

Regulation of the Promyelocytic Leukemia Protein and Its Role in Premature Senescence

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Abstract

Premature senescence functions as a tumor suppressor mechanism in response to oncogenic stimuli. It is characterized by irreversible cell cycle arrest mediated by tumor suppressors such as p53, Rb and the Promyelocytic Leukemia (PML) protein. PML mainly localizes in sub-nuclear structures known as PML nuclear bodies. These nuclear bodies accumulate in senescent cells largely due to increased PML gene transcription driven by p53 and/or the interferon pathways. PML exerts its pro-senescence activity by modulating both the p53 and Rb pathways, the major regulators of the cellular senescence program. Mechanistically, PML binds and promotes p53 modifications to generate a positive feedback loop, thereby triggering the senescence program. In addition, PML associates with Rb and may function in Rb/E2F-mediated gene silencing in senescent cells. Moreover, PML bodies recruit DNA damage sensing and repair proteins, thereby linking PML to the activation of the DNA damage response pathway, a pathway frequently activated in senescence. Therefore, elucidation of key factors controlling PML protein abundance will help to better understand how cells become transformed by avoiding senescence and allowing continued cellular proliferation in the presence of oncogenic signals. These findings will also be crucial in aiding scientists and

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physicians in the development of novel anti-cancer therapies by restoring PML to trigger senescence.

Introduction

Cellular senescence is the phenomenon by which cells undergo an irreversible cell cycle arrest, losing the ability to further divide and proliferate. Several stresses have been implicated in the induction of senescence, including oxidative damage, telomerase dysfunction, aberrant oncogene-dependent proliferative signaling, and DNA damage (Collado and Serrano 2010). Together, these mechanisms converge on the two major tumor suppressor genes *p53* and *Rb*, whose mutations or inactivation are most common in human cancers. Biologically, the onset of the senescence program limits excessive or aberrant cellular proliferation, such that the state of senescence protects against the development of cancer (Vijg and Campisi 2008), and could have further impact on organismal aging and contribute to the development of age-related pathologies (Baker et al. 2011). One particularly relevant source of stress in tumor cells is derived from the aberrant proliferative signals of oncogenes, which may trigger premature senescence through a process termed as oncogene-induced senescence (OIS). Recent studies from multiple groups clearly showed that OIS functions as a potential tumor suppressor mechanism (Di Micco et al. 2006).

Notably, in response to various oncogenic stimuli, such as Ras signaling, there is increased expression of the promyelocytic leukemia protein (PML) (Ferbeyre et al. 2000; Pearson et al. 2000; de Stanchina et al. 2004), which subsequently results in the onset of a premature senescent state (Ferbeyre et al. 2000). PML is a protein initially identified as a fusion product with retinoic acid receptor alpha (RAR α) in acute promyelocytic leukemia (APL) (de The et al. 1991; Goddard et al. 1991; Pandolfi et al. 1991). Consistent with a tumor suppressor role for PML, disruption of PML in vivo sensitizes mice to tumorigenesis induced by physical or chemical carcinogens, and accelerates tumor onset in sev-

eral mouse cancer models (Scaglioni et al. 2006). PML is expressed ubiquitously and is usually accumulated at PML nuclear bodies. Importantly, PML nuclear bodies are found to be significantly elevated in response to several cellular stresses as well as when cells enter the senescent state. Mechanistically, PML plays a key role in senescence mainly acting through the Rb and p53 pathways, two proteins known to physically interact with PML and accumulate in PML nuclear bodies. It was observed that PML levels in senescent cells are largely dependent on DNA damage signals and accumulate near unrepaired DNA damage regions, indicating that senescence is coupled to the incapacity of cells to deal with unrepaired lesions in the genome (Dellaire et al. 2003). In further support of a possible link between senescence and pre-malignancies, PML bodies were frequently found in benign prostate tumors but not in prostate cancers (Vernier et al. 2011). Moreover, PML nuclear bodies are absent in cancer cells (Shen et al. 2006). The intrinsic connection between PML and the onset of senescence suggests a general and central role for PML in tumor suppression.

