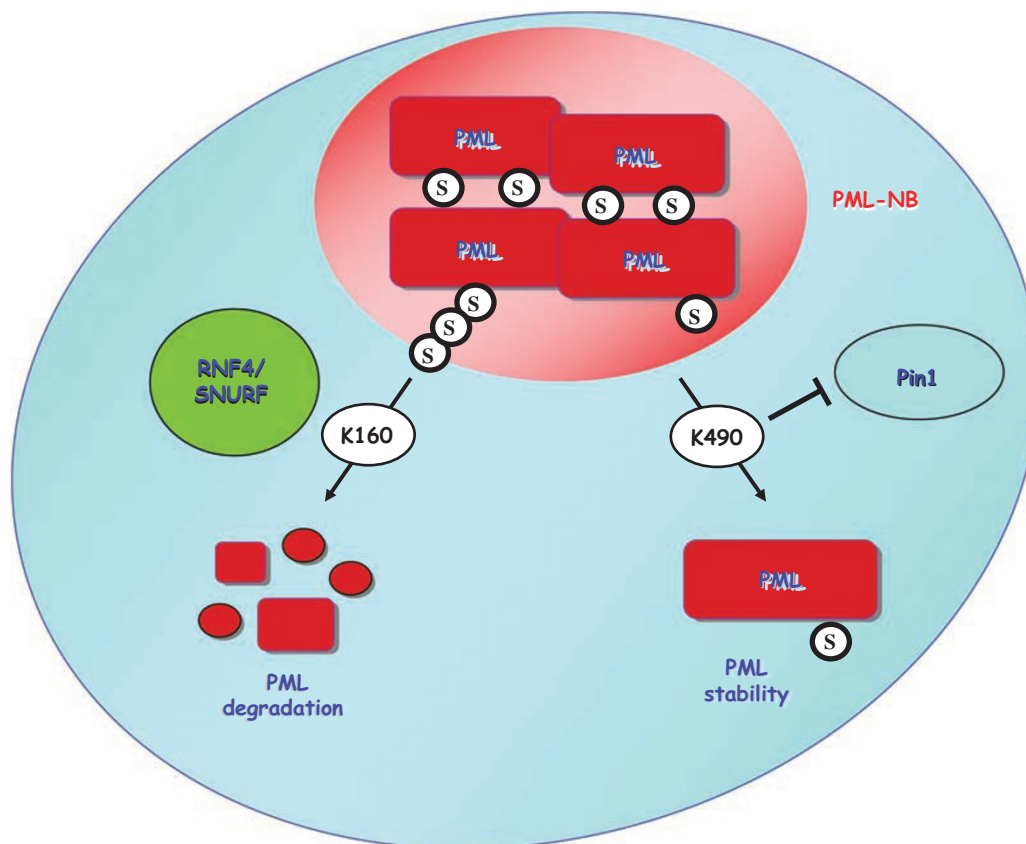


Figure 2 PML stability is regulated by PML sumoylation. Recent data have shown that PML sumoylation, once thought to be necessary mostly for the nucleation of PML-NBs, is a tightly regulated process that impacts also on PML degradation. In a recently proposed model, sumoylation of PML on different lysines has been suggested to have different outcomes on PML stability (Petrie and Zelent, 2008). In this interesting model, which remains to be proven experimentally, polysumoylation on lysine 160 by SUMO2 and SUMO1 promotes PML degradation by RNF4/SNURF, whereas sumoylation on lysine 490 by SUMO1 inhibits PML degradation by Pin1. PML, promyelocytic leukemia; PML-NBs, PML nuclear bodies.

Finally, another group has recently found that HMGA2, a member of a family of architectural proteins and regulators of gene transcription, also promotes PML degradation by the proteasome (Cao *et al.*, 2008). Intriguingly, sumoylation is again brought into the picture because only sumoylated HMGA2 can promote PML degradation (Cao *et al.*, 2008). Furthermore, arsenic trioxide stimulates HMGA2 sumoylation and the localization of HMGA2 in rings that surround PML-NBs (Cao *et al.*, 2008). As in the case of RNF4/SNURF, whether HMGA2 mediates PML degradation upon CK2 phosphorylation remains to be addressed.

In turn, these findings lead to many interesting developments and new questions. For example, if PML sumoylation is necessary for both PML-NBs formation and turnover, how is this functional switch regulated? As already mentioned, it is safe to hypothesize that the sumoylation of PML is a process much more tightly regulated than what we had anticipated until not long ago. It will be important in the near future to better understand how the pathways regulating PML sumoylation and ubiquitination diverge or interconnect in regulating PML abundance in different physiological conditions and how this impacts on the regulation of

apoptosis by PML and on the loss of PML in human tumors.

Apoptotic pathways regulated by PML

In the past decade, PML and the PML-NBs have been implicated in the control of many cellular functions, including but not limited to, the regulation of transcription, neoangiogenesis, DNA damage responses, cellular senescence and apoptosis (Zhong *et al.*, 2000b; Borden, 2002; Bernardi and Pandolfi, 2007). Amid these many functions, the role of PML in regulating apoptosis is perhaps the best-established owing especially to numerous studies conducted by various laboratories on *Pml* null mice and primary cells derived from these mice (Bernardi and Pandolfi, 2003; Hofmann and Will, 2003; Takahashi *et al.*, 2004).