Transcriptional Modulation of the PML Tumor Suppressor

Recent studies indicate that PML is accumulated in senescent cells in part due to enhanced *PML* mRNA translation (Ferbeyre et al. 2000; Scaglioni et al. 2012). *PML* mRNA expression is induced by interferon (IFN) via the Jak/Stat signaling pathway and functions through IFN-stimulated response elements and IFN γ -activated sites present in the PML gene promoter (de Stanchina et al. 2004). Interferon also regulates many other components of PML nuclear bodies to mediate their anti-viral activities (Regad and Chelbi-Alix 2001). The induced expression of IFN-target genes during the establishment of senescence suggest that the IFN pathway might also control the senescent program. In keeping with this notion, treatment of primary human cells with β -IFN leads to the elevated expression of p53, PML, and subsequent onset of premature

senescence (Moiseeva et al. 2006). In addition, constitutive STAT5 signaling, a critical signaling component downstream of Jak can also enhance PML expression and induce senescence (Mallette et al. 2007). These findings are in agreement with the idea that cytokine-mediated PML expression is associated with the onset of the senescence program.

Notably, the first intron of the *PML* gene also contains p53 responsive elements that physically associate with p53 and activate PML transcription during Ras-induced senescence or the DNA damage response (de Stanchina et al. 2004). These findings suggest that PML also functions downstream of the p53 tumor suppressor to mediate senescence, a process that may also involve the activation of the Rb/E2F pathway to halt normal cell cycle progression (Vernier et al. 2011). However, the Scaglioni group recently showed that oncogenic K-RAS could induce PML upregulation even in p53-null cells, suggesting the presence of a p53-independent mechanism of PML induction. Further studies revealed that under these experimental conditions, the PML 5' untranslated mRNA region (5' UTR) mediates selective uploading of PML mRNA onto polyribosomes and its selective translation, leading to p53 independent PML upregulation (Scaglioni et al. 2012). In addition to these upstream regulatory routes, PML gene expression can also be activated by β -catenin in a LEF/TCF-independent manner (Shtutman et al. 2002). Hence, it has been proposed that β -catenin may operate on the basal transcription machinery on the PML promoter. However, more studies are required to further dissect the potential crosstalks or synergies among various transcription factors in governing PML transcription in different stresses or tissue contexts.

Post-Translational Regulation of PML

Besides transcriptional induction of the *PML* gene during the onset of senescence, PML is also regulated via several posttranslational modifications (Fig. 9.1). For example, PML is phosphory-

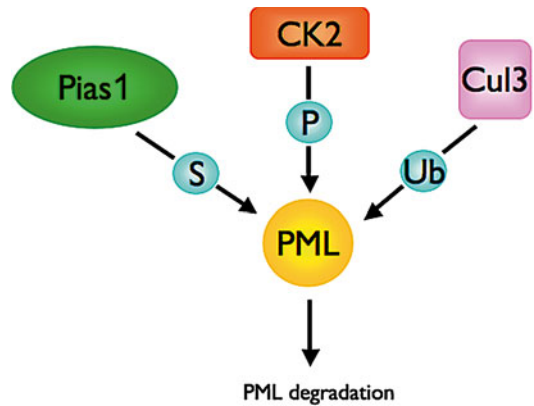


Fig. 9.1 Mechanisms controlling PML protein turnover. Several post-translational modifications trigger PML ubiquitination and proteasomal degradation. *S* SUMOylation, *P* Phosphorylation, *Ub* Ubiquitination. Arrow indicates potential cross-talk

lated by ERK2 on several residues and these modifications have been implicated in promoting the degradation of PML upon treatment with arsenic trioxide. Furthermore, ATR and CHK2 also phosphorylate PML in a DNA damage-dependent pathway (Bernardi et al. 2004). In addition, Big MAP kinase 1 (BMK1) was also found to drive suppression of PML function through its phosphorylation of PML on S403 and T409 (Yang et al. 2010). Moreover, phosphorylation by CK2 induces PML ubiquitination and subsequent degradation by the 26S proteasome (Scaglioni et al. 2006), while the physiological E3 ligase responsible for CK2-induced PML ubiquitination still remains uncharacterized.

Besides phosphorylation-dependent modifications, PML is also modified by the covalent binding of ubiquitin like modifiers, such as SUMO1 and SUMO2/3. A recent study showed that PML is SUMOylated by the E3 SUMO ligase Pias1 (Rabellino et al. 2012). Importantly, SUMOylation of PML has been previously reported to regulate the formation of PML nuclear bodies (Shen et al. 2006). On the other hand, the SUMOylation of PML is also controlled by SUMO proteases (Gong et al. 2000), whose role in senescence remains unknown. Interestingly, overexpression of the SUMO protease SENP1 in the prostate leads to the development of prostatic intraepithelial neoplasia (PIN) at an early age

(Cheng et al. 2006). Further studies showed that androgen suppresses PML protein levels in prostate cancer cells and induces the expression of SENP1, which in turn, enhances prostate epithelial cell proliferation (Bawa-Khalife et al. 2010). Notably, acetylation of PML has been linked to an increase in PML SUMOylation, suggesting that acetylation primes PML for SUMOylation (Hayakawa et al. 2008). However, additional studies are required to further reveal the intrinsic interaction between SUMOylation and acetylation in modulating PML stability.

Consistent with its tumor suppressive role, down-regulation of PML is frequently observed in diverse types of human tumors and is correlated with tumor progression. Because no major noticeable changes in *PML* transcripts were observed in human cancers, it has been proposed that aberrant degradation of the PML protein may be the major mechanism accounting for the reduction of PML abundance in tumors. Moreover, arsenic trioxide, an effective APL treatment, induces PML proteasomal degradation through a process that requires PML SUMOylation by Pias1 (Rabellino et al. 2012). In a broad spectrum of cancers, PML was found to undergo ubiquitin-mediated degradation primed by CK2 phosphorylation, but the responsible E3 ligase for this process was unidentified. However, this group did determine that Pias1-dependent SUMOylation of PML promoted CK2 interaction, triggering PML ubiquitination and turnover by the proteasome (Rabellino et al. 2012). Interestingly, the SUMO-targeting ubiquitin E3 ligase RNF4 has been found to ubiquitinate poly-SUMO chains on PML leading to degradation of the protein (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008). However, it is still unknown whether Pias1, CK2 and RNF4 are part of an integrated cellular network that leads to aberrant degradation of PML in cancer cells. Furthermore, RNF111/Arkadia has also been identified to be a SUMO-targeting E3 ligase that is involved in PML degradation in response to arsenic treatment (Erker et al. 2013). Unfortunately, similar to RNF4, it is still unknown whether Arkadia functions with Pias1 and CK2 to promote PML destruction in cancer

cells. Nevertheless, inhibition of this proteolytic process enhances the tumor suppressor properties of PML (Scaglioni et al. 2006), supporting the rationale for developing anti-CK2 therapies for cancer.

In further identifying upstream regulatory pathways that may control PML stability, the E3 ubiquitin ligase E6AP was also found to interact with PML and can promote PML degradation in a proteasome-dependent manner (Louria-Hayon et al. 2009). Remarkably, E6AP-deficient cells show an increase of DNA damage-dependent apoptosis, due to an accumulation of PML in the nucleus causing an increase in PML-dependent responses. Additionally, partial loss of E6AP attenuates MYC-induced B-cell lymphomagenesis. In this model, tumor suppression is achieved by the induction of cellular senescence but not apoptosis. Accordingly, partial loss of E6AP leads to PML restoration and subsequent induction of PML-dependent senescence (Wolyniec et al. 2012).