As soon as a KO model for *Pml* was created, it became evident that *Pml* null mice and cells are protected from multiple and diverse apoptotic stimuli (Figure 1). *Pml* null mice are resistant to the lethal effects of both γ -irradiation and CD95/Fas (Wang *et al.*, 1998). In addition, primary cells from *Pml* null mice,

such as splenocytes, thymocytes, mouse embryonic fibroblasts and hematopoietic cells are resistant to apoptosis induced by CD95/Fas or γ -irradiation, and many other stimuli, including ceramide, TNF, IFN, UV light and chemotherapeutic drugs (Wang *et al.*, 1998; Louria-Hayon *et al.*, 2003; Wu *et al.*, 2003; Bernardi *et al.*, 2004). In agreement with the dominant negative role of PML-RAR α on PML, hematopoietic progenitors from PML-RAR α transgenic mice are resistant to similar apoptotic stimuli (Wang *et al.*, 1998).

As described in detail in the next few paragraphs, the reason why *Pml* null cells are resistant to many apoptotic stimuli is because PML acts as a pleiotropic factor that regulates the function of several pro- and antiapoptotic factors such as p53, Daxx and c-Jun among others. Different aspects of the biology of these proteins are regulated by PML, mostly through the recruitment into PML-NBs; for example, their transcriptional function or post-translational modification as well as availability of their essential cofactors.

PML and p53

Early evidence that PML collaborates with p53 in the induction of apoptosis was obtained by experiments with thymocytes from *Pml* KO mice. γ -Radiation-induced apoptosis in thymocytes is the prototypical p53-dependent apoptotic stimulus. *Pml* null thymocytes are resistant to γ -radiation-induced apoptosis although to a lesser extent than *p53*^{-/-} thymocytes, suggesting that *Pml* is an important mediator of p53 proapoptotic functions *in vivo* though clearly not the only one (Guo *et al.*, 2000).

From these early studies, consistent evidence has accumulated to support the concept that PML is an important regulator of p53 function upon certain kinds of DNA damage or cellular stress (Ferbeyre *et al.*, 2000; Guo *et al.*, 2000; Pearson *et al.*, 2000; Louria-Hayon *et al.*, 2003; Bernardi *et al.*, 2004; Alsheich-Bartok *et al.*, 2008). Under these circumstances, for reasons that we still do not fully understand and could be related to the modification of PML by kinases involved in DNA-damage signaling pathways (Yang *et al.*, 2002; Bernardi *et al.*, 2004), PML-NBs increase in quantity and quality and start to transiently accommodate p53 and many factors regulating p53 activity.

In detail, PML was first implicated in the control of p53 activity as a regulator of p53 acetylation in the context of cellular senescence, a condition induced by DNA damaging agents or overexpression of oncogenes (Halazonetis *et al.*, 2008). It was shown that upon expression of a strong oncogene such as Ras^{V12}, full activation of p53 depends on the presence of PML, which recruits p53 to the PML-NBs alongside the acetyltransferase p300 and in doing so facilitates p53 acetylation (Figure 3) (Ferbeyre *et al.*, 2000; Pearson *et al.*, 2000).

Later on, it became clear that PML regulates not only p53 acetylation but also p53 stabilization, by interfering with the ubiquitination of p53 by Mdm2 (Figure 3; Kurki *et al.*, 2003). PML can bind to both p53 and Mdm2

(Wei *et al.*, 2003; Zhu *et al.*, 2003) and it has been suggested that PML inhibits Mdm2-mediated ubiquitination of p53 either by forming a trimeric complex with Mdm2 and p53 (Kurki *et al.*, 2003), or by sequestering Mdm2 away from p53 (Bernardi *et al.*, 2004), or yet by promoting p53 deubiquitination by the ubiquitin protease HAUSP (Everett *et al.*, 1997; Li *et al.*, 2002). In addition, PML blocks p53-Mdm2 interaction also by promoting p53 phosphorylation by Chk2 and CK1 (Louria-Hayon *et al.*, 2003; Alsheich-Bartok *et al.*, 2008). PML may additionally stimulate p53 phosphorylation by Chk2 also indirectly, by promoting Chk2 activity. In fact, it was recently shown that upon DNA damage, PML facilitates Chk2 autophosphorylation and activation (Yang *et al.*, 2006). Finally, PML promotes the phosphorylation of p53 by HIPK2 (Moller *et al.*, 2003), a modification that increases p53-mediated transcription of a subset of promoters and is associated with UV-induced apoptosis (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002).

Despite the number of publications addressing the issue of the regulation of p53 by PML, caution needs to be raised about the fact that the majority of these studies have been conducted *in vitro*. Since p53 is a protein regulated by many factors and cofactors, the physiological relevance of these data remains to be established *in vivo*. Furthermore, it would be also important to understand whether tumors that have lost PML expression but retain wild-type (wt) p53 can be treated with PML-inducing drugs to reestablish sensitivity to proapoptotic chemotherapy.