In a recent paper, Yuan and colleagues reported that hypoxia induces PML proteasomal degradation by the Cul3^{KLHL20} ubiquitin ligase complex (Yuan et al. 2011). Sequential modifications of PML by CDK1/2 and Pin1 facilitate the recruitment of PML to Cul3^{KLHL20} for ubiquitination. Under hypoxic conditions, the drastic up-regulation of KLHL20 leads to a robust PML ubiquitination through this pathway, thereby reducing PML abundance. Biologically, PML down-regulation correlates with HIF-1 α , Pin1, and KLHL20 up-regulation in human prostate cancer, and hyperactivation of this pathway correlates with high-grade tumors (Yuan et al. 2011). These studies also clearly showed that Pin1 binds to PML and induces PML down-regulation. This association largely depends on phosphorylation of PML by the MAP-kinase ERK2 (Lim et al. 2011), therefore revealing a CK2-independent pathway controlling PML ubiquitination. Given that the MAP-kinase cascade is activated by several extracellular signals, including oncogenic stress, future studies are needed to elucidate its role in PML-induced senescence. Additionally, it remains to be addressed whether the CDK1/2-Pin1 and the ERK2/Pin1 signaling pathways that mediate the timely ubiquitination of PML are

complementary, mutually exclusive, or tumor type specific.

While the proteasome has been well documented to control intracellular levels of PML, autophagy can also promote the degradation of PML-RAR α , the oncogenic fusion version of PML implicated in the pathogenesis of acute promyelocytic leukemia. Induced by treatment with all-trans retinoic acid (ATRA) or arsenic trioxide (As₂O₃), the ubiquitin-binding adaptor protein p62/SQSTM1 directs ubiquitinated PML-RAR α to autophagosomes for subsequent degradation (Wang et al. 2011). Unfortunately, additional studies are still required to elucidate whether non-fused PML, which functions largely as a tumor suppressor, is similarly targeted for autophagy, but these findings do suggest that other cellular degradation pathways may also regulate PML stability.

PML Induces the p53 Tumor Suppressor Pathway

The role of PML in activating the p53 pathway was first discovered in the context of Ras-induced senescence (Ferbeyre et al. 2000; Pearson et al. 2000). These studies suggested that PML stimulated p53 transcriptional activity by recruiting p53 into PML bodies, inducing p53 modifications. Specifically, PML promotes the acetylation of p53 at lysine 382 in a process catalyzed by the acetyl transferase CBP, which localizes to PML bodies (Pearson et al. 2000). In addition, PML induces the phosphorylation of p53 at serine 15, a modification that is usually catalyzed by the DNA damage response kinases ATM and ATR (Ferbeyre et al. 2000). Interestingly, proteins that inhibit senescence can reverse these modifications. For example, SIRT1, a NAD-dependent deacetylase, inhibits PML-induced senescence by deacetylating p53 at lysine 382 in PML bodies (Langley et al. 2002). The adenoviral oncoprotein E1A blocks cellular senescence by impairing the ability of PML to form PML bodies and preventing p53 phosphorylation in cells expressing oncogenic *ras* (Ferbeyre et al. 2000).

Notably, p53 activation requires the whole PML body macromolecular complex. The site of

interaction between p53 and PML was mapped to the DNA binding domain of p53 (Fogal et al. 2000). Therefore, p53 may not bind simultaneously to PML and DNA. Taking all the data together, a plausible model is that p53 recycles between promoters and PML bodies undergoing cycles of reversible post-translational modifications (Fig. 9.2). Among the PML isoforms, PML IV binds p53 more efficiently than PML I or PML-RAR α , a fusion oncoprotein found in acute promyelocytic leukemia. Consistent with this finding, only PML IV relocalizes p53 into PML bodies when co-transfected with p53 (Fogal et al. 2000). However, expression of PML IV is not sufficient to induce senescence in *PML*^{-/-} MEFs, suggesting that other PML isoforms might be required for this process. It is possible that a particular combination of PML isoforms, possibly with a predominant role of PML IV drives p53 recycling and/or modification. Further studies are required to fully understand the contribution of each PML isoform in the onset of premature senescence.

Furthermore, PML may activate p53 by binding MDM2, an E3 ligase that targets p53 for ubiquitination and proteasomal degradation (Wei et al. 2003). MDM2 can shuttle PML from the nucleus to the cytoplasm in transfected cells, showing preferential binding to the non-SUMOylated form of PML. Trimeric complexes between PML, p53 and MDM2 were found in unstressed cells. Interestingly, upon DNA damage, these complexes segregated into PML-p53 and PML-MDM2 complexes (Kurki et al. 2003). Given that PML was unable to activate a p53 fusion protein lacking its PML binding domain, PML's ability to promote p53 activity seems to rely primarily on the formation of a p53-PML complex. An alternative mechanism proposed is that PML recruits MDM2 into the nucleolus, thereby promoting the stabilization and activation of p53 (Bernardi et al. 2004). This activity of PML depends on PML phosphorylation by ATR and interaction of PML with the nucleolar protein L11. ARF, a positive regulator of p53 and senescence that can also sequester MDM2 into the nucleolus, does not seem to be required for PML and MDM2 translocation (Weber et al. 1999). On the contrary, PML

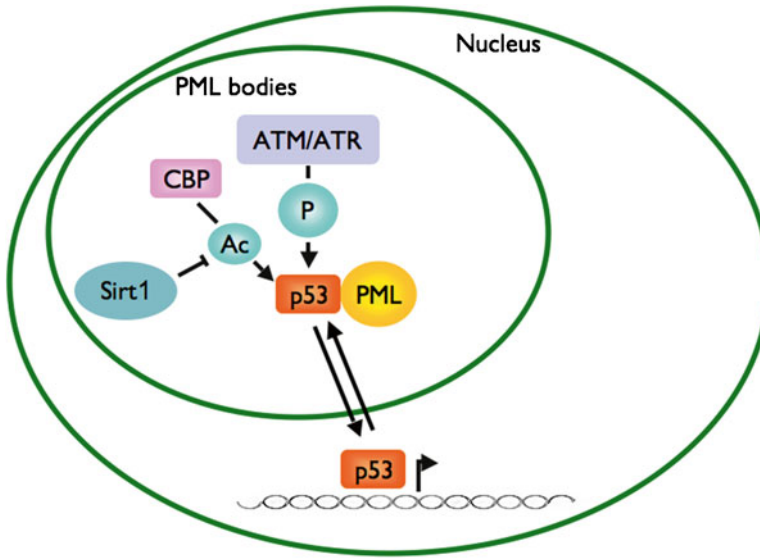


Fig. 9.2 Schematic representation of p53 recycling between PML bodies and DNA promoters. P53 is acetylated in the PML bodies by CBP in a process reversed by

the NAD-dependent deacetylase Sirt1. In addition, PML promotes the phosphorylation of p53 by the DNA damage responsive kinases ATM and ATR

activates transcription of the ARF promoter by forming a complex with β -catenin, which potentially explains its role in activating p53. Finally, previous work also suggested that PML controls the nucleo-cytoplasmic transport of several growth-promoting mRNAs, including MDM2 (Culjkovic et al. 2006).

In summary, these studies clearly demonstrate that PML promotes p53 transcriptional activity. Given that p53 induces the expression of PML, these two tumor suppressors are interlinked in a positive feedback mechanism that potentiates the functions of p53 and/or PML (Fig. 9.2). Additionally, PML was found to suppress growth and tumorigenesis in the absence of p53, indicating that other pathways are also involved in the functions of PML, especially in the onset of cellular senescence.