On a final note, most of the functions of PML described so far depend on the ability of PML to recruit p53 and/or p53-modifying proteins to the PML-NBs. Consistently with this notion, it was recently reported that cytoplasmic PML mutants inhibit p53 activity by acting in a dominant-negative fashion on wt nuclear PML (Bellodi *et al.*, 2006). Mutations introducing a stop codon upstream the nuclear localization signal of PML have been identified in patients with aggressive forms of APL, where mutations are found in the allele of *PML* not involved in the t(15:17) translocation (Gurrieri *et al.*, 2004b). In cells expressing wt PML, mutant PML accumulates in the cytoplasm, delocalizes nuclear PML into cytoplasmic aggregates and inhibits p53 transcriptional activation (Bellodi *et al.*, 2006). It must be underscored that wt cytoplasmic PML isoforms have been characterized and found to be critical in regulating TGF- β signaling and antiviral responses (see also following paragraphs; Lin *et al.*, 2004; McNally *et al.*, 2008). Although the above-mentioned studies do not address the function of wt cytoplasmic PML isoforms in the modulation of cytoplasmic p53 function, they are nonetheless important, because they indicate that patients bearing these or similar PML 'nuclear exclusion' mutations could prove insensitive to proapoptotic drugs acting through p53.

PML and c-Jun

Another apoptotic pathway regulated by PML is UV irradiation-induced cell death. In primary mouse

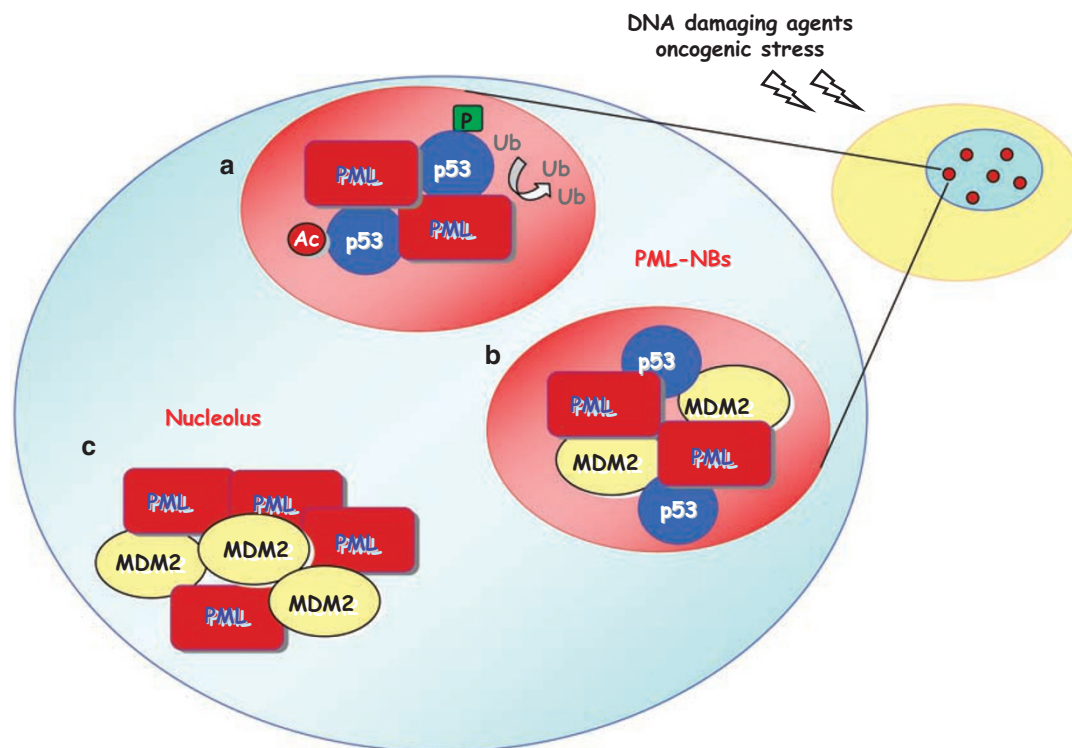


Figure 3 PML activates p53 through various mechanisms. Upon DNA damage induced by irradiation, chemotherapeutic agents or overexpression of oncogenes, PML amplifies p53 activity by several means: (a) by mediating several post-translational modifications of p53 such as acetylation, phosphorylation by various kinases and deubiquitination; (b) by interacting with p53 and Mdm2 and preventing p53 ubiquitination; (c) by sequestering Mdm2 away from p53 in the nucleolus. PML, promyelocytic leukemia.

fibroblasts, UV light-induced apoptosis is p53-independent but JNK/c-Jun-dependent, as demonstrated by the fact that p53 and p21 null fibroblasts are not protected but rather hypersensitized to UV-induced apoptosis (Waldman *et al.*, 1996; Smith and Fornace, 1997; Bissonnette and Hunting, 1998; Lackinger and Kaina, 2000), whereas JNK null fibroblasts are resistant to UV-induced apoptosis (Tournier *et al.*, 2000). Primary mouse fibroblasts lacking *Pml* are also protected from apoptosis upon exposure to UV light indicating that PML regulates apoptosis in a p53-independent manner in these settings. Indeed, while c-Jun becomes transcriptionally activated by UV light in wt cells, the transcriptional activation of c-Jun is impaired in the absence of *Pml* (Salomoni *et al.*, 2005).

Interestingly, UV irradiation causes the dispersal of PML-NBs into a myriad of small nuclear dots, and PML and active, phosphorylated, c-Jun physically interact and colocalize into these novel UV-induced PML nuclear structures (Salomoni *et al.*, 2005). The precise mechanism by which PML regulates the transcriptional activity of c-Jun has not been revealed yet. Similarly to p53, c-Jun could be modified and activated within UV-induced PML bodies, or alternatively UV-induced PML bodies could create a favorable chromatin milieu for the activation of transcription by c-Jun.

More work is necessary to better understand the molecular mechanisms of c-Jun regulation by PML.

However, these data indicate that PML is an important mediator of DNA damage-induced apoptosis and that different types of DNA damage cause different spatial and biochemical changes in the PML-NBs, leading to the regulation of specific transcription factors, p53 and c-Jun being two important examples of this regulation.

PML and Daxx

The death domain-associated protein, Daxx, is a multifaceted protein that localizes to various cellular compartments and participates in different apoptotic pathways, at times with opposite outcomes. Daxx was originally identified by virtue of its interaction with Fas (Yang *et al.*, 1997) and was shown to work as a proapoptotic factor. In detail, it was found that cytoplasmic Daxx is an adaptor protein that interacts with Fas and the kinase ASK1. Daxx interaction with ASK1 causes the activation of ASK1 and JNK and finally culminates in the induction of apoptosis (Yang *et al.*, 1997; Chang *et al.*, 1998).

Later on, it became clear that besides its first characterized cytoplasmic function, Daxx also localizes to the nucleus and has nuclear functions. In the nucleus, Daxx is found both in the nucleoplasm, where it associates with chromatin, and also in the PML-NBs (Torii *et al.*, 1999; Li *et al.*, 2000; Zhong *et al.*, 2000c; Lehenbre *et al.*, 2001). In the absence of PML, Daxx becomes delocalized from PML-NBs and accumulates

in chromatin-dense nuclear regions (Zhong *et al.*, 2000c). Nuclear Daxx has been mostly attributed the functions of transcriptional corepressor (Torii *et al.*, 1999; Li *et al.*, 2000; Zhong *et al.*, 2000c), although in some cases, it can also behave as a transcriptional coactivator (Emelyanov *et al.*, 2002). Regarding the functional consequence of Daxx localization to PML-NBs, it was demonstrated that the ability of Daxx to accumulate in PML-NB correlates with an increase in its proapoptotic function. Specifically, in the PML-NBs, Daxx sensitizes cell lines to Fas-induced apoptosis (Torii *et al.*, 1999) and spleen lymphocytes to apoptosis induced by mitogenic activation (Zhong *et al.*, 2000c). At the same time, recruitment of Daxx to PML-NBs was suggested to block Daxx-mediated transcriptional repression (Li *et al.*, 2000).

On the basis of these observations, two models can be proposed to explain the proapoptotic function of Daxx in PML-NBs: in one scenario, Daxx could repress the expression of antiapoptotic genes localized in the vicinity of PML-NBs (Figure 4). Interestingly, it was recently shown that Daxx represses the expression of a subset of antiapoptotic genes such as cIAP2 (Croxtan *et al.*, 2006). This model, however, seems to be contra-

dicted by the evidence that Daxx localization to PML-NBs inhibits its transcriptional corepressor function (Li *et al.*, 2000). Nonetheless, it could still be postulated that the role of PML in mediating Daxx transcriptional repression is promoter-specific and that we still have not identified the genes repressed by Daxx in the PML-NBs. Alternatively, and in a more far-fetched scenario, in the PML-NBs, Daxx could act as a transcriptional coactivator of proapoptotic genes, although so far, no experimental evidence supports this hypothesis (Figure 4). More work is necessary to better elucidate the collaboration of PML and Daxx in inducing apoptosis and also to understand whether the cytoplasmic and nuclear activities of Daxx are in any way related and coordinated or rather are independent functions.

Intriguingly, in addition to its proapoptotic role, Daxx has also been shown to exert antiapoptotic functions, both during development and in cell lines (Michaelson *et al.*, 1999; Michaelson and Leder, 2003). And indeed, contrary to what described so far, a recent study has shown that in rheumatoid arthritis fibroblasts, Daxx recruitment to PML-NBs contributes to the resistance to Fas-induced apoptosis (Meinecke *et al.*,