PML Promotes Rb-Dependent Senescence

While PML was initially found to regulate senescence via p53, further studies demonstrated that PML was also capable of inducing cell cycle

arrest and senescence in cells where the p53 pathway was inactivated by the papillomavirus oncoprotein E6 or the dominant negative p53 allele p53H175R. These studies suggested that PML might engage an Rb-dependent senescence program that does not require p53. Further work demonstrated that PML associates with Rb and that the PML-Rb complex formation requires the pocket region of Rb, and the B boxes and the C-terminal region of PML (Alcalay et al. 1998). Rb was also found to be essential for the formation of senescence associated heterochromatin foci (SAHF). Although SAHF does not colocalize with PML bodies, the Adam's group identified PML bodies as key components of SAHF formation, as the heterochromatin protein HP1 and the histone chaperone HIRA transiently localize to PML bodies before moving to SAHF (Zhang et al. 2005). These events were observed upon replicative senescence, enforced expression of p16^{INK4A}, or Ras-induced premature senescence, before cell cycle arrest or detection of senescence markers. Recently, the Ferbeyre group reported that Rb/E2F complexes were relocalized into PML bodies along with heterochromatin proteins when senescence was induced by

forced PML expression by retroviral vector, or by induction of endogenous PML by β -interferon, oncogenic *ras*, or short telomeres (Vernier et al. 2011). Relocalization of Rb/E2F complexes into PML bodies resulted in the inhibition of E2F transcriptional activity and a corresponding reduction in E2F regulated genes. Consequently, blocking the expression of E2F target genes leads to cell cycle arrest and accumulation of DNA damage signals that are essential for activation of p53 and the senescence process. Additionally, PML bodies may also be the site of “nucleation” or initiation of chromatin condensation on E2F target genes that later become the detectable sites of SAHF (Vernier et al. 2011).

Furthermore, indirect connections were also identified between the Rb pathway and PML through the regulation of the Myc and Cyclin D1 oncoproteins. In this regard, it is well known that Myc and Cyclin D1 promote inactivation of Rb via phosphorylation catalyzed by the CDK4/6-Cyclin D complex. PML enhances the inhibitory function of Mad, an antagonist of Myc (Khan et al. 2001). In addition, PML controls the protein stability of Myc, possibly by binding and recruiting it into PML bodies along with the 26S proteasome (Buschbeck et al. 2007). On the other hand, PML prevents the expression of Cyclin D1 in part by blocking the transport of *Cyclin D1* mRNA to the cytoplasm (Lai and Borden 2000).

It is noteworthy that PML cannot induce senescence in the absence of functional Rb and p53 pathways. However, PML can still slow cellular growth and inhibit tumor formation in these conditions. It is plausible that PML activity must be carried out by PML in association with other cellular factors apart from the p53 and Rb pathways, as well as translocation of PML to the nucleolus, or defects in PML body formation, among other mechanisms.

PML Contributes to the DNA Damage Response

Several proteins involved in DNA damage response have been found to localize to PML bodies. These proteins range from DNA damage

sensing proteins such as p53, ATM, ATR, topoisomerases and helicases, to DNA repair proteins involved in both double-strand breaks and homologous recombination. It has been reported that treatment with DNA damaging agents increases the number and size of PML bodies and causes relocalization and/or modification of PML bodies-associated proteins. Studies by Carbone and colleagues proposed that PML bodies function as sensors of DNA damage lesions contributing to the recognition of the sites of damage and/or their processing (Carbone et al. 2002). It has been postulated that the increase in PML bodies is part of the cellular response to DNA damage.

Given that the formation of DNA damage foci and the DNA damage response pathway are important features of the onset of senescence (Di Micco et al. 2006), it is possible that the association of PML bodies with DNA damage sensing or DNA repair proteins contributes to the activation of the DNA damage response pathway detected in senescence and to the proper onset of permanent cell cycle arrest to avoid the transference of damaged DNA to daughter cells.