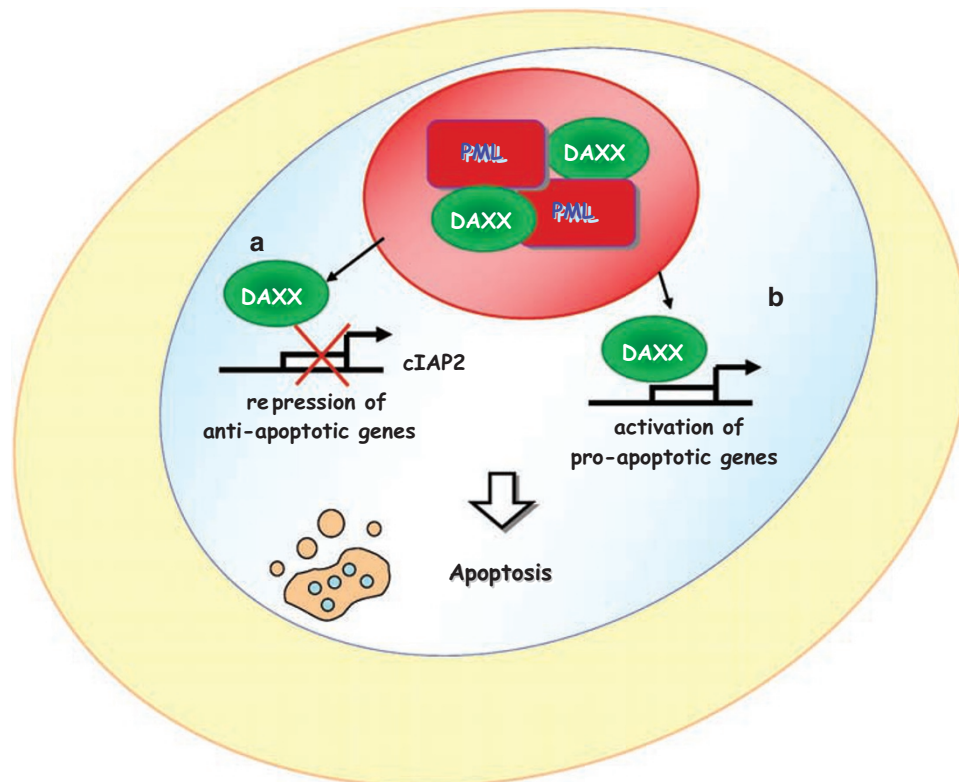


Figure 4 Models of PML/Daxx cooperation in the induction of apoptosis. Daxx is a transcriptional corepressor localizing to the cytoplasm and the nucleus, where it accumulates into PML-NBs. The localization of Daxx to PML-NBs has been proposed to mediate its ability to induce apoptosis upon Fas ligand. Daxx works mostly as a transcriptional corepressor, although transcriptional coactivation by Daxx has also been described. Two opposite models are presented to explain the functional cooperation of PML and Daxx in inducing apoptosis: (a) the localization of Daxx in PML-NBs mediates its ability to repress the transcription of antiapoptotic genes; (b) Daxx localization to PML-NBs mediates its ability to activate the transcription of proapoptotic genes. Both models are theoretical. Moreover, it has not been elucidated yet whether the PML-dependent function of Daxx in apoptosis occurs within the nuclear bodies or Daxx is somehow modified in the PML-NBs and performs its proapoptotic function elsewhere in the nucleus. PML, promyelocytic leukemia; PML-NBs, PML nuclear bodies.

2007). If confirmed in other cell types or different tissues, these data imply that, depending on the cell context, Daxx may exert pro- or antiapoptotic functions from within the PML-NBs, or after transiting through the PML-NBs.

It will be, therefore, very interesting to understand what are the molecular determinants of such different specificity: whether different cofactors differentially colocalize with Daxx in PML-NBs, or whether the specific post-translational modification of Daxx or PML may shift the balance from a proapoptotic to an antiapoptotic factor.

Pml and death receptor-associated proteins

Besides the functional interaction of PML with Daxx in mediating Fas-induced apoptosis, it was recently shown that PML may impact on apoptosis induced by Fas ligand also through another more direct mechanism, specifically by regulating the availability of the adaptor protein FLASH (Krieghoff *et al.*, 2007; Milovic-Holm *et al.*, 2007). FLICE-associated huge protein (FLASH) was originally identified in a two-hybrid screen as a caspase-8 interacting protein (Imai *et al.*, 1999) and was proposed to activate caspase-8 at the DISC (death-inducing signaling complex), a multiprotein complex localized at the cell membrane that mediates the induction of apoptosis by death receptors such as Fas. Recent studies determined that FLASH interacts also with Sp100, a constitutive component of PML-NBs, and that in steady-state conditions, FLASH localizes both to the cytoplasm as well as to the PML-NBs (Krieghoff *et al.*, 2007; Milovic-Holm *et al.*, 2007). In response to Fas activation, FLASH is released from PML-NBs and accumulates in the cytoplasm, at mitochondria, where it colocalizes with a fraction of procaspase-8 and is suggested to promote its activation to caspase-8 (Krieghoff *et al.*, 2007; Milovic-Holm *et al.*, 2007). The relevance of this newly discovered mechanism of caspase-8 activation is shown in experiments with leptomycin B, an inhibitor of nuclear export, where blocking FLASH translocation to the cytoplasm with leptomycin B significantly inhibited Fas-induced apoptosis (Krieghoff *et al.*, 2007; Milovic-Holm *et al.*, 2007). Although intriguing, these data pose some difficulty of interpretation about the role of PML in Fas-induced apoptosis. If PML-NBs were simply nuclear storage centers sequestering FLASH away from cytoplasmic procaspase-8, one would expect Pml null cells to have more cytoplasmic FLICEs and to be more prone to apoptosis induced by Fas, whereas on the contrary, Pml null cells are resistant to Fas-inducing apoptosis (Wang *et al.*, 1998). This apparent contradiction indicates that FLASH localization into PML-NBs may not be a simple matter of sequestration. One possible hypothesis is that FLASH needs to be somehow 'activated' or modified before exiting the nucleus and activating procaspase-8, and that this modification occurs in the PML-NBs when the appropriate stimulus is received. More work will hopefully reveal the molecular details of this regulation.

Similarly to FLICE, another adaptor protein originally implicated in apoptosis induced by death receptors and also found to localize to PML-NBs is the protein TRADD (Morgan *et al.*, 2002). TRADD is a death domain protein associated with TNF-R1 that works as an adaptor molecule for the recruitment of FADD and caspase-8 to the TNF-R1 DISC (Kischkel *et al.*, 2000). Besides its best-described cytoplasmic function, TRADD shuttles between the nucleus and the cytoplasm and can promote apoptosis also from within the nucleus (Morgan *et al.*, 2002). In the nucleus, TRADD localizes to PML-NBs, and TRADD-induced apoptosis is Pml-dependent (Morgan *et al.*, 2002). Interestingly, nuclear TRADD activates an apoptotic pathway that is fundamentally distinct from the cytoplasmic TNF-R1-dependent pathway; in that it requires PML and p53, but does not require caspases (Morgan *et al.*, 2002).