Targeted Therapies to Restore PML Tumor Suppressor Function

PML is slowly emerging as a key tumor suppressor whose expression is down regulated in multiple types of human carcinomas (Gurrieri et al. 2004; Lee et al. 2007; Vincenzi et al. 2010). As such, identifying pharmacological means to restore its expression becomes increasingly more vital. In this review, we detail the proteasomal degradation pathway as a major route by which PML expression can be regulated, thereby making it an attractive therapeutic target when attempting to manipulate cellular PML levels. Currently, multiple small molecule inhibitors have been designed that may prove to be useful for future therapeutic regimens. As E6AP has been identified to be an E3 ligase that promotes the ubiquitination and degradation of PML (Louria-Hayon et al. 2009), inactivation of this ligase’s function may enhance expression of PML in pathological conditions where PML

levels may be compromised. Interestingly, a product-like macrocyclic *N*-methyl peptide inhibitor has been designed that can inhibit E6AP-catalyzed polyubiquitination of target proteins. While this peptide has not been tested on PML ubiquitination mediated by E6AP, it has shown robust inhibitory activity against E6AP-catalyzed polyubiquitination of two characterized target proteins, Prx1 and p53 (Yamagishi et al. 2011). Although obstructing the E3 ligase activity of E6AP may be the most direct means to prevent PML degradation, inhibition of upstream signaling pathways may also prove to have merit as pharmacological interventions. As PML must first be phosphorylated by upstream kinases before it can be recognized by E3 ligases, inhibiting these kinases may also elevate PML expression and function. Scaglioni et al. demonstrated in a lung cancer xenotransplant model that emodin, a pharmacological inhibitor of CK2 kinase, could re-establish PML function in vivo (Scaglioni et al. 2006). Furthermore, a recent study elegantly described the in vivo anti-tumor effects of using a newly developed BMK1 inhibitor (XMD8-92) to restore PML activity (Yang et al. 2010). CK2, BMK1, and E6AP are three proteins known to play integral roles in PML degradation. Based on the studies described in this review, other attractive targets may also include additional E3 ligases such as KLHL20 or the SUMO E3-ligase Pias1.

While there has been some headway made in treating cancers deficient in the PML tumor suppressor, this field is still in its infancy. Additional studies identifying new and novel upstream regulators of PML function would significantly improve our understanding of this protein while also identifying new nodes of intervention when attempting to treat tumors deficient in PML.

Conclusion

The promyelocytic leukemia tumor suppressor PML plays a critical role in the onset of premature senescence, preventing the proliferation of cells with malignant potential. Mechanistically,

the contribution of PML to senescence involves the p53 and Rb tumor suppressor pathways. PML physically interacts with the DNA-binding domain of p53 to promote p53 modifications. Additionally, p53 activation is driven by PML binding to MDM2, an E3 ligase that catalyzes the ubiquitination and subsequent degradation of p53, and by PML-mediated sequestration of MDM2 into the nucleolus. PML also interacts with Rb and recruits the Rb/E2F complex into PML bodies. These interactions may play a role in Rb/E2F-mediated gene silencing and heterochromatin formation during senescence. Likewise, PML bodies associate with DNA damage response and repair proteins, which might be components of the DNA damage response frequently found aberrantly activated in senescent cells. Importantly, replicative senescence has been proposed as a result of accumulated chronic DNA damaging signals and tumorigenesis may occur when the DNA damage response is inactivated. Therefore, bypassing senescence or reversion of this process is considered a critical step towards cellular transformation. Consistent with the notion of PML being a tumor suppressor, *PML* inactivation in mice leads to cancer susceptibility, and PML deficiency occurs in a broad spectrum of human cancers through mechanisms that involve its aberrant ubiquitination and turnover. Therefore, understanding the molecular mechanisms of PML degradation during tumorigenesis should provide the rationale for developing novel therapeutic strategies to trigger PML restoration as an approach to induce premature senescence to suppress tumor formation.

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