The role of PML in TRADD-induced apoptosis is fundamentally different from its role in FLICE-induced apoptosis. While in the case of FLICE, the PML-NBs seem to participate to a reinforcing mechanism that converges into the same apoptotic pathway initiated by Fas at the membrane, PML-NBs-dependent TRADD-induced apoptosis is different from the classical cytoplasmic apoptotic pathway initiated at the DISC. Although the physiological relevance and the details of the molecular mechanisms by which PML regulates these pathways remain to be established, it is evident that cytoplasmic cell death may have an important nuclear component of which PML is one mediator.

PML and caspases

The findings described in the previous paragraphs depict PML as an important regulator of apoptosis initiation, as PML controls the activation of many factors that trigger apoptosis upstream of the cell death machinery. Interestingly, recent evidence is beginning to suggest that PML could also regulate the activity of proteins that more directly execute the apoptotic program, such as caspases. Caspases are cysteine proteases synthesized as inactive precursors, or procaspases, which become sequentially activated by proteolytic processing and lead to the execution of apoptosis through a cascade of successive caspase activation. Two major classes of caspases have been described: initiator caspases (caspase-8, -10, -9 and -2) and effector caspases (caspase-3, -6 and -7). Initiator caspases process/activate other caspases and are early regulators of the caspase cascade, whereas effector caspases process various protein substrates that directly execute the apoptotic process (Sanchez-Pulido *et al.*, 2007).

Early studies had shown that the overexpression of PML can induce apoptosis in the absence of caspase-mediated apoptotic features (Quignon *et al.*, 1998). For example, DNA condensation and induction of caspase-3 activity were not observed upon PML overexpression, and broad-spectrum caspase inhibitors such as z-VAD-fmk did not prevent PML-induced cell death, implying

that PML may trigger cell death in a caspase-independent manner (Quignon *et al.*, 1998).

While caspase-independent apoptosis may be induced by the overexpression of PML in specific cell types, substantial evidence significantly implicates PML in pathways leading to caspase-dependent cell death, such as the ones discussed in the previous paragraphs. Moreover, more recent findings suggest that PML can possibly also directly modulate caspase activation (Sanchez-Pulido *et al.*, 2007).

The most direct link between PML and caspases is being provided by the observation that caspase-2 colocalizes with PML in PML-NBs (Tang *et al.*, 2005; Sanchez-Pulido *et al.*, 2007). Caspase-2 is an initiator caspase activated by various apoptotic signals, but perhaps most prominently implicated in apoptosis induced by DNA damage, where it is important for the mitochondrial release of cytochrome C (Guo *et al.*, 2002; Lassus *et al.*, 2002; Paroni *et al.*, 2002). Unlike caspase-3, -4, -8 and -10 that localize mainly to the cytosol and caspase-6, -7 and -9 that localize both to the cytoplasm and the nucleus, caspase-2 is found in nuclear filaments in some cells and in nuclear dots in other cells (Tang *et al.*, 2005). Interestingly, the nuclear dots of caspase-2 colocalize with PML-NBs, an association that requires the protease domain of the active caspase (Tang *et al.*, 2005). Although the consequences of ablating the localization of caspase-2 to PML-NBs on its ability to induce apoptosis have not been investigated yet, caspase-2 has been reported to induce apoptosis from within the nucleus (Paroni *et al.*, 2002). These data, though very preliminary, suggest that the induction of apoptosis by nuclear caspase-2 may require its localization to PML-NBs and urge a more thorough analysis of the role of PML in caspase-2 activity.

Besides caspase-2, other caspases have also been connected to PML and the PML-NB. For example, it has been recently shown that SATB1, a protein that associates to scaffold/matrix attachment regions and regulates chromatin remodeling, is sumoylated and then cleaved by caspase-6 in a process occurring within or facilitated by PML-NBs (Tan *et al.*, 2008). Although it remains to be established whether SATB1 cleavage by caspase-6 is part of an apoptotic program or a nonapoptotic developmental processes, this is another example of a caspase-mediated event that has been positioned in proximity of the PML-NBs. It is also not clear from this work whether caspase-6 directly localizes to PML-NBs (Tan *et al.*, 2008), although caspase-6 can localize to the nucleus (Tang *et al.*, 2005).

Finally, another caspase with nuclear and cytoplasmic localization is caspase-7 (Tang *et al.*, 2005). It has been reported that when SUMO modified, caspase-7 localizes to dot-like subnuclear structures (Hayashi *et al.*, 2006). These structures have not been clearly identified as PML-NBs, but it is known that many different proteins acquire PML-NBs localization when SUMO modified (Bernardi and Pandolfi, 2007). In conclusion, although the precise nuclear localization of caspases-6 and -7 has not been established yet, altogether these data hint at the possibility that nuclear PML mediates caspase function

in a direct way, a possibility that should be carefully examined.

Another intriguing possibility that remains to be explored, as discussed in the following paragraph, is whether cytoplasmic forms of PML may directly or indirectly regulate apoptosis, perhaps impinging on caspase activity, although to date no data support this hypothesis.

Open questions and future directions

Despite the number of publications accumulated in recent years on the role of PML in apoptosis, some important issues have so far gone unaddressed. In the near future, we should focus our attention on answering these questions. For example, we should attempt to better understand the physiological significance of the regulation of apoptosis by specific PML isoforms and also, we should examine whether cytoplasmic PML may exert any role in mediating apoptosis.

The PML gene is heavily regulated at the level of alternative splicing, with PML isoforms differing predominantly in their C-terminal and central regions (Jensen *et al.*, 2001; Bernardi and Pandolfi, 2007). A better characterization of the specificity of expression and function of PML isoforms has recently begun, thanks to the generation of isoform-specific antibodies (Condemine *et al.*, 2006). Several important and somewhat surprising findings have been made. One of these findings is that the most abundant isoform of PML in human cells and tissues is PML I, an isoform that is also conserved in the mouse, whereas other isoforms such as PML IV are expressed less than PML I and seem not to be perfectly conserved in mouse (Condemine *et al.*, 2006). This observation raises intriguing questions. For example, PML IV has been the most studied isoform of PML so far, especially in studies addressing the role of PML in apoptosis, because PML IV has been shown to interact specifically with p53 and to mediate p53-dependent senescence and apoptosis (Fogal *et al.*, 2000; Guo *et al.*, 2000; Bischof *et al.*, 2002). As PML IV is one of the least abundant isoforms of PML, and it is reported to be poorly conserved in the mouse (Condemine *et al.*, 2006), it remains to be established whether other PML isoforms can mediate p53-dependent apoptosis in the mouse, or whether even the less conserved and less expressed PML IV may have an important physiological impact *in vivo*. This question could be addressed experimentally by knocking down PML IV as well as other PML isoforms by using specific shRNA either *in vitro* or, more convincingly, *in vivo*.

Moreover, besides the role of PML isoforms in regulating p53-dependent apoptosis, another question that needs to be addressed is whether there is isoform-specificity also in other PML apoptotic functions, such as the regulation of Daxx, c-Jun and caspases.

Another interesting point raised by the publication of Condemine *et al.* (2006) is that there could be a surprising variability in the levels of PML isoform I,

and perhaps also of other isoforms, in different tissues. The ratio of isoform I mRNA compared to total PML mRNA was analysed in a few tissues, and the surprising result is that brain and liver show very different expression levels of PML I, with PML I contributing to around 80% of total PML in the brain and less than 30% in the liver (Condemine *et al.*, 2006). These data imply first of all that different PML isoforms could be regulating developmental or differentiation processes. Secondly and more relevant to the current discussion, if there is physiological significance to isoform specificity in regulating apoptosis, it can be postulated that tumors arising in different tissues have different sensitivity to apoptosis by specific stimuli depending on the isoform of PML that is mostly expressed. At the same time, if PML isoforms are differentially lost in tumors, this could imply that tumors become more resistant to specific apoptotic stimuli depending on the isoform that is mostly lost. Interestingly, data by Condemine *et al.* (2006) indicate that PML isoform I could be specifically downregulated in some tumors. These data, however, are still preliminary and a more thorough analysis of the expression of PML isoforms in tumors is necessary to understand whether the downregulation of PML I is common to all tumors and also whether other isoforms may be specifically downregulated.

Besides the role of nuclear PML, another interesting aspect that has not been thoroughly addressed so far concerns the role of cytoplasmic PML in apoptosis. Cytoplasmic aggregates of PML can be generated through the expression of bona fide cytoplasmic PML isoforms, which lack the nuclear localization signal, but also by nuclear PML isoforms, which localize both to the nucleus and partly to the cytoplasm when expressed in *Pml* null cells (Condemine *et al.*, 2006). Therefore, given that cytoplasmic PML bodies may be expressed in many cell types, it will be important to understand whether PML can modulate apoptotic programs also from the cytoplasm, and whether from the cytoplasm PML can perhaps interact more directly with the apoptosis execution machinery.

Notably, a recent publication has shown that cytoplasmic PML I isoforms, and specifically an isoform lacking exons 5 and 6, is specifically enriched in cells exposed to herpes simplex virus-1 and participates to cellular antiviral responses by sequestering viral proteins (McNally *et al.*, 2008). It will be very important to understand whether some apoptotic conditions may also promote the accumulation of specific cytoplasmic isoforms of PML.

Finally, a central question that needs to be earnestly dealt with in the near future is the physiological relevance of all the described functions of PML in regulating apoptosis. Of all the studies that have been described in this review, few of them have been validated by *in vivo* observations, as most have been performed *in vitro* and in conditions of overexpression. While it is generally preferred that the overexpression studies be interpreted with caution and be used in conjunction with other experimental approaches, this concept is particularly imperative for proteins that have the

tendency to aggregate, such as PML, and that for this reason could trigger nonspecific cellular stress responses when overexpressed. Evidence that PML regulates apoptosis is overwhelmingly solid, because it has been proven by many different approaches and validated in *Pml* KO animals. What remains to be established with more accuracy is which one of the various pathways that PML seems to regulate *in vitro* plays an important role in the modulation of apoptosis *in vivo*, and, particularly, which of these pathways is relevant to the physiological consequence of the loss of PML in solid tumors. For example, it would be useful to test *in vivo* the sensitivity of tumors that have lost PML expression (Gurrieri *et al.*, 2004a) to proapoptotic stimuli that are currently utilized in chemotherapy to understand whether the loss of PML may lead to resistance to selective agents and which of the pathways described so far is more relevant to the specific *in vivo* condition.

Conclusions and therapeutic implications

One clear concept that is emerging from the current overview of the role of PML in apoptosis is that although *Pml* null mice and cells are resistant to a wide variety of apoptotic stimuli both *in vitro* and *in vivo*, there appears to be no common mechanism to explain the general resistance of these cells to apoptosis. Instead, PML-NBs emerge as signaling centers that regulate the availability, post-translational modification and activation of many and diverse proteins implicated in apoptotic pathways. Most of these proteins are upstream regulators of apoptosis, in other words 'decision makers' that evaluate whether the conditions are right to trigger apoptosis. Whether or not PML might also be involved more directly in the execution of apoptosis is an attractive hypothesis that is starting now to be explored; thanks to the recent findings that PML could regulate the activity of caspases.

Given the general and important role of PML as a mediator of apoptosis, one goal we should actively pursue is to find therapies that increase PML expression in tumors, in the hope of making tumor cells more sensitive to chemotherapy. As PML is rarely mutated in tumors and it is more often downregulated at the post-transcriptional level (Gurrieri *et al.*, 2004a), the concept of developing 'PML-enhancing' therapies is not far-fetched. These drugs could on the one hand restore PML expression in tumors that have decreased or lost PML expression (Gurrieri *et al.*, 2004a; Chang *et al.*, 2007), but perhaps they also could be of use in tumors expressing PML, as overexpression of PML has been reported to cause apoptosis or growth inhibition also in cells that have not lost its expression (Liu *et al.*, 1995; Mu *et al.*, 1997; Le *et al.*, 1998).

Inhibitors of CK2, a kinase that mediates PML degradation by the proteasome, represent one such class of drugs. Indeed, pharmacological inhibition of CK2 has already been shown to enhance PML tumor-suppressive properties *in vivo* (Scaglioni *et al.*, 2006).

In addition, as we are currently shedding more light into the mechanisms regulating PML sumoylation and degradation, these studies will hopefully lead to new strategies for increasing PML expression.

A recent report identified 2,5-MeC, an inhibitor of tyrosine kinases, as an inducer of PML protein and PML-NBs (Komura *et al.*, 2007). The consequences of PML upregulation were analysed in wt and *p53* null cell lines and PML upregulation was found to induce apoptosis only in the presence of *p53*, either endogenous or reexpressed in *p53*-negative cells (Komura *et al.*, 2007). Although these experiments were conducted in a limited number of cell lines and more experimental work is necessary to verify whether PML upregulation induces apoptosis only in the presence of *p53*, these data are further proof for the fact that drugs that upregulate PML might have clinical utility. In the future, *in vivo* experiments and preclinical trials will be essential to understand whether agents that promote PML expression may inhibit tumor growth, either alone or in combination with other chemotherapeutic drugs.

Of great relevance to targeting PML for cancer therapy, a seemingly contradictory study has recently found that a PML-inhibiting rather than a PML-promoting agent cooperates with chemotherapy in suppressing leukemia (Ito *et al.*, 2008). PML was identified as a gene that is robustly expressed in hematopoietic stem cells and that promotes stem cell quiescence. As a consequence, loss of *Pml* results in increased cycling and consequent exhaustion of hematopoietic stem cells. Intriguingly, the same results were found to apply to leukemia stem cells in a model of chronic myeloid leukemia: leukemia stem cells generated in *Pml* null mice cycle more than *Pml* wt cells, exhaust earlier in serial transplantation experiments and are more sensitive to chemotherapeutic drugs that kill proliferating cells (Ito *et al.*, 2008). On the basis of these experiments, it was tested whether mimicking the loss of *Pml* by using arsenic trioxide, a drug known to trigger the degradation of PML as well as that of PML-RAR α , would result in sensitization of chronic myeloid

leukemia stem cells to killing by chemotherapy. This was found to be the case and led to the implication that the use of arsenic trioxide in conjunction with current chemotherapy in chronic myeloid leukemia patients should cause exhaustion of leukemia stem cells and eradication of chronic myeloid leukemia.

However exciting, these data could be considered at odd with the idea of promoting PML expression for treating cancer. Instead, these findings suggest that both negative and positive pharmacological manipulations of PML levels through the use of distinct drugs, if carefully designed and timed, could be tremendously advantageous for cancer treatment. For example, PML-inhibiting drugs could be used as adjuvant therapy to increase the cycling of cancer stem cells and the efficacy of chemotherapy in early phases of tumor treatment, whereas in later stages, and if cancer stem cells are exhausted, one could switch to PML-enhancing agents to sensitize more committed tumor cells to apoptosis. Alternatively, PML-lowering or PML-enhancing agents could be used in a tumor-specific manner, depending on the dependency of different tumors on cancer stem cells and also on the expression levels on PML in various tumor compartments. Evidently, further work is necessary to determine in detail the expression levels and the function of PML in different tumor types and tumor compartments. On the basis of these findings, we will be able to better understand the impact of targeting PML for cancer therapy.

In conclusion, this is undoubtedly a very exciting moment in PML research, as it is now possible to translate critical biological information into effective treatment modalities.

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